A morpho-molecular prognostic model for hepatocellular carcinoma

S Srivastava 1, KF Wong 1, CW Ong 1,2, CY Huak 3, KG Yeoh 4, M Teh 2, JM Luk 1,5,6 and M Salto-Tellez * 2,7

1Cancer Science Institute, National University of Singapore, Singapore; 2Department of Pathology, National University Health System, National University of Singapore, Singapore; 3Biostatistics Unit, National University Health System, National University of Singapore, Singapore; 4Department of Medicine, National University Health System, National University of Singapore, Singapore; 5Department of Pharmacology, National University Health System, National University of Singapore, Singapore; 6Department of Surgery, National University Health System, National University of Singapore, Singapore; 7Centre for Cancer Research and Cell Biology, Queen’s University Belfast, 97, Lisburn Road, Belfast BT9 7BL, UK

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 U133 Plus array data in a separate cohort of 228 HCC patients.

RESULTS: On immunohistochemical evaluation and multivariate Cox regression analysis p53, alpha fetoprotein (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 < 0.0001). The OS and RFS of HCCC high was higher (104 and 78 months) as compared with HCCC low (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 HCCC 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

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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 sub-Saharan 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). Conventional, 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 immuno-expression 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

*Correspondence: Professor M Salto-Tellez; E-mail: manuel_salto-tellez@nuhs.edu.sg

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°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 follow-up. The RFS data was censored for patients without tumour recurrence. The cut-off percentage for determining positive expression of each protein was determined by receiver-operating characteristics 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 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°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 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).

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 these 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 | Cohort 1, 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)      |
| Alcohol*                          |                 |                |
| 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                 |                 |                |
| 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. *Alcoholic intake of approximately ≥60 mg per day for prolonged period. aCases 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 ± 8.05 months. The mean RFS was 60.11 ± 8.05 months. The OS for 1-, 3-, 5-year were 81%, 65% and 50%, respectively, for cohort 1 and 2.
MMPM for hepatocellular carcinoma
S Srivastava et al

Molecular Diagnostics

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tissue microarrays (original magnification P(21.10

43 months) (45

by tumour size and TNM stage the MMPM could be a robust

and the clinicopathological features alone as shown in the ROC

higher than the individual markers (p53, CD44, AFP and CD31)

it was not significant. The prognostic power of the MMPM was

P against clinicopathological factors, however for RFS (P = 0.074)

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\textsuperscript{high}

had a significant shorter OS and RFS (43.2 and 26.3 months) as

compared with HCC\textsuperscript{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\textsuperscript{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, \textbeta-catenin, CD31, CD44, CD117, Ki-67, TP53) were overexpressed

in > 50% of tumour samples. Significant overexpression in tumour

samples was observed in \textbeta-catenin by immunohistochemistry in tumour

tissue microarrays (original magnification × 400).

respectively. The RFS for 1-, 3-, 5-year were 61%, 44% and 29%,

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

to model for multivariable 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\textsuperscript{high} (score 3.240) and

HCC\textsuperscript{low} (score < 3.240). The OS and RFS in HCC\textsuperscript{high} was signifi-

antly higher (104 and 78 months) than in HCC\textsuperscript{low} (73 and 43 months)

(P < 0.0001 for OS and RFS, respectively) (Figure 2). The HCC\textsuperscript{high}

e also expressed higher serum AFP (ng/dl\textsuperscript{-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\textsuperscript{low}. Patients with higher stage (III + IV), > 5 cm tumour size,

positive vascular invasion and satellitosis belonged to HCC\textsuperscript{high}

group as compared with HCC\textsuperscript{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

DISCUSSION

Hepatocellular carcinoma is one of the most common malignant

tumours worldwide and has poor prognosis and high recurrence

Figure 1 Representative positive expression of protein markers: (A) HE; (B) epidermal growth factor receptor (EGFR); (C) CD117; (D) E-cadherin;

(F) CD31; (G) CD10; (H) cyclin D1; (I) CD117; (J) CD44; (K) P53; (L) CD133; (M) Hepar; (N) \textbeta-catenin by immunohistochemistry in tumour

tissue microarrays (original magnification × 400).
Clinicopathological 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 overall 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 analyses (Yamashita et al., 2008). Based on a standard scoring system derived from Cox Regression analysis, we stratified the study cohort into HCClow and HCHigh groups, with considerable differences in OS and RFS between them. When stratified by TNM stage and tumour size, MMPM stayed 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.
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 ribavirin 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 DFS 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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