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Original Article

Significance of incorporation of DNMT1 and HLA-DRα with TNM staging in patients with hepatocellular carcinoma after curative resection

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Abstract: Hepatocellular carcinoma (HCC) is the most common type of hepatic cancer and is particularly a problem in China. Bio-molecular markers have been demonstrated to be of prognostic significance and might help predict tumor behavior. In our study, we aimed to assess the prognostic values of DNA methyltransferase 1 (DNMT1), HLA-DRα, and β-catenin, as well as the combined use of molecular biomarkers, clinicopathological parameters and the TNM staging system to find a method for superior prognostic performance for HCC by analyzing a Chinese HCC cohort. We revealed the significant prognostic roles of DNMT1 (OR: 2.570; 95% CI: 1.401-4.715; P = 0.002) and HLA-DRα (0.350; 0.189-0.616; 0.001), and further developed an estimation formula to predict prognosis in HCC patients after curative resection, based on TNM staging, operative blood loss, abnormal total bilirubin, DNMT1 and HLA-DRα. The receiver operating characteristic curve analysis showed that prediction from the multivariate logistic regression had an area of 0.847 and performed better than the conventional TNM staging system, as well as other current HCC staging systems. Our study demonstrated the prognostic values of DNMT1 and HLA-DRα in HCC patients after curative resection. Additionally, we developed a prognostic estimation formula featured better stratification ability than the conventional TNM staging and provided a practicable stratification method for HCC patients after curative resection.

Keywords: Hepatocellular carcinoma, tumor-node-metastasis staging, HLA-DRα, DNA methyltransferase 1, prognosis

Introduction

Hepatocellular carcinoma (HCC) is the one of the most frequently diagnosed cancers and one of the leading causes of cancer-related deaths worldwide [1-6]. Over 50% of the deaths were expected to occur in China partly due to the raised prevalence of hepatitis B virus [7-10]. Hepatectomy was considered the treatment of choice and presented the chance for cure for HCC patients, especially for those in the absence of hepatic cirrhosis [11, 12]. Yet, the prognostic outcome is not fully satisfactory. Furthermore, unlike other malignancies, prognostic assessment and long-term outcomes of HCC rely on not only the stage of the tumor but also multiple confounding factors [13]. Given the complexity and importance of prognostic assessment, multiple staging systems have been developed and proposed [14]. Although there is still a lack of broad consensus on the most reliable staging system to use [15, 16], the Tumor-Node-Metastasis (TNM) staging system [17] has been the most prevalent method and acknowledged as authoritative, which was proven to be better than the Cancer of the Liver Italian Program (CLIP) score [18], the Chinese University Prognostic Index (CUPI) [19], and the Okuda staging [20] systems with regard to stratification and prediction of prognosis [21-23]. Despite of its superiority over the other systems, the TNM staging system focuses mainly on clinicopathological characteristics and excludes other potential prognostic factors such as molecular biomarkers of HCC, liver function, or surgery-related factors that definitely would affect post-operative prognosis of HCC.

With the development of translational medicine, findings from basic research largely deepened the understanding of tumor biology. Biomolecular markers from biopsy, serum sample, or postoperative specimen might be able to further predict tumor behavior, thus helping inform the patient and clinician in the aspects of either decision-making process or prognosis-predicting efficacy. Of note, in the past decade, DNA methyltransferase 1 (DNMT1), HLA-DRα, and/or β-catenin have been shown to get implicated in tumorigenesis and their prognostic values have been widely explored in various tumors [24-33]. However, with respect to HCC, the prognostic impacts of these molecular markers have still to be elucidated, especially in Chinese subjects.

In the present study, we aimed to assess the prognostic values of these biomarkers, as well as the combined use of molecular biomarkers, clinicopathological parameters and the TNM staging system to find a method for superior prognostic performance for HCC by analyzing a Chinese cohort with definite pathological diagnosis, relatively consistent treating strategies and complete follow-up data.

Materials and methods

Patients and data collection

The study protocol was approved by the Medical Ethics Committee of the Chinese PLA General Hospital (Beijing, China). Our study enrolled a 10-year duration (from January 1991 to June 2002) patient cohort with pathologically confirmed HCC who underwent radical resection as initial treatment at the Institute of Hepatobiliary Surgery, Chinese PLA General Hospital. To ensure the reliability and verifiability of our analysis, following exclusion criteria were applied to patient selection: *(i)* evidence of distant metastasis based on clinical examination, *(ii)* evidence of residual tumor loci or tumor thrombus in the major veins based on intraoperative ultrasonography, *(iii)* evidence of cancer cells in the surgical margins, *(iv)* evidence of tumor loci within 30 days postoperatively based on computed tomography or ultrasonography, and *(v)* patient lost to follow-up. Patients meeting any of the 5 exclusion criteria were excluded. Additionally, as the present study was primarily designed to identify the prognostic factors for HCC resection, those patients who had severe perioperative complications, died after surgery (within 30 days postoperatively), or died from causes unrelated to HCC were also excluded.

For all the patients enrolled in the study, demographic data and clinicopathological parameters were collected and analyzed by reviewing the medical computerized database of the cohort. To ensure accuracy, data input and assessments of tumor staging (TNM, CLIP, CUPI and Okuda) were carried out separately by two operators.

Pathological analysis

Two experienced pathologists who were blinded to the present study independently performed pathological analysis. If no agreement was obtained between the two pathologists, a third experienced pathologist, who was also blinded to the study, performed further analysis. All archival formalin-fixed, paraffin-embedded sections were carefully reviewed and selected from corresponding hematoxylin and eosin stained sections to identify and mark representative tumorous areas. Tissue microarray and immunohistochemistry studies were performed as previously reported to detect the expressions of molecular markers on HCC tissues [34-37]. Briefly, the tumor tissue samples were embedded in tissue microarray sets. The diameter of the spots was 1.0 mm and the distance between spots was 0.5 mm. The tissue samples were arranged regularly and had even thicknesses, with no folds, migrations, or cracks. Healthy liver tissue samples were used as controls.

The Envision immunohistochemistry assay kit (DAKO, Kyoto, Japan) was used. The primary antibodies were goat anti-DNMT1 polyclonal antibody (sc-10219), rabbit anti-HLA-DRα polyclonal antibody (sc-25614), and anti-β-catenin monoclonal antibody (sc7963), all from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA). Samples stained without primary antibodies were used as negative staining controls.

The immunohistochemistry results were evaluated by the percentage of positively stained neoplastic cells under light microscopy. The percentages of positive staining cancer cells were graded by the following criteria: negative, less than 10% of cancer cells stained; and positive,

Table 1. Identification of prognostic predictors by univariate cox regression model

Variable	Coefficient	Wald	OR (95% CI)	*P* value
Demographics				
Age	0.002	0.049	1.002 (0.988-1.015)	0.824
Gender	0.712	3.854	0.491 (0.241-0.999)	0.050
AFP	0	3.514	1.000 (1.000-1.001)	0.061
HBV infection	0.262	1.961	1.299 (0.901-1.874)	0.161
Hepatic cirrhosis	0.046	5.395	1.501 (1.065-2.114)	0.020
Liver function				
ALT	-0.001	0.947	0.999 (0.996-1.001)	0.330
AST	0.001	0.444	1.001 (0.998-1.004)	0.505
ALP	0.004	21.056	1.004 (1.002-1.006)	<0.001
GGT	0.001	9.573	1.001 (1.001-1.002)	0.002
TBIL	0.736	19.141	2.087 (1.501-2.902)	<0.001
Child-Pugh classification	0.835	26.365	2.305 (1.675-3.172)	<0.001
Clinical factors related to surgery				
Operative blood loss	0.880	27.281	2.410 (1.733-3.353)	<0.001
Operative time	0.594	10.170	1.812 (1.257-2.611)	0.001
PV blockage	-0.106	0.402	0.899 (0.648-1.248)	0.526
Clinicopathological factors				
Edmonson-Steiner grade	0.277	5.640	1.319 (1.050-1.657)	0.018
TNM staging	0.585	33.112	1.796 (1.471-2.192)	<0.001
Tumor size	0.370	5.558	1.448 (1.065-1.968)	0.018
Tumor number	0.256	2.310	1.303 (0.926-1.833)	0.129
Molecular markers				
HLA-DRα	-0.747	12.530	0.474 (0.313-0.716)	<0.01
DNMT1	0.849	20.845	2.337 (1.623-3.364)	<0.01
β-catenin	-4.474	4.467	0.622 (0.401-0.966)	0.035

AFP = alpha-fetoprotein, HBV = Hepatitis B virus, ALT = Alanine transaminase, AST = Aspartate transaminase, ALP = Alkaline phosphatase, GGT = Gamma-glutamyl transpeptidase, TBIL = Total bilirubin, PV = Portal vein, TNM = Tumor-Node-Metastasis, DNMT1 = DNA methyltransferase 1.

more than or equal to 10% of cancer cells stained [38, 39].

Model establishment

Survival outcomes were retracted from the database of the patient cohort. A survival time longer than the median survival time was defined as a "good prognosis"; a survival time shorter than the median survival time was defined as a "poor prognosis". The results from the multivariate logistic regression analysis were used for prognosis prediction. The area under the receiver operating characteristic (ROC) curve (AUC), which served as an effective tool to evaluate clinical diagnostic tests and prognostic models [40], was used to compare this prediction model with traditional staging systems (TNM, CLIP, CUPI and Okuda) in prognostic prediction.

Statistical analysis

All statistical analyses were performed using SPSS 15.0 statistical software (SPSS Inc., Chicago, IL, USA). The Kaplan-Meier method was used to calculate survival rates. Normally and non-normally distributed continuous data were expressed as a mean with standard deviation and a median with range, respectively. The differences between groups were tested with the independent two-sample t-test and the nonparametric Mann-Whitney test. Categorical variables were expressed as a count with percentage. Differences between groups were compared using Fisher's exact test. Univariate and multivariate Cox regression models were performed to develop a prediction model for prognosis. For model selection, variables with a *P* value <0.1 in the univariate logistic regres-

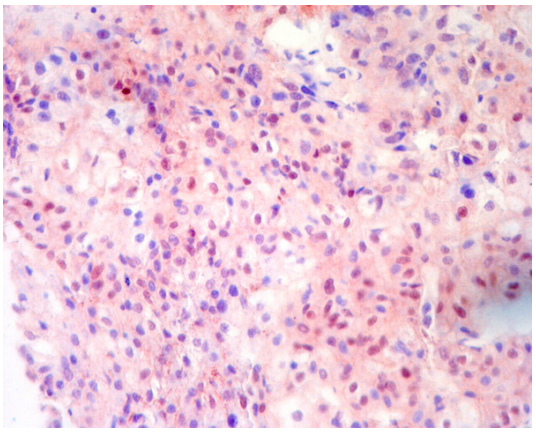

Figure 1. Representative immunohistochemistry results for DNMT1.

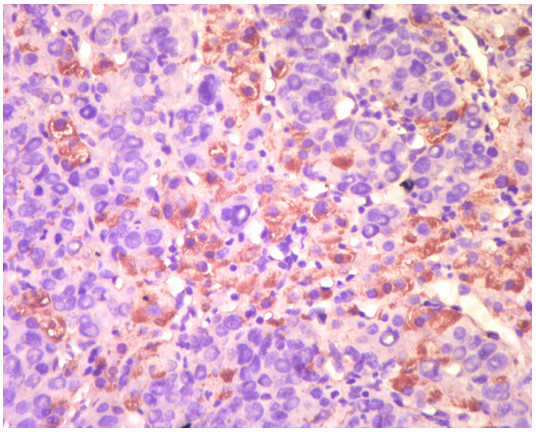

Figure 2. Representative immunohistochemistry results for HLA-DRα.

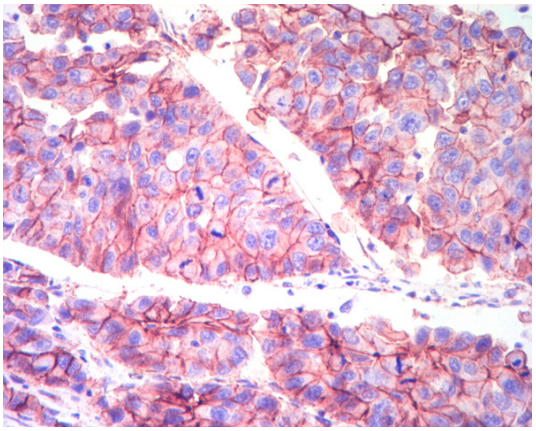

Figure 3. Representative immunohistochemistry results for β-catenin.

sion model were included stepwise in the multivariate logistic regression model using the backward conditional method. Similarly, the AUC was used to compare different methods of predicting HCC prognosis and it was compared by computing z-value that was then used to determine *P* values. A *P* value less than 0.05 was considered statistically significant.

Results

Patients' demographics and survival rates

A total of 234 HCC patients (214 males, 20 females) with a mean age of 48.7 ± 11.4 years were enrolled in the study. The mean follow-up time was 30 ± 26 months. The median survival time was 26.4 months. The 1-year, 2-year, 3-year, and 5-year survival rates were 66.7%, 52.2%, 42.9%, and 29.1%, respectively. Demographic data and clinicopathological characteristics of the patients were presented in **Table 1**.

Immunohistochemical staining of molecular markers

The representative immunohistochemistry results from tissue samples positive for DNMT1, HLA-DR or β-catenin are shown in **Figures 1**-**3**. DNMT1-positive cells had small yellow-brown granules in the nucleus or cytoplasm (**Figure 1**). HLA-DRα-positive cells were yellow-brownish in the cytoplasm or plasma membrane; some lymphocytes in the interstitium were also positive for HLA-DRα (**Figure 2**). β-catenin-positive cells had a yellow-brown color; positive staining was observed mainly in the plasma membrane, but some cells had staining in the cytoplasm and nucleus (**Figure 3**).

Risk factors associated with prognosis

Univariate analysis identified 15 variables with *P* values less than 0.05 as possible independent prognostic factors. These variables were included in a stepwise Cox multivariate regression analysis, and the multivariate analysis identified 5 independent predictors associated with prognosis: TNM stage (stages II and III) (odds ratio (OR): 1.849; 95% confidence interval (CI): 1.293-2.645; P = 0.001), operative blood loss over 1 L (1.987; 1.083-3.643; 0.027), abnormal total bilirubin (TBIL) (2.539; 1.430-4.509; 0.001), and DNMT1 positive staining (2.570; 1.401-4.715; 0.002) were associated with higher risk of poor prognosis after HCC resection, whereas HLA-DRα positive staining (0.350; 0.189-0.616; 0.001) was associated with lower risk (**Table 2**).

Table 2. Identification of prognostic predictors by multivariate cox regression model

Variable	Coefficient	Wald	OR (95% CI)	*P* value
TNM staging	0.615	11.331	1.849 (1.293-2.645)	0.001
Operative blood loss	0.686	4.923	1.987 (1.083-3.643)	0.027
TBIL	0.932	10.115	2.539 (1.430-4.509)	0.001
HLA-DRα	-1.051	13.239	0.350 (0.198-0.616)	<0.001
DNMT1	0.944	9.300	2.570 (1.401-4.715)	0.002

TNM = Tumor-Node-Metastasis, TBIL = Total bilirubin, DNMT1 = DNA methyltransferase 1.

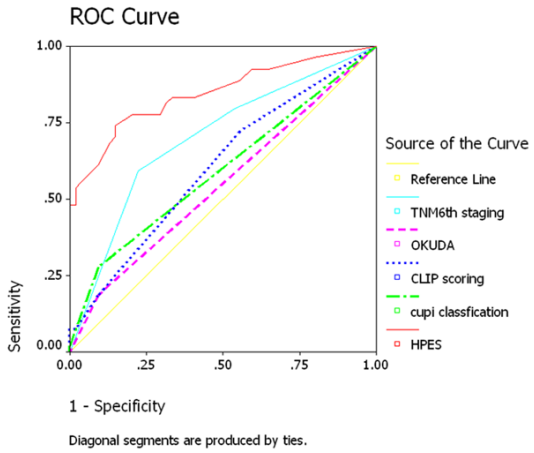

Figure 4. Receiver operating characteristic curves for prediction based on the multivariate logistic regression model and the TNM, CLIP, CUPI, and Okuda systems. *indicates a significant difference in AUC with regards to the predicted probability.

Establishment of the HCC prognostic estimation formula and prediction of survival probabilities

The HCC prognostic estimation formula (HPEF) was developed based on the regression coefficients for the 5 independent prognostic risk factors reported in **Table 2**.

HPEF = 0.686× Operative blood loss (≥1 L: 1; <1 L: 0) + 0.932×TBIL (abnormal: 1; normal: 0) + 0.615×TNM (Stage I: 1; Stage II: 2; Stage III: 3) + 0.944×DNMT1 (positive: 1; negative: 0) - 1.051×HLADRα (positive: 1; negative: 0).

To obtain the probability of survival after hepatectomy, S(t) can be computed using the following equation: $S(t) = S_0(t)^{\exp(HPEF-HPEF0)}$, where HPEF0 is the risk score of the average patient in the series, namely, 1.329.

Performance of the multivariate logistic regression, TNM, CLIP, CUPI, and Okuda systems

We then used ROC analysis to compare the performance of predictions from the multivariate logistic regression with the conventional TNM staging system, the CLIP score, the CUPI staging system, and the Okuda staging system. The prediction from the multivariate logistic regression had the largest AUC (0.847), which was better than prognosis based on TNM (0.700), CLIP (0.604), CUPI (0.593), or Okuda (0.546) ($z = 0.794$, $P<0.001$; **Figure 4**).

Discussion

In this study, we revealed significant prognostic values of DNMT1 (OR: 2.570; 95% CI: 1.401-4.715; P = 0.002) and HLA-DRα (0.350; 0.189-0.616; 0.001), and further developed an estimation formula to predict prognosis in HCC patients after curative resection, based on TNM staging, operative blood loss, TBIL, DNMT1 and HLA-DRα in a Chinese HCC cohort. ROC analysis showed that prediction from the multivariate logistic regression featured the AUC of 0.847 and performed better than the conventional TNM staging system, as well as other current HCC staging systems.

The definite mechanisms involved in our results are beyond the scope of the current study but might be explained by the following considerations.

Our microarray results indicate that DNMT1 expression is strongly and independently associated with poor prognosis. As for DNMT1, it is a DNA methyltransferase widely expressed in somatic cells [41]. In particular, DNMT1 binds to DNA replication sites via a special functional domain to maintain methylation. In addition, DNMT1 can interact with proliferating cell nuclear antigens. Thus, DNMT1 plays an important role in HCC pathogenesis and is a key molecular marker that influences the prognosis of HCC [42]. In reference to HLA-DR, it is a cell surface receptor with α and β chains that functions in tumor antigen presentation [43]. Iizuka

et al. used oligonucleotide microarrays to identify 12 genes related to the early recurrence of HCC after radical resection, one of which was HLA-DRα [44]. The role of HLA-DRα in cancer pathogenesis is incompletely understood, but three possible mechanisms have been proposed in previous reports [43, 45]: *(i)* during early tumor transformation, cancer cells express HLA-DRα, so it is an important surface marker for early cancer development; *(ii)* cancer cells stimulate an inflammatory reaction and then T cells release cytokines, which thereupon induce expression of HLA-DRα; or *(iii)* HLA-DR might be a membrane surface marker at the early stage of tissue differentiation so that when normal cells de-differentiate and become malignant, HLA-DRα is expressed. We found that HLA-DRα expression significantly correlated with good prognosis. This might be because loss of HLA-DRα expression leads to immune escape of cancer cells, resulting in early recurrence and metastasis of HCC [46].

On the other hand, the TNM system is extensively applied in HCC patients for tumor staging after hepatectomy; advanced TNM staging of HCC is widely accepted as being associated with a poor prognosis [47]. Hence, it is expected that TNM would be a significant component of HPEF. Similarly, intra-operative blood loss more than 1 L was associated with poor prognosis. As has been documented in previous studies, operative blood loss may promote tumor spillage and spread during the operation, which could thusly accelerate tumor recurrence [48, 49]. With regard to TBIL, multivariate logistic regression analysis also indicated that elevated preoperative TBIL was independently linked with poor prognosis, which is in concordance with the earlier findings; TBIL, to some degree, provides a simple, objective, and cost-effective method of evaluating liver function in HCC [50]. Patients with elevated TBIL often have obvious liver injury, and liver function has been proven to be a critical factor in the prognosis of patients with HCC [19, 51].

Since its introduction for HCC staging in the year of 1977, TNM has currently been recognized as one of the most generally accepted staging systems. However, TNM did not take some potential prognostic factors, like molecular biomarkers, into account to stage HCC. In the era of "from bench to bedside", certain molecular markers with prognostic values have already been incorporated into some staging or classification systems [52, 53]. As to HCC, recent studies have proposed molecular biomarkers for the prediction of HCC prognosis to overcome the limitations of the traditional staging systems [44, 54, 55]; similarly, certain common clinicopathological parameters have also been used for prognosis prediction in HCC after curative surgery [56]. Of note, one of the successful utilities is the development of the Japan Integrated Staging score [57] which integrated the TNM staging and scores obtained using the Child-Pugh classification; simple and effective as it is, it carried the limitation of rare evaluation in patient population outside Japan [58, 59]. Meanwhile the CLIP score also enrolled biomarkers in its algorithm, while alpha-fetoprotein lone seemed obviously inappropriate. Furthermore, three previous research groups have used gene microarrays to screen for molecular markers in the prognostic prediction of patients with HCC [44, 54, 55]. However, molecular markers alone cannot fully produce a clear prognostic prediction and some of their results were even conflicting due to small sample size, inconsistency of inclusion criteria, possible covariance between molecular markers or between markers and clinical factors, and false positive errors. In addition, Hao and colleagues found a combination of 6 common clinicopathological parameters including tumor size, number of tumor nodules, tumor stage, venous infiltration status, serum alpha-fetoprotein and total albumin levels, that were significantly associated with the overall survival and disease-free survival of HCC patients [56]. However, their model excluded molecular biomarkers and their AUC was only 70%. Comparatively speaking, our study features two strengths: *(i)* The predictions from our multivariate logistic regression model had an AUC of approximately 85%, which illustrates that the predictions from the multivariate logistic regression model performed better than the conventional TNM staging system in predicting prognosis of HCC patients after resection and also performed better than the CLIP, CUPI, and Okuda systems. *(ii)* Our results provide a feasible strategy for the translation of molecular markers and clinical characteristics from bench to bedside and confirm the feasibility of establishing HCC prognosis based on clinical factors, molecular markers and TNM staging. Despite of the improvements aforementioned, multicenter prospective

studies are required to confirm or refine this formula and clarify risk classification.

In conclusion, our study demonstrated the prognostic values of DNMT1 and HLA-DRα in HCC patients after curative resection. Additionally, we developed a prognostic estimation formula featured better stratification ability than the conventional TNM staging and provided a practicable stratification method for HCC patients after curative resection.

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Disclosure of conflict of interest

None.

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