British Journal of Cancer (2012) 107, 334–339

www.bjcancer.com

A morpho-molecular prognostic model for hepatocellular carcinoma

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BACKGROUND: Hepatocellular carcinoma (HCC) is the third common cause of cancer-related deaths and its prognostication is still suboptimal. The aim of this study was to establish a new prognostication algorithm for HCC.

METHODS: In all, 13 biomarkers related to the etiopathogenesis of HCC were evaluated by immunohistochemistry using tissue microarrays containing 121 primary HCC resection cases, and validated in subsequent cohort of 85 HCC cases. The results were compared with Affymetrix Gene Chip Human Genome U133Plus microarray data in a separate cohort of 228 HCC patients.

RESULTS: On immunohistochemical evaluation and multivariate Cox regression analysis p53, alpha fetaprotein (AFP), CD44 and CD31, tumour size and vascular invasion, were significant predictors for worse survival in HCC patients. A morpho-molecular prognostic model (MMPM) was constructed and it was a significant independent predictor for overall survival (OS) and relapse-free survival (RFS) ($P<0.000$). The OS and RFS of HCC^{low} was higher (104 and 78 months) as compared with HCC^{high} (73 and 43 months) ($P<0.0001$for OS and RFS). Hepatocellular carcinoma patients with higher stage (III + IV), >5 cm tumour size, positive vascular invasion and satellitosis belonged to HCC^{high} group. The validation group reproduced the same findings. Gene expression analysis confirmed that 7 of the 12 biomarkers were overexpressed in >50% of tumour samples and significant overexpression in tumour samples was observed in *AFP*, *CD31*, *CD117* and *Ki-67* genes.

CONCLUSION: The MMPM, based on the expression of selected proteins and clinicopathological parameters, can be used to classify HCC patients between good *vs* poor prognosis and high *vs* low risk of recurrence following hepatic resection.

British Journal of Cancer (2012) **107,** 334–339. doi:10.1038/bjc.2012.230 www.bjcancer.com
Published online 19 June 2012

Keywords: p53; microvessel density; prognosis; liver cancer; survival

Hepatocellular carcinoma (HCC) is one of the most common cancers in men and the third most common cause of cancer-related death worldwide (Parkin, 2000). Regions of high incidence are areas of subSaharan Africa and South-east Asia (World Health Organization, http://www.who.int/whois), mainly linked to the presence of risk factors such as chronic Hepatitis B and/or Hepatitis C infection (Lai *et al*, 2003). Surgical resection followed by liver transplant is the mainstay treatment; however, this treatment is available only for a subset of patients, and even though hepatic resection is curative, the long-term prognosis is still poor (Chen *et al*, 2006). Conventionally, Barcelona Clinic Liver Cancer (BCLC) (Llovet *et al*, 1999) and Tumour-Node-Metastasis (TNM) (Vauthey *et al*, 2002) staging systems, serum alpha fetoprotein (AFP) level and tumour size are used for the prognostication of HCC patients. These staging systems incorporate histopathological features of HCC tumours such as tumour size, number of tumours, vascular invasion and satellitosis. Recently, a significant number of tissue-based markers have been studied in relation to prognosis (survival and tumour recurrence). However, none of these biomarkers, alone or in combination with other clinicopathological conventional features, are used in the routine clinical practice. We selected a panel of tissue-based molecular markers on the basis of their role in hepatocarcinogenesis and following the recent published reports on molecular/genomic classification of HCC. Briefly, we selected p53 (TP53), Ki-67 (PCNA), cyclin D1 (CCND1), (related to proliferation and cell cycling, G3 (Boyault *et al*, 2007) cluster A (Lee *et al*, 2004); β-catenin, E-cadherin (Wnt signalling pathway, S1, G5, G6) (Hoshida *et al*, 2009; Boyault *et al*, 2007); CD44 (HB subtype); cancer stem cell-related (CD133, CD117); angiogenesis-related (CD31) and hepatocyte functional markers (AFP, Hepar, CD10) (Yang *et al*, 2010). We performed immunohistochemical analysis on 121 pairs of human HCC tissues and their corresponding non-tumour hepatic tissues followed by confirmation of immunoexpression on 50 full sections. We then constructed a morpho-molecular prognostic model (MMPM) based on the prognostic power of the histological parameters and the relative expression of the immunohistochemical markers. The resulted MMPM predicted patient outcome (death/relapse) more powerfully than any

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Received 2 February 2012; revised 3 April 2012; accepted 25 April 2012; published online 19 June 2012

molecular markers. The robustness of MMPM was corroborated and reproducible on a separate cohort of 85 HCC cases.

MATERIALS AND METHODS

Three independent cohorts of patients were included in the study. For immunohistochemical analysis, formalin-fixed paraffin-embedded tissues from surgically resected specimens of HCC patients who had undergone curative hepatectomy between 1990 and 2003 (cohort 1, $n=121$) and from 2004 to 2009 (cohort 2, $n=85$) at National University Hospital, Singapore were taken. For gene expression analysis, an independent cohort of 228 patients with HCC (cohort 3) was recruited from Queen Mary Hospital, Hong Kong, between 1993 and 2007 as described previously (Luk *et al*, 2006). For the latter, the tumour and adjacent non-tumourous tissues were collected after hepatectomy, and were immediately snap frozen and stored at $-80\,^{\circ}C$ prior to analysis. Cohort 1 and 2 were analysed for immunohistochemistry in a tissue microarray (TMA) format as described previously (Das *et al*, 2008). Clinicopathological information was obtained from the medical records and included ethnicity, age, gender, tumour number, tumour size, stage, histological grading, vascular invasion, satellitosis and preoperative serum AFP (Table 1). Tumour differentiation was defined according to the Edmondson grading system (Edmondson and Steiner, 1954). Tumour staging was defined according to the sixth edition of the TNM classification of the International Union against Cancer (Sobin and Wittekind, 2002). Patients were followed up for death/relapse. Overall survival (OS) was defined as the interval between surgery and death or date of last observation. The death data was censored at the last follow-up for living patients. Patients of both cohorts were followed up until May 2010. Relapse-free survival (RFS) was defined from the date of surgery until the detection of recurrent tumour or the date of last follow-up. The RFS data was censored for patients without tumour recurrence. Ethics approval for this study was obtained from National University Singapore-Institutional Review Board (NUS-IRB; 10–133).

Immunoreactivity for each marker was assessed semi quantatively by evaluating the extent and intensity of the staining; extent was recorded by the percentage of positive tumour cells in relation to the total number of tumour cells; intensity was recorded as absent, weak, moderate or strong. No or weak staining was considered as negative and moderate or strong staining was considered as positive. Immunohistochemical staining was assessed by two independent and trained viewers (SS and OCW). The cut-off percentage for determining positive expression of each protein was determined by receiver-operating characteristics (ROC) analysis against the OS as described previously (Heagerty *et al*, 2000). Using values derived from the area under the ROC, values above 0.5 indicate significant discriminatory power for survival (Zlobec *et al*, 2007). Gene expression profiling was performed by cDNA microarray using AffyU133Plus assay (Affymetrix, Santa Clara, CA, USA) containing 47 000 probes. All procedures for hybridisation, labelling and scanning of gene chips were as described previously in accordance with the manufacturer's recommendations (Burchard *et al*, 2010). Raw gene expression profiling data were deposited to GEO database with the accession number of GSE25097.

Statistical analysis was performed using SPSS v 15.0 for Windows (SPSS, Armonk, NY, USA). Cumulative OS and RFS was calculated by the Kaplan–Meier method and analysed by the log-rank test followed by multivariate analyses using Cox proportional hazard regression model for statistically significant factors. A P-value $\leqslant 0.05$ was considered statistically significant.

RESULTS

Immunohistochemical expression of the markers

All the samples were assessed for the immunohistochemical expression of the 13 protein markers (Figure 1). The subcellular localisation of the expression (cytoplasmic/membranous/nuclear) along with the cut-off values and frequency of positive expression of the markers are given in Supplementary Table 1 (S1). To establish the reliability of TMAs for this analysis, we further analysed the expression in full sections of these markers in 50 HCC cases randomly chosen from cohort 1. The results were concordant in 96% (48/50) of the cases. Two cases showed nuclear staining of p53 (50, 30%) in the TMA, but weak staining (less than 10%) in the corresponding full tumour sections. These cases were taken as p53 negative according to the results on full tumour sections.

Table 1 Clinicopathological features of two cohorts with hepatocellular carcinoma

Clinical and pathological features	Cohort1, *n* (%)	Cohort 2, *n* (%)
Age (years)		
<50	27 (22.3)	19 (22.4)
>50	70 (57.9)	66 (77.6)
Sex		
Male	104 (86)	64 (75)
Female	17 (14)	21 (25)
Ethnicity		
Chinese	99 (81.8)	63 (74.1)
Others	22 (18.2)	22 (25.9)
Serum AFP (ng dl^{-1})		
<20	35 (28.9)	33 (38.8)
>20	59 (48.8)	37 (43.5)
HbsAg		
Yes	68 (56.2)	52 (61.2)
No	33 (27.3)	28 (32.9)
Alcoholic[a]		
Yes	32 (26.4)	27 (31.8)
No	55 (45.5)	45 (52.9)
Tumour differentiation		
I+II	104 (86)	71 (83.5)
III+IV	17 (14)	14 (16.5)
TNM stage		
I+II	85 (71.2)	68 (80)
III+IV	21 (17.4)	17 (20)
Tumour number		
Solitary	75 (62)	64 (75.3)
Multiple	46 (38)	21 (24.7)
Size (cm)		
<5	46 (38)	46 (54.1)
>5	75 (62)	38 (44.7)
Vascular invasion[b]		
Yes	52 (43)	17 (20)
No	41 (33.9)	60 (70.6)
Satellitosis		
Yes	15 (12.4)	12 (14.1)
No	106 (87.6)	73 (85.9)

Abbreviations: AFP = α-fetoprotein; HbsAg = hepatitis B surface antigen; TNM = tumour-node-metastasis. [a]Alcoholic intake of approximately >60 mg per day for prolonged period. [b]Cases with complete clinical information were included in the analysis.

Prognostic significance of 13 protein markers expression and clinicopathological characteristics

The mean OS was 93.51 ± 9.88 months. The mean RFS was 60.11 ± 8.05 months. The OS for 1-, 3-, 5-year were 81%, 65% and 50%,

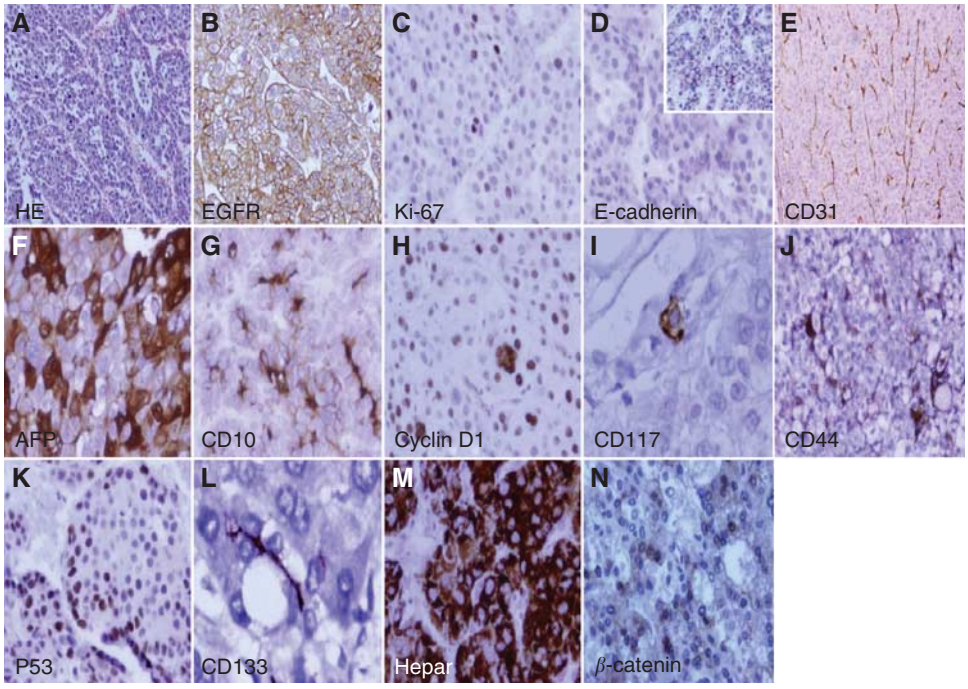

Figure 1 Representative positive expression of protein markers: (**A**) HE; (**B**) epidermal growth factor receptor (EGFR); (**C**) Ki-67; (**D**) E-cadherin; (**E**) CD31; (**F**) AFP; (**G**) CD10; (**H**) cyclin D1; (**I**) CD117; (**J**) CD44; (**K**) P53; (**L**) CD133; (**M**) Hepar; (**N**) β-catenin by immunohistochemistry in tumour tissue microarrays (original magnification × 400).

respectively. The RFS for 1-, 3-, 5-year were 61%, 44% and 29%, respectively. On univariate analysis p53, CD44, AFP, CD31, Ki-67, E-cadherin and cyclin D1 were unfavourable predictors of OS and RFS. Among the clinicopathological parameters higher TNM stage, more than 5 cm tumour size, positive satellitosis and vascular invasion were poor prognostic factors for OS or RFS (Table 2). Multivariate Cox regression model showed that p53, AFP, CD31 and CD44, vascular invasion and tumour size are statistically significant, independent factors for prognosis (Table 3).

Morpho-molecular prognostic model

The risk scores for MMPM were calculated using Cox regression model for multivariate analysis, and it was as follows: (0.800 × CD31) + (0.597 × p53) + (0.662 × AFP) + (0.485 × CD44) + (0.583 × size) + (1.001 × vascular invasion). The protein marker represents the expression level (positive = 1, negative = 0), and the histological features can be present (= 1) or absent (= 0). The median of the final score was 3.240. Accordingly, the 121 cases were dichotomised in two groups, HCC^{high} (score > 3.240) and HCC^{low} (score < 3.240). The OS and RFS in HCC^{low} was significantly higher (104 and 78 months) than in HCC^{high} (73 and 43 months) ($P < 0.0001$ for OS and RFS, respectively) (Figure 2). The HCC^{high} also expressed higher serum AFP ($ng\,dl^{-1}$) (3706 ± 9199 *vs* 346 ± 1625; $P = 0.006$) and higher MVD (21.10 ± 12.65 *vs* 15.95 ± 13.33; $P = 0.015$) as compared with HCC^{low}. Patients with higher stage (III + IV), > 5 cm tumour size, positive vascular invasion and satellitosis belonged to HCC^{high} group as compared with HCC^{low} group ($P < 0.001$, $P < 0.001$, $P < 0.001$ and $P = 0.022$, respectively). On multivariate analysis, the MMPM was an independent prognostic factor for OS ($P = 0.008$) against clinicopathological factors, however for RFS ($P = 0.074$) it was not significant. The prognostic power of the MMPM was higher than the individual markers (p53, CD44, AFP and CD31) and the clinicopathological features alone as shown in the ROC curve in Supplementary Figure 1 (SF1). Further, when stratified by tumour size and TNM stage the MMPM could be a robust predictor of OS (*P*-value 0.002 and 0.006, respectively) and RFS (*P*-value 0.016 and 0.000) (Figure 2).

Validation of the MMPM

Validation for the predictive power of the MMPM was done in another cohort of 85 HCC patients. Patients classified as HCC^{high} had a significant shorter OS and RFS (43.2 and 26.3 months) as compared with HCC^{low} (63.7 and 58.5 months) ($P = 0.032$ and 0.000 for OS and RFS, respectively) (Figure 3). Similar to cohort 1, we observed that the patients with higher TNM stage (III + IV) (14/16; $P < 0.001$), > 5 cm tumour size (23/35; $P = 0.009$), positive vascular invasion (17/17; $P < 0.001$) and satellitosis (9/11; $P = 0.048$) belonged to the HCC^{high} group. Higher MVD was also observed in high-risk group as compared with low-risk group (20 ± 2 *vs* 18 ± 2). However, it was not significant ($P = 0.551$). Similarly, the serum level of AFP was of borderline significance ($P = 0.060$). The *P*-values of early TNM stage (I + II) and tumour size less than 5 cm were 0.004 and 0.027, respectively, for RFS. For OS, early TNM stage (I + II) and tumour size < 5 cm were not significant, $P = 0.29$ and 0.095, respectively, which is likely to be owing to small sample size.

Validation of MMPM by gene expression of prognostic markers

Of 12 of the 13 prognostic markers represented in the microarray (Hepar-1 had no corresponding gene), 7 of them (i.e., AFP, β-catenin, CD31, CD44, CD117, Ki-67, TP53) were overexpressed in > 50% of tumour samples. Significant overexpression in tumour samples was observed in *AFP*, *CD31*, *CD117*, *Ki-67* genes.

DISCUSSION

Hepatocellular carcinoma is one of the most common malignant tumours worldwide and has poor prognosis and high recurrence

Table 2 Univariate overall and recurrence-free survival analysis for clinicopathological features and protein markers

	Overall survival			Recurrence-free survival		
Variable	**HR**	**95% CI**	***P*-value**	**HR**	**95% CI**	***P*-value**
Clinicopathological						
Gender (male *vs* female)	1.44	0.81–2.55	NS	0.90	0.54–1.52	NS
Age, years (>50 *vs* <50)	1.19	0.65–216	NS	1.00	0.62–1.62	NS
Ethnicity (Chinese *vs* non-Chinese)	0.70	0.33–1.49	NS	0.88	0.50–1.53	NS
Serum AFP, ng dl^{-1} (>20 *vs* <20	1.66	0.96–2.85	0.066	1.62	1.02–2.55	0.057
Grade (I+II *vs* III+IV)	0.70	0.30–1.64	NS	0.91	0.50–1.64	NS
TNM stage (I+II *vs* III+IV)	2.42	1.38–4.22	0.002	2.52	1.56–4.06	0.000
Tumour size, cm (>5 *vs* <5)	1.68	1.00–2.83	0.048	2.20	1.44–3.37	0.000
Number (solitary *vs* multiple)	1.21	0.70–2.68	NS	1.49	0.96–2.31	NS
Satellitosis (yes *vs* no)	3.01	1.58–5.72	0.001	3.34	1.93–5.79	0.000
Vascular invasion (yes *vs* no)	3.39	1.91–6.01	0.000	2.89	1.82–4.58	0.000
Protein expression						
P53 (positive *vs* negative)	2.38	1.42–4.0	0.001	1.83	1.18–2.84	0.006
Cyclin D1 (positive *vs* negative)	1.67	0.99–2.82	0.049	1.04	0.68–1.60	NS
CD31 (positive *vs* negative)	1.85	1.04–3.29	0.033	1.67	1.06–2.63	0.026
EGFR (positive *vs* negative)	1.08	0.63–1.84	NS	0.97	0.63–1.51	NS
Ki-67 (positive *vs* negative)	3.16	1.11–8.99	0.031	2.05	0.74–5.65	NS
β-catenin (positive *vs* negative)	1.12	0.65–1.90	NS	1.10	0.72–1.68	NS
E-cadherin (positive *vs* negative)	1.99	1.00–3.97	0.045	1.37	0.83–2.26	NS
AFP (positive *vs* negative)	2.21	1.26–3.87	0.005	2.13	1.35–3.36	0.001
Hepar-1 (positive *vs* negative)	0.58	0.34–1.00	NS	0.71	0.46–1.09	NS
CD10 (positive *vs* negative)	1.13	0.66–1.94	NS	1.28	0.82–1.98	NS
CD117 (positive *vs* negative)	1.82	0.66–5.0	NS	1.16	0.74–3.48	NS
CD133 (positive *vs* negative)	1.41	0.56–3.55	NS	0.47	0.15–1.5	NS
CD44 (positive *vs* negative)	2.21	1.28–3.83	0.004	1.34	0.88–2.04	NS

Abbreviations: AFP = α-fetoprotein; CI = confidence interval; EGFR = epidermal growth factor receptor; HR = hazard risk ratio; NS = not significant; TNM = tumour-node-metastasis. Univariate analysis, Cox proportional hazards regression model.

Table 3 Multivariate analysis for the protein markers and clinicopathological features for OS and RFS

	OS			RFS		
Variable	**HR**	**95% CI**	***P*-value**	**HR**	**95% CI**	***P*-value**
Clinicopathological						
TNM stage (I+II *vs* III+IV)	1.92	0.68–5.42	NS	0.98	0.38–2.53	NS
Tumour size, cm (>5 *vs* <5)	2.38	1.18–4.81	0.013	2.98	1.70–5.23	0.000
Satellitosis (yes *vs* no)	0.77	0.25–2.41	NS	2.25	0.81–6.23	NS
Vascular invasion (yes *vs* no)	2.98	1.38–6.41	0.004	1.89	1.06–3.362	0.030
Protein expression						
P53 (pos *vs* neg)	1.93	1.03–3.63	0.039	1.93	1.03–3.63	0.030
Cyclin D1 (pos *vs* neg)	1.82	0.88–3.76	NS	1.11	0.56–2.19	NS
CD31(pos *vs* neg)	2.41	1.10–5.31	0.028	1.70	0.97–3.00	0.060
Ki-67 (pos *vs* neg)	3.16	1.11–8.99	NS	1.50	0.49–4.56	NS
E-cadherin (pos *vs* neg)	1.99	1.00–3.97	NS	0.64	0.32–1.26	NS
AFP (pos *vs* neg)	2.22	1.03–4.78	0.041	1.68	0.81–3.46	NS
CD44 (pos *vs* neg)	2.12	1.02–4.41	0.044	0.94	0.49–1.82	NS

Abbreviations: AFP = α-fetoprotein; CI = confidence interval; HR = hazard ratio; neg = negative; OS = overall survival; pos = positive; RFS = recurrence-free survival; TNM = tumour-node-metastasis. Multivariate analysis, Cox proportional hazard regression model. Variables were adopted for their prognostic significance by univariate analysis.

rate, regardless of the treatment. Therefore, it is imperative for clinicians and scientists to find new ways to stratify patients for appropriate treatment. Previous reports have attempted to build a model based on the prognostic value of putative hepatic stem cell biomarkers in HCC (Yang *et al*, 2010). Traditionally, however, tumour staging system (TNM and BCLC staging), tumour size and serum AFP levels are used to predict the outcome of HCC patients, which sometimes cannot accurately predict the outcome of all HCC patients (Qin and Tang, 2004). Till date, there is neither any molecular marker routinely incorporated to staging systems, nor there is a molecular prognostic model. The present study was undertaken to identify a morpho-molecular prognosticator of HCC patients. The criteria for selection of the molecular markers evaluated in this study were tissue-based markers that are routinely available in Pathology Department with a strong basis for their role in hepatocarcinogenesis. Our results showed that the poor prognostic value of the overexpression of these markers was highest when all the four biomarkers and two histological parameters with individual prognostic significance were taken into consideration together. TP53 is a tumour suppressor gene, with a well-known function in DNA repair and apoptosis (Hu *et al*, 2003) and has been implicated in both hepatocarcinogenesis and HCC tumour recurrence. CD44 has been identified as a tumour stem cell marker in various epithelial cancers, including HCC. It is also a marker for tumour progression and has been previously reported to predict worse survival in HCC patients (Endo and Terada, 2000). CD31 is involved in angiogenesis and microvessel density previously shown in lung cancer and also in HCC (Giatromanolaki *et al*, 1996; Frachon *et al*, 2001). Alpha fetoprotein is an oncofetal marker traditionally used to prognosticate and follow up HCC patients (Kawai *et al*, 2001). We validated our findings in a separate cohort of 228 HCC patients and observed a significant overexpression in tumour samples in *AFP*, *CD31*, *CD117* and *Ki-67* genes. In other studies, significant overexpression of *TP53* gene (subgroup G3) (Boyault *et al*, 2007) and *CD44* gene (Yang *et al*, 2010) were observed in HCC patients. Therefore, the gene expression analysis in these studies was both confirmatory of the protein expression and, despite the possible transcriptional modifications, the overall relevance of the elements forming our proposed MMPM.

Classifications of HCC based on genetic profiles have been reported previously; however in a routine clinical set-up, high-throughput analyses have problems of reproducibility and affordability. Immunohistochemical analysis can provide cheaper, faster and more reproducible results. Few studies have reported HCC stratification based on immunohistochemical analysis (Yamashita *et al*, 2008). Based on a standard scoring system derived from Cox Regression analysis, we stratified the study cohort into HCC^{low} and HCC^{high} groups, with considerable differences in OS and RFS between them. When stratified by TNM stage and tumour size, MMPM stood as a good predictor of OS and RFS, regardless of the tumour stage and size ($P<0.05$). Of interest, the MMPM was valuable in predicting the outcome in early-stage HCC and small size tumour, which are usually difficult to predict by conventional indices (Qin and Tang, 2004). Although the MMPM was validated in a smaller second cohort, a larger independent cohort is required to validate this scoring system.

Hepatocellular carcinoma has a 5-year recurrence rate of approximately 80–90% and currently, size of hepatic nodules, vascular invasion and serum AFP level are used for risk estimation

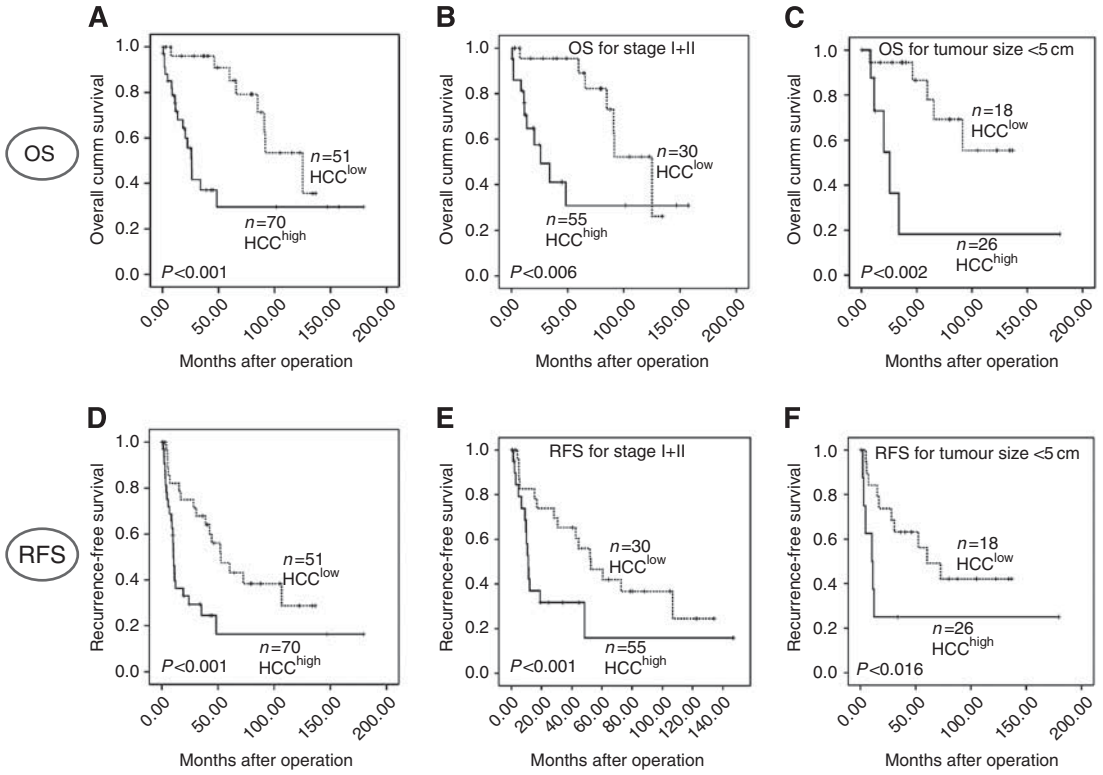

Figure 2 Kaplan–Meier survival analysis of low- and high-risk HCC patients by MMPM in cohort 1: overall survival (**A**), stratification of OS of cohort 1 for early TNM stage (I + II) (**B**), and tumour size <5 cm (**C**), recurrence-free survival (**D**), stratification of RFS for early TNM stage (I + II) (**E**) and tumour size <5 cm (**F**).

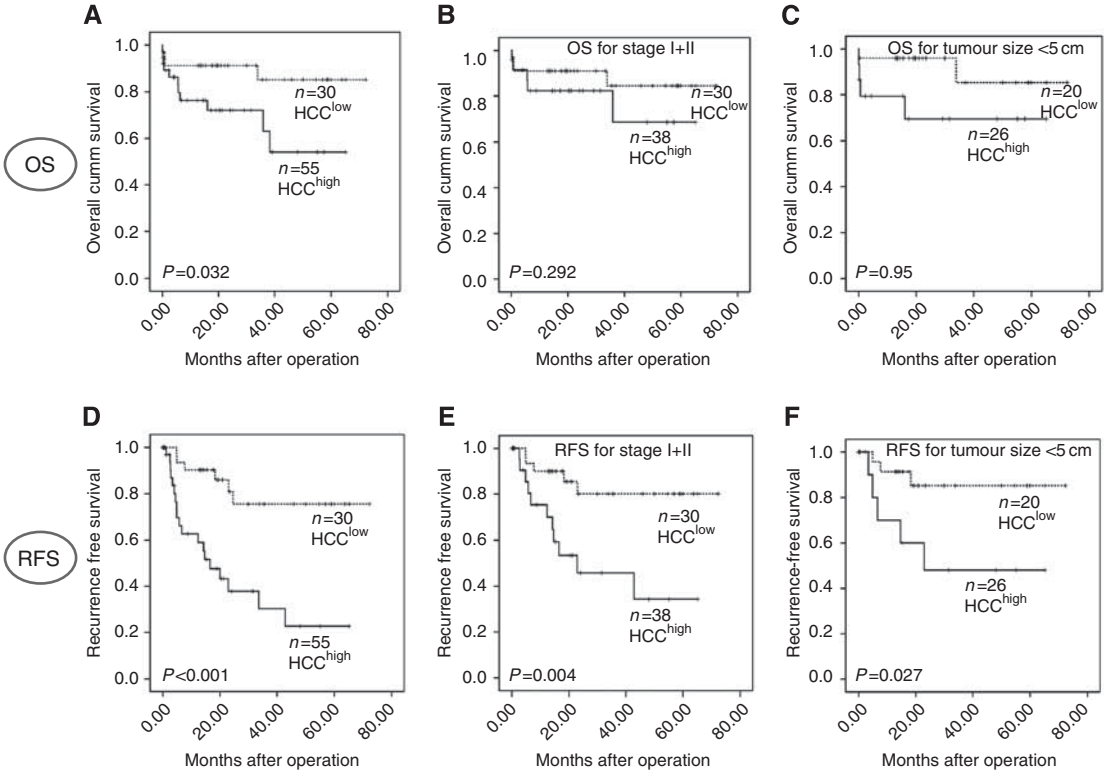

Figure 3 Kaplan–Meier survival analysis of low- and high-risk HCC patients by MMPM in cohort 2: overall survival (**A**), stratification of OS of cohort 2 for early TNM stage (I + II) (**B**), and tumour size <5 cm (**C**), recurrence-free survival (**D**), stratification of RFS for early TNM stage (I + II) (**E**) and tumour size <5 cm (**F**).

of tumour recurrence. Sorefanib and combination of ribavarin and interferon are few treatment options available for such HCC patients. It is also known that, the HCC recurrence is most frequently observed in the first 1–2 years after curative treatment. Our model shows that HCC patients with score more than 3.240 have shorter RFS period (43 *vs* 78 months, $P<0.0001$). Therefore MMPM could be useful in stratifying HCC patients for early recurrence so that a timely intervention could be made.

Immunohistochemical studies have been sometimes criticised by their subjectivity due to their qualitative interpretation. Because of this, and also because of the relatively small amount of tissue analysed per case in the TMA format, we chose two forms of validation. The remarkable concordance between TMA cores and large sections, already reported in other cancer types (Zhang *et al*, 2003) is highly reassuring of the technical robustness of this approach.

In conclusion, our study identifies p53, CD44, CD31 and AFP as powerful predictors of OS and RFS in HCC patients, and, as such, our proposed MMPM represents a powerful discriminator of prognosis and has implications in future in patient management.

ACKNOWLEDGEMENTS

We thank Madam Cheong Sok Lian for providing the clinicopathological information and Madam Choo Shoonian for help in immunohistochemistry. This study is funded by Singapore Cancer Syndicate, Agency for Science, Technology and Research, Singapore, and the Grants numbers are MN005, MN005R and MN077.

Conflict of interest

The authors declare no conflict of interest.

Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)

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