Research Articles

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Diagnostic and prognostic significance of cell death and macrophage activation markers in patients with hepatocellular carcinoma

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Background & Aims: The serum cell death parameters M30 and M65 and the macrophage activation marker sCD163 (soluble CD163) are elevated in patients with acute and chronic liver diseases. However, their diagnostic and prognostic potential in patients with hepatocellular carcinoma (HCC) has not yet been investigated.

Methods: Serum levels of M30, M65, and sCD163 were measured in two cohorts of HCC patients and a cohort of cirrhotic patients. The parameters were compared between patients with and without HCC and the overall survival (OS) times according to M30, M65, and sCD163 were assessed.

Results: M30 and M65 levels were higher in HCC patients than in cirrhotic patients (both p <0.001). M65 was an independent parameter for non-invasive identification of HCC patients by logistic regression analysis and could supplement AFP (alpha-fetoprotein) and abdominal ultrasound in non-invasive detection of HCC patients. High M65 serum levels as well as high sCD163 concentrations were associated with an impaired prognosis in univariate Cox regression analysis. The sCD163 level was associated with OS independently of the CLIP (Cancer of the Liver Italian Program) score, the BCLC (Barcelona Clinic Liver Cancer) stage, and the CRP (C-reactive protein) level in a multivariate Cox regression model.

Conclusions: Serum M65 has the potential as a new diagnostic parameter for HCC and serum sCD163 is a new prognostic parameter in HCC patients.

Keywords: Hepatocellular carcinoma; Cell death; M30; M65; CD163.

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Abbreviations: sCD163, soluble CD163; HCC, hepatocellular carcinoma; OS, overall survival; AFP, alpha-fetoprotein; CLIP, Cancer of the Liver Italian Program; BCLC, Barcelona Clinic Liver Cancer; CRP, C-reactive protein; NASH, non-alcoholic steatohepatitis; CK, cytokeratin; RFA, radiofrequency ablation; LITT, laser interstitial thermal therapy; TACE, transarterial chemoembolisation; MELD, model of end stage liver disease; ROC, receiver operating curve; ELISA, enzyme-linked immunosorbent assay; AUROC, area under the receiver operating curve.

Introduction

Hepatocellular carcinoma (HCC) is an increasing burden in the world with very limited treatment options [1]. The majority of HCC cases develop in chronically inflamed livers due to chronic viral hepatitis, alcohol abuse and, with rapidly increasing incidence, in patients with non-alcoholic steatohepatitis (NASH) [2]. Therefore, treatment and prognosis of patients with HCC are governed by the stage and aggressiveness of the tumor disease and the incidence of complications related to end stage liver disease. Curative treatment options are confined to a small proportion of HCC patients [3]. Therefore, early diagnosis as well as estimation of survival and risk stratification is an important issue for HCC patients.

A commonly used laboratory screening parameter is alpha-fetoprotein (AFP) [4]. However, it has low sensitivity and specificity and its use for HCC surveillance is not recommended anymore as ultrasound imaging has a more favorable efficiency [5]. In former times, it was used for non-invasive diagnosis of HCC in combination with dynamic imaging. In the present guidelines, AFP is completely replaced as diagnostic tool by dynamic imaging due to its low sensitivity as single blood based parameter [5]. However, dynamic imaging is a cost-intensive technique and therefore, new blood-based parameters with higher sensitivity and specificity for HCC diagnosis are desirable.

The stratification of HCC patients is currently performed by several scoring systems, including Cancer of the Liver Italian Program (CLIP) score and Barcelona Clinic Liver Cancer (BCLC) staging scores [6,7].

During HCC progression, hepatic cell death occurs through invasion of the cancer cells into non-tumor tissue and by necrotic and apoptotic death of the tumor cells due to lack of nutrients or cytopathic immune responses. Epithelial cell death can be assessed by measurement of cytokeratin (CK) 18 fragments in the blood of patients. CK 18 based serum cell death biomarkers reflecting apoptosis (M30) and necrosis (M65) are useful for the estimation of disease severity in patients with acute [8,9] and chronic liver disease [10–12].

Kupffer cells, the resident macrophages in the liver, play an important role in hepatic homeostasis, but also in carcinogenesis.

Upon activation by different stimuli including hepatocyte death, they release reactive oxygen species, interleukins and cytokines, which modulate hepatocellular growth, but may also further sustain inflammation and hepatic cell death [13,14]. Soluble CD163 (sCD163) can be regarded as a marker for activated macrophages of which 80% reside in the liver as Kupffer cells [15–18]. sCD163 is indicative for the outcome of patients with acute liver failure [19] and correlates with the severity of portal hypertension in patients with liver cirrhosis [20].

Enhanced cell turnover in association with inflammation of the liver are hallmarks of hepatocarcinogenesis. Alternative cell death markers such as M30 and M65 have the potential to identify and quantify forms of cell death which are not recognized by standard cell death parameters such as aminotransferases. Enhanced cell death may lead to macrophage activation, which in turn may potentiate cell turnover. Recently, sCD163 was shown to reflect macrophage activity in serum [17]. In the present study, we investigated if cell death and macrophage activation markers have the potential to improve diagnosis and prediction of prognosis of patients with HCC. The diagnostic potential of M30, M65, and sCD163 was investigated in a case control study with two cohorts of patients with HCC and patients with liver cirrhosis without HCC. Furthermore, patients with HCC were followed prospectively and the potential of the markers to predict survival was investigated.

Materials and methods

Selection of patients

Between February 2009 and August 2011, patients with confirmed HCC presenting at the Department of Internal Medicine 1 of the Goethe University Hospital and patients presenting between April and December 2010 at the University Hospital Freiburg were consecutively enrolled into the present prospective cohort study. The diagnosis of HCC was made according to the EASL practice guidelines by histopathology or by dynamic imaging with characteristic hypervascularity in the arterial phase and washout in the portal venous phase [5]. All patients gave written informed consent prior to inclusion. The study was performed in accordance with the 1975 Declaration of Helsinki and the REMARK guidelines [21] for prospective biomarker studies. Exclusion criteria were an age below 18, history of cancer other than HCC in the last five years, history of solid organ transplantation and local or systemic treatment for HCC within the last 28 days. Treatment of HCC was carried out according to BCLC stage. In brief, patients with early HCC eligible for liver transplantation were listed at Eurotransplant. HCC patients not eligible for liver transplantation or with intermediate or advanced disease received treatment of HCC with local ablative therapy including radiofrequency ablation (RFA) and laser interstitial thermal therapy (LITT), resection, transarterial chemoembolisation (TACE) or systemic treatment as recommend by the BCLC criteria. Patients with end stage HCC received best supportive care [22]. The diagnostic accuracy of parameters of hepatocyte death and macrophage activation was assessed at the day of study inclusion to diagnose HCC in a cross sectional approach by using age- and gender-matched patients with liver cirrhosis with comparable liver function as a control cohort.

The CLIP score, BCLC stage, MELD (model of end stage liver disease) score and Child Pugh stage were assessed by clinical examination, laboratory parameters and the results of abdominal ultrasound examination, computer tomography or magnetic resonance imagining at the time of inclusion in the study [6,7,23,24].

The control cohort consisted of patients with liver cirrhosis who were treated in our department. The patients participated in a prospective cohort study. Inclusion criteria were liver cirrhosis, proven by histopathological examination of liver biopsy material or explicit morphological criteria of liver cirrhosis in ultrasound, computer tomography or magnetic resonance imaging and an age >18 years. Exclusion criteria were a history of malignant disease within the last five years and former solid organ or bone marrow transplantation. All patients gave their informed consent to participate in the study. The study was approved by the local ethics committee of the Goethe University Hospital.

The validation cohort consisted of patients with HCC who were recruited from July 2011 until July 2012 at the J.W. Goethe University Hospital, Frankfurt, and from December 2010 until September 2011 at the University Hospital Freiburg, Germany. Inclusion and exclusion criteria were the same as in the test cohort.

Blood sampling

Blood was taken from each individual at the day of inclusion into the study. Standard blood parameters for clinical chemistry, hematology, and AFP were analyzed at the central laboratories of the Goethe University Hospital and Freiburg University Hospital. Serum tubes for scientific analyses were centrifuged at 1500*g* for 10 min at 4 °C. An additional centrifugation step at 2000*g* 4 °C was performed to completely remove any remaining cells. Serum samples were aliquoted and stored at −80 °C until further use.

Detection of apoptotic and necrotic neoepitope levels and macrophage activation with M30, M65, and Macro163 enzyme-linked immunosorbent assays

Total and apoptotic cell death of cytokeratin 18 positive cells was measured in serum samples from all patients using the M30 Apoptosense and M65 ELISA two-side enzyme-linked immunosorbent assays (PEVIVA AB, Bromma, Sweden) as described previously [10]. Serum sCD163 levels were determined with the Macro163 sandwich ELISA (Trillium Diagnostics, Bangor, Maine, USA) according to the recommendation of the manufacturer. All measurements were performed in duplicates on a Tecan SLT Rainbow plate reader (Tecan, Männedorf, Switzerland).

Statistical analysis

The present study was a prospective cohort study. HCC patients in the two cohorts were included into the study from the day of written informed consent and were followed up until death, liver transplantation or last contact. Patients who underwent liver transplantation were excluded from further analysis from the day of transplantation. The primary end point was overall survival. Continuous variables are expressed as mean ± standard deviation whereas categorical variables are reported as frequencies and percentages.

The diagnostic accuracy for the diagnosis of HCC was estimated using the receiver operating curve (ROC) analysis. Independent discriminative factors for HCC diagnosis were assessed with a logistic regression model with backward elimination. Differences in the serum biomarker values between different patient cohorts were determined using the non-parametric Wilcoxon-Mann-Whitney and Kruskal-Wallis tests. For sub-analysis of a statistically significant Kruskal-Wallis test, the Bonferroni correction was used. p values <0.05 were considered to be significant. In the box plots, the vertical lines indicate the range, the horizontal boundaries of the boxes represent the first and third quartile. The correlation coefficient r between different parameters was calculated by using the Spearman correlation.

Predictors of survival were determined using a univariate Cox regression hazard model. Death was recorded as event. For assessment of independent predictors of survival, a multivariate Cox regression hazard model with forward stepwise (likelihood ratio) entry was used. Survival curves with the estimated hazards were calculated with the Cox regression model. The patients at risk at the individual time points are shown in the figures. Statistical analyses were performed with SPSS (Version 22.0, IBM, New York, USA) and BiAS (Version 9.02, Epsilon-Verlag, Darmstadt, Germany).

Results

The HCC cohorts consisted of 142 patients in the test and 125 patients in the validation cohort. The patients were prospectively enrolled into the present study. Patient characteristics are summarized in Table 1. In 91 patients of the test cohort and in 67 patients of the validation cohort, HCC was diagnosed by histopathological examination of biopsies, whereas in 51 patients of the test and 58 patients of the validation cohort, the diagnosis of HCC was obtained by dynamic imaging [25]. The tumor stages of the patients according to the CLIP and BCLC score and patient characteristics are shown in Table 1. Forty-one patients (28.9%) in the