Observational Study

Medicine®

OPEN

The transcriptomic G1–G6 signature of hepatocellular carcinoma in an Asian population

Association of G3 with microvascular invasion

John Carson Allen Jr , PhD[a], Jean-Charles Nault, PhD[b,c], Guili Zhu, MD[a], Andrew Yu Keat Khor, MD[a], Jin Liu, PhD[a], Tony Kiat Hon Lim, MBBS, FRCPATH, FRCPA[d], Jessica Zucman-Rossi, PhD[b,c], Pierce K.H. Chow, MBBS, MMed (Surgery), FRCS, FAMS, PhD[e,f,g,*]

Abstract

In this study, a transcriptomic group classification based on a European population is tested on a Singapore cohort. The results highlight the genotype/phenotype correlation in a Southeast Asian population. The G1–G6 transcriptomic classification derived from hepatocellular carcinoma (HCC) resected from European patients, robustly reflected group-specific clinical/pathological features. We investigated the application of this molecular classification in Southeast Asian HCC patients.

Gene expression analysis was carried out on HCC surgically resected in Singapore patients who were grouped into G1–G6 transcriptomic categories according to expression of 16 predictor genes (illustrated in Supplementary Table 1, http://links.lww.com/MD/B413 and Supplementary Fig. 1, http://links.lww.com/MD/B413) using quantitative reverse transcription polymerase chain reaction (RT-PCR). Univariate and multivariate polytomous logistic regression was used to investigate association between clinical variables and pooled transcriptomic classes G12, G3, and G456.

HCC from Singapore (n = 82) were distributed (%) into G1 (13.4), G2 (24.4), G3 (15.9), G4 (24.4), G5 (14.6), and G6 (7.3) subgroups. Compared to the European data, the Singapore samples were relatively enriched in G1–G3 versus G4–G6 tumors (53.7% vs 46.3%) reflecting the higher proportion of hepatitis B virus (HBV) patients in Singapore versus Europe samples (43% vs 30%). Pooled classes were defined as G12, G3, and G456. G12 was associated with higher alpha-fetoprotein (AFP) concentrations (OR = 1.69, 95% CI: 1.30–2.20; $P < 0.0001$) and G3 with microvascular invasion (OR = 4.91, 95% CI: 1.06–24.8; $P = 0.047$).

The European and Singapore cohorts were generally similar relative to associations between transcriptomic groups and clinical features. This lends credence to the G1–G6 transcriptomic classifications being applicable regardless of the ethnic origin of HCC patients. The G3 group was associated with microvascular invasion and holds potential for investigation into the underlying mechanisms and selection for therapeutic clinical trials.

Abbreviations: AFP = alpha-fetoprotein, HBV = hepatitis B virus, HCC = hepatocellular carcinoma, HCV = hepatitis C virus, INSERM = Institut National de la Santé et de la Recherche Médicale, OS = overall survival, RT-PCR = reverse transcription polymerase chain reaction.

Keywords: G1–G6, genomic, hepatocellular carcinoma (HCC), INSERM, microvascular invasion, transcriptomic classification

1. Introduction

1.1. Background

Hepatocellular carcinoma (HCC) is the most predominant type of primary liver cancer and the third leading cause of cancer-related mortality worldwide.[1–5] There are multiple etiologies of HCC, the most important being chronic viral hepatitis B and C which account for 80% to 90% of all HCC worldwide.[3,6,7] The prevalence and etiology of HCC vary considerably by geographic location with East Asia ranking

Editor: Abderrahim Oussalah.

Funding: This work was supported by the Ligue Contre le Cancer (Equipe Labellisée) and INCa (Institut National du Cancer).

Authors' declaration: All authors included in this paper have made substantial contribution to the manuscript in accordance with the Annals of Surgery's guideline. All authors have given final approval of the version to be published.

The authors have no conflicts of interest to disclose.

Supplemental Digital Content is available for this article.

[a] Centre for Quantitative Medicine, Duke-NUS Graduate Medical School, Singapore, Singapore, [b] INSERM, UMR-1162, Génomique Fonctionnelle des Tumeurs Solides, IUH, [c] Université Paris Descartes, Labex Immuno-oncology, Sorbonne Paris Cité, Faculté de Médecine, Paris, France, [d] Department of Pathology, Singapore General Hospital, Singapore, [e] Department of Surgical Oncology, National Cancer Centre, [f] Office of Clinical Sciences, Duke-NUS Medical School, Singapore, [g] Department of HPB and Transplant Surgery, Singapore General Hospital, Singapore, Singapore.

[*] Correspondence: Pierce K.H. Chow, Office of Clinical Sciences, Duke-NUS Graduate Medical School, Academia, Level 6, 20 College Road, Singapore 169856, Singapore (e-mail: pierce.chow@duke-nus.edu.sg).

Medicine (2016) 95:47(e5263)

Received: 20 May 2015 / Received in final form: 16 September 2016 / Accepted: 30 September 2016

http://dx.doi.org/10.1097/MD.0000000000005263

among the regions of highest prevalence owing to the high incidence of hepatitis B virus (HBV).[8,9] This is in contrast to hepatitis C virus (HCV) and high alcohol intake which are the primary etiological factors in the West.[10,11]

Surgical resection, radiofrequency ablation, and liver transplantation in selected cases are the main modalities of curative treatment for HCC. Nevertheless, these are applicable in fewer than 30% of cases and outcomes are impaired by high recurrence rates (up to 70%) and tumor-related deaths (30–50% at 5 years).[12,13] Microvascular invasion is specifically associated with poorer overall survival (OS) and shorter time to tumor recurrence.[14,15] In more advanced HCC, locoregional ablative therapy such as transarterial chemoembolization and radioembolization are useful for prolonging survival in an additional 20% of patients.[16] The only proven systemic therapeutic agent is sorafenib for which efficacy is limited and the mechanism of action remains unclear.[17–19]

HCC is heterogeneous at the molecular level,[20,21] which makes prognostication based on anatomical staging difficult and the selection of systemic therapeutic agents challenging. Although the complete etiological mechanisms of HCC molecular carcinogenesis are not fully understood,[22,23] various molecular alterations have been proposed as playing significant roles in HCC tumorigenesis—these include chromosomal aberrations, mutations in TP53, and aberrations in the Wnt, TGFβ, and Ras signaling pathways.[24–28] Studies employing genome-scale analysis of gene expression suggest that HCC is a heterogeneous disease represented by several different subtypes of liver cancer defined by distinct gene expression profiles implicating different molecular mechanisms.[24,29] Conceptually, distinct gene expression profiles and their biological pathways may be associated with particular clinical features, response to therapy and natural history. Genomic markers may thus provide insight into the molecular pathways involved, predict treatment response to targeted therapies and potentially improve prognostication.

1.2. The INSERM G1–G6 transcriptomic classification

In a study on patients from a European population, Boyault et al analyzed 57 HCC tissue samples using Affymetrix HG-U133A GeneChip arrays and identified 6 groups (labeled G1–G6) based on unsupervised hierarchical clustering of transcriptomic profiles using a 6712 probe set.[30] Moreover, tumors could be classified into the 6 groups, G1–G6, using a minimal subset of 16 genes. Validation of the associations identified in the Affymetrix analysis was performed on a second sample of 63 independent tumors using quantitative reverse transcription polymerase chain reaction (RT-PCR).

In the G1–G6 transcriptomic classification scheme, groups G1–G3 were generally characterized by chromosomal instability and association with the mitotic cell cycle. They found that G1 and G2 HCC tumors were associated with HBV (low and high copy, respectively) and were very distinct from other HCCs. G1 and G2 were both linked to protein kinase B (AKT) pathway activation—G1 through IGF2 overexpression and G2 through *PIK3CA* mutations, and developmental and imprinting genes. G1 included HBV-related tumors from younger patients relative to other HBV HCCs, frequent *AXIN1* mutations, absence of *TP53* mutation, and overexpression of genes normally controlled by parental imprinting. In contrast, G3 subgroup HCC tumors were generally characterized by mutation of TP53 but without HBV infection. G3 was associated with overexpression of genes encoding proteins implicated in nucleus import/export, and overexpression of genes controlling the cell cycle and cell cycle checkpoints. G3 tumors were also associated with the worst prognosis.

Groups G4–G6 were generally characterized by chromosomal stability. G4 was a heterogeneous subgroup of tumors including *TCF1*-mutated hepatocellular adenomas and carcinomas. G5 and G6 were strongly related to β-catenin mutations that lead to Wnt pathway activation.[31] G6 tumors were characterized by satellite nodules, higher activation of the Wnt pathway, and E-cadherin under expression.

A subset of the clinical features listed by Boyault et al appears to be relevant to the Southeast Asian population. We hypothesized that the G1–G6 transcriptomic classification and associations with clinical, pathological, and biological tumor features could apply to Southeast Asian HCC patients and might potentially be useful for prognostication of response to therapy.

2. Methods

2.1. G1–G6 group classification

HCC tumor specimens of patients surgically resected by a single surgeon at the Singapore General Hospital (SGH) and collected between May 2001 and October 2012 were snap frozen within liquid nitrogen, and stored in the SingHealth Tissue Repository (STR) at −80°C. All tissues were collected with patient consent and the study was approved by the Institutional Review Board (CIRB Ref 2012/387/B). Frozen HCC tissues were retrieved from the STR and sent to the Institut National de la Santé et de la Recherche Médicale (INSERM) laboratory for gene expression analysis. Patients were grouped into G1–G6 transcriptomic categories according to the 16-gene predictor using quantitative RT-PCR as previously described.[30] All the transcriptomic analyses were performed blindly from clinical and pathological data. Clinical and pathological features of patients and tumors were obtained from an established database.[14,32] Satellite tumors were defined as nodules located less than 2 cm from the primary tumor.

2.2. Statistical analysis

All statistical analyses were performed using SAS V9.3 (SAS, Inc., Cary, NC). Statistical significance was set at $P \leq 0.05$.

Standard methods were used in the statistical analysis. Patients were classified into transcriptomic groups according to the INSERM tissue analysis. Boyault et al used Fisher exact test to investigate association of G1–G6 groups and patient clinical features on 2 independent patient cohorts, an initial cohort using the Affymetrix GeneChip™ in which the 6 transcriptomic groups were identified and defined ("Initial" cohort), and an independent follow-up validation cohort using RT-PCR ("Validation" cohort).[30] Our analysis was consistent with that of Boyault et al in which individual transcriptomic classifications were dichotomized as G1 versus non-G1, G2 versus non-G2, etc. and then afterward cross-tabulated with dichotomized Singapore clinical and demographic variables. Fisher exact test for association was applied to the resulting 2×2 tables. Not all variables analyzed by Boyault et al were available for the Singapore cohort.

Variables defined and analyzed by Boyault et al which were also available for the Singapore cohort were serum alpha-fetoprotein (AFP) >100 IU/mL, gender, HBV status, age, and presence/absence of satellite nodules. Additional clinical–pathological variables available for all patients from Singapore

were tumor burden as defined by the Milan criteria (single HCC ≤5 m, or ≤3 lesions each ≤3 cm, no macrovascular invasion, no distant metastasis) and the presence or absence or microvascular invasion. Test results for the European and Singapore analyses were compared for agreement based on *P* values and significance level.

Selected demographic and clinical baseline variables reflective of biological tumor parameters were investigated for as potential transcriptomic group patient classifiers using logistic regression. Model stability and improved precision for parameter estimation was achieved by pooling patients from selected G1–G6 groups to create 3 pooled classes. Pooling rationale was based on shared inherent genetic and biological pathway features described previously: G12 was created from G1 and G2 while retaining G3 as a separate class and G456, which served as the reference class, was created from G4, G5, and G6. Univariate and multivariate generalized (polytomous) logistic regression analysis was performed to identify associations of G12, G3, and G456 with baseline variables reflective of inherent biological tumor features. The idea is that evidence of association can link transcriptomic classes with clinical variables reflecting biological tumor parameters and tumorigenic pathways, thus suggesting treatment strategies—as opposed to a focus on survival prognosis.

OS and disease-free survival (DFS) for G12, G3, and G456 was estimated using the Kaplan–Meier method and compared among the 3 pooled classes using the log-rank test.

3. Results

3.1. Tissue sample disposition for G1–G6 group classification

A total of 113 Singapore HCC tumor samples were analyzed at the INSERM Research Laboratory for HCC transcriptomic classification. Eighty-two samples with sufficient tissue quantity and RNA of good quality were analyzed by quantitative RT-PCR. Data from these samples were used in the statistical analysis. The 82 samples corresponded to 82 patients characterized by HBV infection in 36 (43.9%), HCV in 22 (26.8%), and both HBV and HCV in 1 (1.22%). Forty-five (55%) patients had tumor burden beyond the Milan criteria and 51 (62%) had AFP levels >200 IU/mL. Microvascular invasion was present in 43.2% of the Singapore cohort. Details of the other major clinical and tumor features are found in Table 1.

Frequency distributions for the G1–G6 classifications were compared between Singapore and European samples. Singapore frequency counts (%) were G1: 11 (13.4), G2: 20 (24.4), G3: 13 (15.9), G4: 20 (24.4), G5: 12 (14.6), and G6: 6 (7.3) (n = 82). Corresponding counts for European patients were G1: 11 (9.2), G2: 17 (14.2), G3: 15 (12.5), G4: 41 (34.2), G5: 24 (20.0), and G6: 12 (10.0) (n = 120). Overall, the 2 distributions did not differ statistically by Fisher exact test ($P = 0.248$). However, 53.7% of Singapore tissue samples were classified as G1–G3 and 46.3% as G4–G6, whereas in the European data 35.8% were classified as G1–G3 and 64.2% as G4–G6. The difference in the G1–G3: G4–G6 ratio for Singapore versus Europe was statistically significant by Fisher exact test ($P = 0.014$) and reflects HBV+ enrichment in the Singapore G1–G3 groups. The percentage of patients with HBV+ versus HBV− was not significantly different for G123 (56.4%) versus G456 (44.1%) ($P = 0.352$). Singapore patient characteristics and HCC features are presented in Table 1 according G1–G6 transcriptomic classification.

3.2. Association of G1–G6 transcriptomic groups with HCC clinical variables: Singapore vs Europe

General agreement was exhibited among the European and Singapore cohorts relative to associations between transcriptomic groups and clinical features.

The association between serum AFP >100 IU/mL and G1 was statistically significant in the Singapore cohort ($P = 0.0006$) and in both European cohorts (Initial, $P = 0.01$; Validation, $P = 0.006$). Significance levels in the European samples supporting association between female gender and G1 approached significance for the Initial cohort ($P = 0.06$) and achieved significance in the Validation cohort ($P = 0.05$). Similarly, for the Singapore samples association between G1 and female gender approached statistical significance ($P = 0.068$). Results assessing association between age <60 and pooled G1 and G2 data were consistent among all 3 cohorts (European: Initial, $P = 0.04$; Validation, $P = 0.09$; Singapore, $P = 0.073$), although $P \leq 0.05$ was not achieved in any cohort. Evidence for association between satellite nodules and G6 was mixed for the European samples (Initial, $P = 0.01$; Validation, $P = 0.30$) but consistent with the nonsignificant Singapore outcome ($P = 0.319$). HBV positivity was the only outcome variable exhibiting inconsistent results between the 3 cohorts (European: Initial, $P = 0.05$; Validation, $P = 0.07$; Singapore, $P = 0.79$) (Table 2).

In an assessment of association between the G1–G6 classification and presence of activating CTNNB1 mutations (coding for β-catenin) we performed quantitative RT-PCR of 2 target genes of the Wnt/β-catenin pathway, GLUL and LGR5. These genes are classically overexpressed when the Wnt/β-catenin pathway is activated due to CTNNB1 mutations and could be used as surrogate markers. We showed that GLUL and LGR5 were significantly overexpressed in the G5 and G6 subgroup (Fig. 1). These results confirmed the association between activation of the Wnt/β-catenin pathway and the G5–G6 subgroup in the Singapore cohort.

3.3. Association of clinical variables and transcriptomic class in the Singapore cohort

In univariate generalized logistic regression analysis on the pooled transcriptomic classes G12, G3, and G456, baseline variables exhibiting statistical significance at $P \leq 0.20$ were ln (AFP), age, microvascular invasion (Y/N), tumor grade (1 and 2/3 and 4), ethnicity (Chinese/non-Chinese), gender, and HBV status (+/−). Multivariate analysis (n = 67 owing to incomplete patient profiles) on these 7 variables identified ln(AFP), age, and microvascular invasion as statistically significant predictors of pooled class membership (Table 3). Multivariate analysis on a parsimonious model incorporating only ln(AFP), age, and microvascular invasion, effectively increasing the sample size to n = 76 owing to fewer incomplete profiles, showed statistical significance for ln(AFP) ($P = 0.0002$), age ($P = 0.015$), and microvascular invasion ($P = 0.018$) with improved precision in odds ratios estimates (Table 4). The odds ratio estimate (95% CI) for ln(AFP) was 1.69 (1.30, 2.20) indicating a 69% increase in odds of G12 membership (relative to G456) per unit increase in ln (AFP). Advanced age was associated with reduced odds of belonging to G12 and G3 (relative to G456) with respective odds ratios (95% CI) of 0.92 (0.87, 0.98) and 0.91 (0.85, 0.98). Hence each additional year of age reduced the odds of belonging to G12 by 7% and to G3 by 8%, with increased odds of belonging to G456. Odds of patients with microvascular invasion were almost

Table 1

Singapore patient characteristics and HCC features by G1–G6 transcriptomic classification.

	Transcriptomic group						
Clinical/HCC variable	**G1 (n = 11)**	**G2 (n = 20)**	**G3 (n = 13)**	**G4 (n = 20)**	**G5 (n = 12)**	**G6 (n = 6)**	**Total (n = 82)**
Gender, n (%)							
Male	7 (10.1)	16 (23.2)	12 (17.4)	19 (27.5)	11 (15.9)	4 (5.8)	69 (54.9)
Female	4 (30.8)	4 (30.8)	1 (7.7)	1 (7.7)	1 (7.7)	2 (15.4)	13 (45.1)
Ethnicity, n (%)							
Chinese	2 (5.7)	7 (20.0)	6 (17.1)	10 (28.6)	6 (17.1)	4 (11.4)	35 (42.7)
Non-Chinese	9 (19.2)	13 (27.7)	7 (14.9)	10 (21.3)	6 (12.8)	2 (4.3)	47 (57.3)
Age, y							
Mean ± SD	53.3 ± 21.8	59.0 ± 8.8	54.7 ± 10.3	63.2 ± 12.2	63.7 ± 8.0	67.5 ± 5.5	59.9 ± 12.6
Age category, n (%)							
≤60 y	7 (17.2)	13 (31.7)	9 (22.0)	7 (17.1)	5 (12.2)	0 (0.0)	41 (50.0)
>60 y	4 (9.8)	7 (17.1)	4 (9.8)	13 (31.7)	7 (17.1)	6 (14.6)	41 (50.0)
Milan criteria, n (%)							
Within	7 (18.9)	6 (16.2)	4 (10.8)	12 (32.4)	5 (13.5)	3 (8.1)	37 (45.1)
Exceeds	4 (8.9)	14 (31.1)	9 (20.0)	8 (17.8)	7 (15.6)	3 (6.7)	45 (54.9)
Microvascular invasion, n (%)							
No	7 (15.2)	13 (28.3)	3 (6.5)	12 (26.1)	8 (17.4)	3 (6.5)	46 (56.8)
Yes	4 (11.4)	7 (20.0)	10 (28.6)	7 (20.0)	4 (11.4)	3 (8.6)	35 (43.2)
Child–Pugh status, n (%)							
A	9 (12.0)	19 (25.3)	11 (14.7)	18 (24.0)	12 (16.0)	6 (8.0)	75 (91.5)
B	2 (28.6)	1 (14.3)	2 (28.6)	2 (28.6)	0	0	7 (8.5)
Tumor size group, n (%)							
≤5 cm	6 (13.6)	9 (20.5)	5 (11.4)	15 (34.0)	5 (11.4)	4 (9.0)	44 (54.7)
>5 cm	5 (13.2)	11 (29.0)	8 (21.1)	5 (13.2)	7 (18.4)	2 (5.3)	38 (46.3)
HBV status, n (%)							
Negative	5 (13.9)	9 (12.3)	3 (8.3)	12 (33.3)	3 (8.3)	4 (11.1)	36 (49.3)
Positive	5 (13.5)	8 (21.6)	9 (24.3)	6 (16.2)	7 (18.9)	2 (5.4)	37 (50.7)
Hepatitis Status, n (%)							
None	4 (22.2)	2 (11.1)	2 (11.1)	7 (38.9)	1 (5.56)	2 (11.1)	18 (22.0)
HBV	5 (13.9)	8 (22.2)	8 (22.2)	6 (16.7)	7 (19.4)	2 (5.6)	36 (43.9)
HCV	1 (4.6)	8 (36.4)	2 (9.1)	7 (31.8)	2 (9.1)	2 (9.1)	22 (26.8)
HBV + HCV	0	0	1 (100)	0	0	0	1 (1.22)
Unconfirmed	1 (20.0)	2 (40.0)	0	0	2 (40.0)	0	5 (6.10)
Serum AFP (IU/mL), n (%)							
Median (IQR)	3230 (599, 13,739)	627 (34.3, 8366)	27 (8.9, 74.3)	16.65 (7.5, 82.5)	19.4 (2.6, 30.1)	20.6 (4.1, 837)	37.0 (8.9, 825)
Serum category, n (%)							
≤200 IU/mL	2 (3.9)	9 (11.8)	11 (21.6)	16 (31.4)	9 (17.7)	4 (7.8)	51 (67.1)
>200 IU/mL	8 (32.0)	10 (40.0)	2 (8.0)	2 (8.0)	1 (4.0)	2 (8.0)	25 (32.9)
Max. tumor size (cm)							
Mean ± SD	5.08 ± 2.47	5.76 ± 3.37	7.67 ± 6.03	5.06 ± 3.25	7.61 ± 5.70	7.47 ± 7.58	6.19 ± 4.53
Median (IQR)	4 (3.2, 6)	5.5 (3.2, 6.75)	6 (1.5, 23)	4 (4, 9.5)	6 (4.5, 9.9)	3.5 (2.6, 12)	4.65 (3.1, 7)
Tumor number, n (%)							
Solitary	9 (14.8)	14 (17.1)	10 (12.2)	15 (18.3)	9 (11.0)	4 (4.9)	61 (74.4)
Multiple	2 (9.5)	6 (28.6)	3 (14.3)	5 (23.8)	3 (14.3)	2 (9.5)	21 (25.6)
Pathological grade, n (%)							
Grade 1, 2	4 (9.5)	10 (23.8)	4 (9.5)	15 (35.7)	7 (16.7)	2 (4.8)	42 (51.9)
Grade 3, 4	7 (18.0)	10 (25.6)	9 (23.1)	4 (10.3)	5 (12.8)	4 (10.3)	39 (48.2)
Extrahepatic invasion, n (%)							
No	11 (13.6)	20 (24.7)	12 (14.8)	20 (24.7)	12 (14.8)	6 (7.4)	81 (98.8)
Yes	0	0	1 (100)	0	0	0	1 (1.2)
Microscopic resection margin, n (%)							
R_0	11 (15.3)	19 (26.4)	11 (15.3)	16 (22.2)	10 (13.9)	5 (6.9)	72 (87.8)
R_1	0	1 (10.0)	2 (20.0)	4 (40.0)	2 (20.0)	1 (10.0)	10 (12.2)
Satellite tumors, n (%)							
No	10 (15.2)	16 (24.2)	9 (13.6)	17 (25.8)	10 (15.2)	4 (6.1)	66 (81.5)
Yes	1 (6.7)	4 (26.7)	4 (26.7)	2 (13.3)	2 (13.3)	2 (13.3)	15 (18.5)
Cirrhosis, n (%)							
No	6 (17.6)	5 (14.7)	7 (20.6)	10 (29.4)	3 (8.8)	3 (8.8)	34 (42.0)
Yes	5 (10.6)	15 (31.9)	6 (12.8)	10 (21.3)	8 (17.0)	3 (6.4)	47 (58.0)

HBV = hepatitis B, HCV = hepatitis C, IQR = interquartile range, R_0 = negative margin, R_1 = positive margin, SD = standard deviation.

5 times greater for belonging to G3 than patients with no microvascular invasion, with odds ratio (95% CI) of 4.91 (1.06, 24.8) (Table 4).

Odds ratios on the continuous variables are risk multipliers rather than measures of absolute risk, hence curves from the fitted regression model showing estimated probability of class membership in G12, G3, and G456 as a function of ln(AFP) and age is informative in an assessment of these variables (Fig. 2A and B). Figure 2A and B shows that the probability curves for G12 and G456 are essentially reflections of one another. Figure 2A shows low probability of G12 for low levels of AFP with a dramatic rise as AFP levels increase. Predicted probability curves for G12 as a function of AFP remain relatively unaffected by presence or absence of microvascular invasion. In the absence of microvascular invasion, probability of G3 remains consistently low. In the presence of microvascular invasion and younger age, the probability of G3 is relatively high for low levels of AFP and drops off dramatically with increasing levels of AFP; however, with increasing age, the probability of G3 is suppressed at all levels of AFP. Figure 2B shows relatively low probability of G3

Table 2

European vs Singapore HCC populations per Boyault et al: associations between transcriptomic groups and clinical variables.

Variable	Transcriptomic groupings	European cohorts[‡]		Singapore[§]
		Identification (Affymetrix, n = 57)	Validation (RT-PCR, n = 63)	(n = 82)
Serum AFP > 100 IU/mL	G1	**0.01**[*]	**0.006**[*]	**0.0006**[*]
Female	G1	0.06[†]	**0.05**[*]	0.068[†]
HBV+	G2	**0.05**[*]	0.07[†]	0.790
Age < 60	G1 and G2	**0.04**[*]	0.09[†]	0.073[†]
Satellite nodules	G6	$<\mathbf{10}^{-2}$[*]	0.30	0.319

AFP = alpha-fetoprotein, HBV = hepatitis B, HCC = hepatocellular carcinoma, RT-PCR = reverse transcription polymerase chain reaction.
Bold values signifies statistically significant at $P \leq 0.05$.
[‡] Extracted from Table 1, Boyault et al; *P* values from Fisher exact test.
[§] Fisher exact test: [*] $P \leq 0.05$; [†] $P \leq 0.10$.

when microvascular invasion is absent with higher probability at younger ages. When microvascular invasion is present there is a pronounced increase in the probability of G3 at younger ages with an accompanying, relatively precipitous, drop off with increasing AFP levels, and a consistent lowering of the curve with increasing age. In the presence of microvascular invasion, the probability of G12 remains suppressed at the lower AFP levels with a modest increase with rising AFP levels.

3.4. Patient survival related to pooled transcriptomic classes

OS curves for the 3 classes did not differ statistically ($P = 0.784$). Estimated 12-month OS was G12: 83%, G3: 90%, and G456: 86%; 24-month OS was G12: 78%, G3: 75%, G456: 75%. DFS did not differ significantly among the 3 classes ($P = 0.307$). Estimated 6-month DFS was G12: 78%, G3: 78%, G456, 100%; 12-month DFS was G12: 70%, G3: 65%, G456: 68%; and 24-month DFS was G12: 56%, G3: 26%, G456: 38%.

4. Discussion

This is the first validation study of the G1–G6 transcriptomic classifications of Boyault et al in a Southeast Asian HCC patient population. Our results were generally consistent with those of Boyault et al and lend credence to the G1–G6 transcriptomic classifications as being applicable regardless of geographic origin of HCC patients. G1 was strongly associated with higher serum AFP in both cohorts. This is not surprising because in Boyault et al,[30]Fig. 1B, AFP was shown to be one of the 16 classifiers with strong weight on G1 ($u = -12.36$). Serum AFP would be expected to be significant because the RNA expression of AFP is one of the classifiers. Female gender was weakly associated with G1 in both cohorts; G1 and G2 were weakly associated with younger age in both; HBV+ infection was associated with G2 in Europe but not in Singapore; and satellite nodules were associated with G6 in Europe but no satellite nodules were found in Singapore. Patients with hemochromatosis or of African origin are uncommon in Southeast Asia, and we found no clinical features in the Southeast Asian population associated with G4, G5, or G6 subgroups.

The relative frequencies among G1–G6 differed somewhat between Singapore (S) and European (E) cohorts with a higher percentage of Singapore patients in the G1–G3 classifications (46%) compared to Europe (36%). The greatest disparities were on the order of 10 percentage points and occurred in G2 (S: 24.4; E: 14.2) and G4 (S: 24.4; E: 34.2). In a previous work,[30] it was found that G1–G3 subgroups were enriched in HBV-related HCC, which could explain the differences of distribution observed in the present study. HBV is the leading etiology of HCC in Singapore as well as other Asia-Pacific countries with a prevalence of approximately 75% to 80%,[33] whereas HCV and alcohol are the leading ones in European and Western populations. The relative proportions of patients with HBV in the Singapore and European cohorts were 43.9% and 30%. Although not statistically significant, Boyault et al found better survival for subgroups G4–G6 than subgroups G1–G3.

In an analysis of pooled classes G12, G3, and G456, our data showed serum AFP as a significant predictor of the G12 pooled transcriptomic class and may reflect biological processes

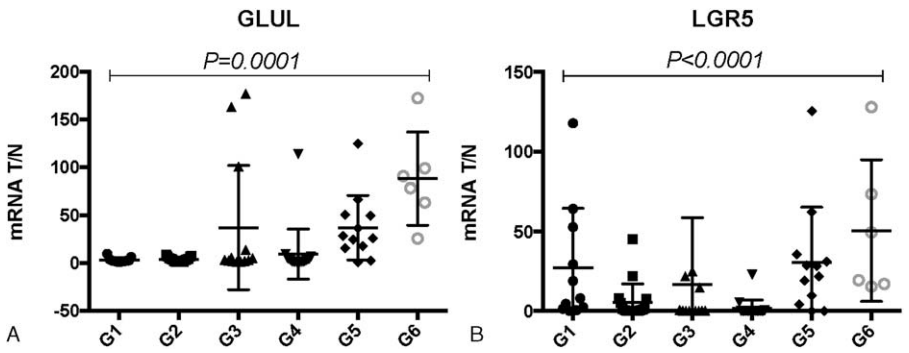

Figure 1. Expression of the genes GLUL and LGRF among the G1–G6 groups.

Table 3

Summary of univariate and multivariate polytomous logistic regression analyses on pooled classes G12, G3, and G456: baseline clinical variables reflecting tumor biological parameters (n = 67).

		Univariate		Multivariate*		
Variable	**n**	**Omnibus *P*†**	**Odds ratio‡ (95% CI)**	**Omnibus p-value**	**Adj. rdds ratio§ (95% CI)**	**Class *P***
ln (AFP) (AFP units), IU/mL	76	**0.0002**	G12: 1.59 (1.27, 2.01)	**0.001**	G12: 1.84 (1.31, 2.57)	**0.0004**
			G3: 1.11 (0.84, 1.46)		G3: 1.08 (0.75, 1.57)	0.673
Age, y	82	**0.026**	G12: 0.95 (0.91, 0.99)	**0.014**	G12: 0.89 (0.83, 0.97)	**0.004**
			G3: 0.94 (0.89, 0.99)		G3: 0.93 (0.85, 1.01)	0.087
Microvascular invasion, Y/N	81	**0.046**	G12: 1.11 (0.41, 2.98)	**0.025**	G12: 0.48 (0.09, 2.48)	0.379
			G3: 0.18 (0.43, 0.78)		G3: 8.19 (1.22, 55.0)	**0.030**
Tumor grade, 1, 2/3, 4	81	0.075		0.575		
Ethnicity, Chinese/non-Chinese	82	0.145		0.858		
Gender	82	0.174		0.822		
HBV status, Y/N	73	0.200		0.177		
Microscopic resection margins, R_0, R_1	82	0.219				
Milan criteria, within/exceeds	82	0.362				
Satellite tumors, Y/N	81	0.477				
Tumor size, ≤5/>5	82	0.510				
Cirrhosis, Y/N	81	0.523				
Child–Pugh status, A/B	82	0.530				

AFP = alpha-fetoprotein, CI = confidence interval, HBV = hepatitis B.

Bold values signifies statistically significant at $P \leq 0.05$.

* Includes variables significant at $P \leq 0.20$ in the univariate analysis.

† Wald Chi-square testing overall significance of G12 or G3.

‡ G12 = pooled G1 and G2; reference is G456 = pooled G4, G5, and G6.

§ Adjusted for 7 variables included in the multivariate analysis.

underpinning HCC in the pooled G12 transcriptomic group.[34] This is consistent with the current understanding of the clinical behavior of HCC. G1 was also weakly associated with the female gender which could be significant as HCCs are generally more prevalent in males.[3,35]

Our data further suggest that microvascular invasion is strongly associated with G3. The odds of patients with microvascular invasion were almost 5 times greater for belonging to G3 than patients with no microvascular invasion, with odds ratio (95% CI) of 4.91 (1.06, 24.8). In the absence of microvascular invasion, probability of G3 remains consistently low.

Microvascular invasion is an independent clinical feature that is negatively associated with OS as well as a major factor affecting metastasis.[14,15] While G3 was found to overexpress proteins of nuclear pore and cell cycle regulators,[30] the underlying mechanism for its association with microvascular invasion is unknown. As the G3 group is associated with aberrant methylation of CDKN2A and aberrant mutation of TP53, these genes may play a major role in the mechanism of microvascular invasion in HCC. CDKN2A produces the protein (alternate reading frame protein product of the CDKN2A), which is an upstream regulator of TP53. This suggests a relationship between aberrant methylation of CDKN2A and the aberrant mutation of TP53, as well as the mechanism leading to microvascular invasion. Previous studies have also shown a positive correlation between TP53 overexpression and microvascular invasion,[36,37] as well as a correlation between aberrant methylation and poor prognosis in HCC.[38–40] Exploring the roles these genes might have in microvascular invasion might elucidate the pathways leading to microvascular invasion and reveal new therapeutic targets for HCC. The G3 group thus provides an enriched group of patients for further investigation of the underlying mechanism of microvascular invasion and eventually, for therapeutic clinical trials.

Table 4

Multivariate polytomous logistic regression analysis on pooled classes G12, G3, and G456: clinical variables AFP, microvascular invasion, and age (n = 76).

					P*	
Variable	**Class**	**Odds ratio†**	**95% Wald confidence interval**		**Class**	**Omnibus**
ln (AFP) (AFP units), IU/mL	G12	1.69	1.30	2.20	**<0.0001**	**0.0002**
	G3	1.08	0.79	1.47	0.634	
Age, y	G12	0.92	0.87	0.98	**0.010**	**0.018**
	G3	0.91	0.85	0.98	**0.011**	
Microvascular invasion, Y/N	G12	0.41	0.10	1.64	0.206	**0.011**
	G3	4.91	1.06	24.8	**0.047**	

nAFP = alpha-fetoprotein.

Bold values signifies statistically significant at $P \leq 0.05$.

* Wald Chi-square.

† G12 = pooled G1 and G2; reference is G456 = pooled G4, G5, and G6.

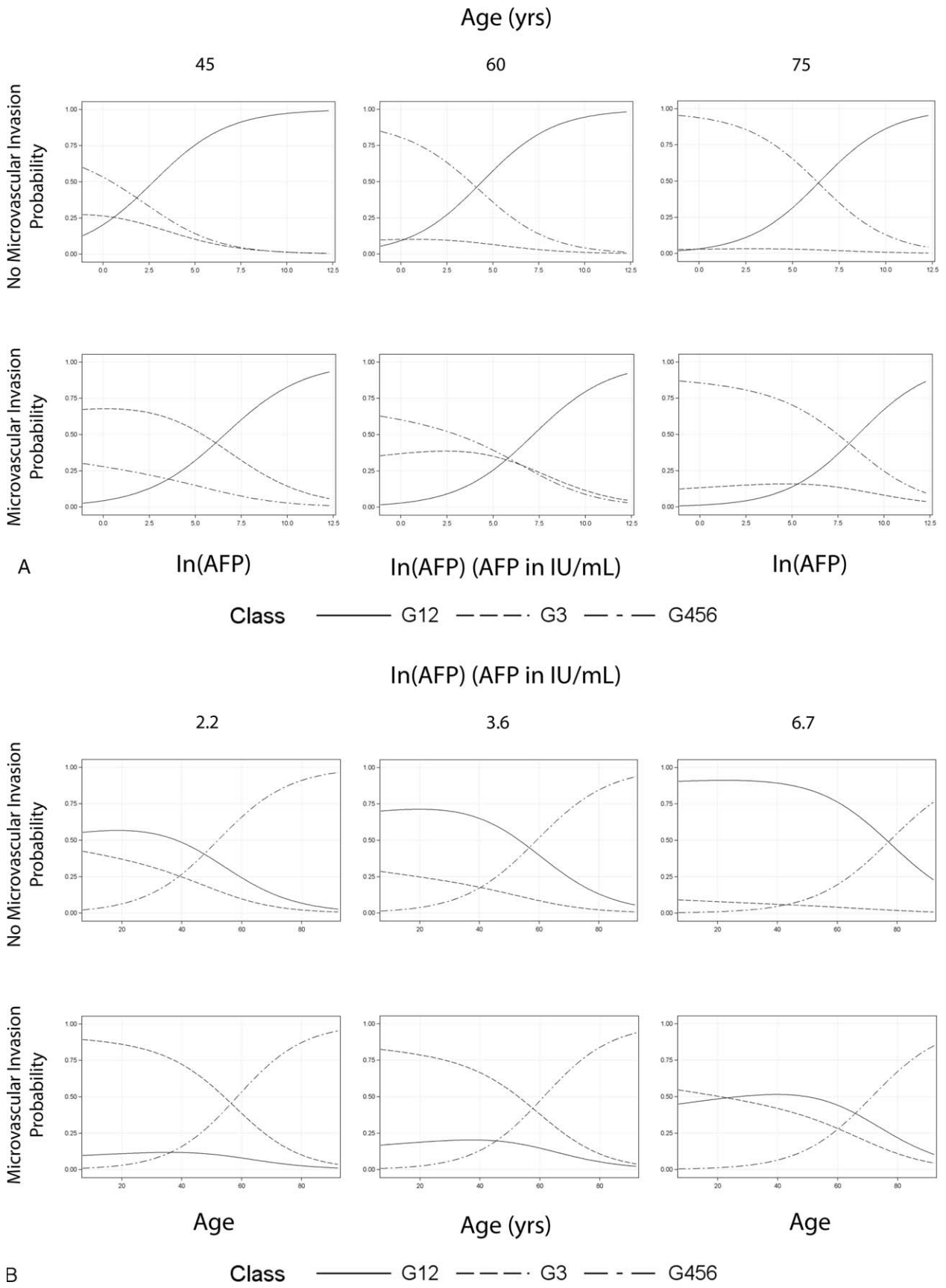

Figure 2. A. Logistic regression model based predicted probabilities of G12, G3 and G456 for selected values of age across the observed range of ln(AFP) for microvascular invasion absent and present. B. Logistic regression model based predicted probabilities of G12, G3 and G456 for selected values of ln(AFP) across the observed range of age for microvascular invasion absent and present.

This classification system allows important biological and pathological parameters (e.g., microvascular invasion), as well as specific molecular pathways (e.g., AKT pathway activation) pertaining to a specific HCC tumor, to be identified through a single biopsy. This will be applicable even in inoperable cases. Patients can thus be potentially be stratified and prioritized in treatment algorithms. The identification of molecular pathways under-pining specific tumors will also potentially allow selection for inclusion in clinical trials.

One limitation of our study was the modest sample sizes in both Singapore and European cohorts. Nevertheless, statistically significant or near-significant consistency of association was demonstrated for comparable variables measured on both populations.

5. Conclusion

General agreement was exhibited among the European and Singapore cohorts relative to associations between transcriptomic groups and clinical features and lends credence to the G1–G6 transcriptomic classifications as applicable regardless of geographic origin of HCC patients.

The G12 pooled class was associated with high AFP levels, while the G3 group was associated with microvascular invasion and holds potential for investigation into the underlying mechanisms and selection for therapeutic clinical trials.

Acknowledgment

The authors would like to thank Singapore Tissue Repository, SingHealth, and National Medical Research Council for their support in this study.

References

[1] El-Serag HB. Hepatocellular carcinoma. N Engl J Med 2011; 365:1118–27.
[2] Ferlay J, Shin HR, Bray F, et al. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 2010;127:2893–917.
[3] Mittal S, El-Serag HB. Epidemiology of HCC: consider the population. J Clin Gastroenterol 2013;47:S2–6.
[4] Parkin DM, Bray F, Ferlay J, et al. Estimating the world cancer burden: Globocan 2000. Int J Cancer 2001;94:153–6.
[5] Shariff MI, Cox IJ, Gomaa AI, et al. Hepatocellular carcinoma: current trends in worldwide epidemiology, risk factors, diagnosis and therapeutics. Expert Rev Gastroenterol Hepatol 2009;3:353–67.
[6] Schutte K, Bornschein J, Malfertheiner P. Hepatocellular carcinoma—epidemiological trends and risk factors. Dig Dis 2009;27:80–92.
[7] Nordenstedt H, White DL, El-Serag HB. The changing pattern of epidemiology in hepatocellular carcinoma. Dig Liver Dis 2010;42: S206–14.
[8] Bosetti C, Turati F, La Vecchia C. Hepatocellular carcinoma epidemiology. Best Pract Res Clin Gastroenterol 2014;28:753–70.
[9] Di Bisceglie AM. Hepatitis B and hepatocellular carcinoma. Hepatology 2009;49:S56–60.
[10] Bosch FX, Ribes J, Diaz M, et al. Primary liver cancer: worldwide incidence and trends. Gastroenterology 2004;127:S5–16.
[11] Fattovich G, Stroffolini T, Zagni I, et al. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. Gastroenterology 2004;127:S35–50.
[12] Forner A, Llovet JM, Bruix J. Hepatocellular carcinoma. Lancet 2012;379:1245–55.
[13] Ishizawa T, Hasegawa K, Aoki T, et al. Neither multiple tumors nor portal hypertension are surgical contraindications for hepatocellular carcinoma. Gastroenterology 2008;134:1908–16.
[14] Lim KC, Chow PK, Allen JC, et al. Microvascular invasion is a better predictor of tumor recurrence and overall survival following surgical resection for hepatocellular carcinoma compared to the Milan criteria. Ann Surg 2011;254:108–13.
[15] Iguchi T, Shirabe K, Aishima S, et al. New pathologic stratification of microvascular invasion in hepatocellular carcinoma: predicting prognosis after living-donor liver transplantation. Transplantation 2014; 99:1236–42.
[16] Bruix J, Sherman M. Management of hepatocellular carcinoma. Hepatology 2005;42:1208–36.
[17] Llovet JM, Ricci S, Mazzaferro V, et al. Sorafenib in advanced hepatocellular carcinoma. N Engl J Med 2008;359:378–90.
[18] Cheng AL, Kang YK, Chen Z, et al. Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. Lancet Oncol 2009;10:25–34.
[19] Shao YY, Shau WY, Chan SY, et al. Treatment efficacy differences of sorafenib for advanced hepatocellular carcinoma: a meta-analysis of randomized clinical trials. Oncology 2015;88:345–52.
[20] Woo HG, Kim SS, Cho H, et al. Profiling of Exome mutations associated with progression of HBV-related hepatocellular carcinoma. PLoS ONE 2014;9:e115152.
[21] Friemel J, Rechsteiner MP, Frick L, et al. Intratumor heterogeneity in hepatocellular carcinoma. Clin Cancer Res 2015;21:1951–61.
[22] Galuppo R, Ramaiah D, Ponte O, et al. Molecular therapies in hepatocellular carcinoma: what can we target? Digestive Diseases and Sciences 2014;59:1688–97.
[23] Weledji EP, Enow Orock G, Ngowe MN, et al. How grim is hepatocellular carcinoma? Ann Med Surg 2014;3:71–6.
[24] Teufel A, Staib F, Kanzler S, et al. Genetics of hepatocellular carcinoma. World J Gastroenterol 2007;13:2271–82.
[25] Buendia MA. Genetics of hepatocellular carcinoma. Semin Cancer Biol 2000;10:185–200.
[26] Nault J-C, Zucman-Rossi J. Genetics of hepatocellular carcinoma: the next generation. J Hepatol 2014;60:224–6.
[27] Thorgeirsson SS, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. Nat Genet 2002;31:339–46.
[28] Shiraha H, Yamamoto K, Namba M. Human hepatocyte carcinogenesis. Int J Oncol 2013;42:1133–8.
[29] Villanueva A, Llovet JM. Targeted therapies for hepatocellular carcinoma. Gastroenterology 2011;140:1410–26.
[30] Boyault S, Rickman DS, de Reynies A, et al. Transcriptome classification of HCC is related to gene alterations and to new therapeutic targets. Hepatology 2007;45:42–52.
[31] Zucman-Rossi J, Benhamouche S, Godard C, et al. Differential effects of inactivated Axin1 and activated beta-catenin mutations in human hepatocellular carcinomas. Oncogene 2007;26:774–80.
[32] Lim KC, Chow PK, Allen JC, et al. Systematic review of outcomes of liver resection for early hepatocellular carcinoma within the Milan criteria. Br J Surg 2012;99:1622–9.
[33] Asia-Pacific Working Party on Prevention of Hepatocellular CarcinomaPrevention of hepatocellular carcinoma in the Asia-Pacific region: consensus statements. J Gastroenterol Hepatol 2010;25:657–63.
[34] Farinati F, Marino D, De Giorgio M, et al. Diagnostic and prognostic role of alpha-fetoprotein in hepatocellular carcinoma: both or neither? Am J Gastroenterol 2006;101:524–32.
[35] Leong TYM, Leong ASY. Epidemiology and carcinogenesis of hepatocellular carcinoma. HPB (Oxford) 2005;7:5–15.
[36] Sung CO, Yoo BC, Koh KC, et al. Prognostic significance of p53 overexpression after hepatic resection of hepatocellular carcinoma. Korean J Gastroenterol 2005;45:425–30.
[37] Park NH, Chung YH, Youn KH, et al. Close correlation of p53 mutation to microvascular invasion in hepatocellular carcinoma. J Clin Gastroenterol 2001;33:397–401.
[38] Dong Y, Wang A. Aberrant DNA methylation in hepatocellular carcinoma tumor suppression (Review). Oncol Lett 2014;8:963–8.
[39] Mah W-C, Lee CGL. DNA methylation: potential biomarker in hepatocellular carcinoma. Biomarker Res 2014;2:5–15.
[40] Csepregi A, Ebert MPA, Röcken C, et al. Promoter methylation of CDKN2A and lack of p16 expression characterize patients with hepatocellular carcinoma. BMC Cancer 2010;10:317.