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Research Paper

Plasma m*SEPT9*: A Novel Circulating Cell-free DNA-Based Epigenetic Biomarker to Diagnose Hepatocellular Carcinoma

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SUMMARY

Background: Patients with cirrhosis are at high risk of hepatocellular carcinoma (HCC). The *SEPT9* gene is a key regulator of cell division and tumor suppressor whose hypermethylation is associated with liver carcinogenesis. The primary aim of this study was to evaluate the diagnostic accuracy of a PCR-based assay for the analysis of *SEPT9* promoter methylation in circulating cell-free DNA (m*SEPT9*) for diagnosing HCC among cirrhotic patients.
Methods: We report two phase II biomarker studies that included cirrhotic patients with or without HCC from France (initial study) and Germany (replication study). All patients received clinical and biological evaluations, and liver imaging according to current recommendations. The primary outcome was defined as the presence of HCC according to guidelines from the American Association for the Study of Liver Diseases. The diagnosis of HCC was confirmed by abdominal contrast-enhanced computed tomography scan and systematically discussed in a multidisciplinary consultation meeting. HCC-free cirrhotic patients were recruited if the screening abdominal ultrasound showed no evidence of HCC at the time of blood sampling for the m*SEPT9* test and on the next visit six months later. The adjudicating physicians were blinded to patient results associated with the m*SEPT9* test.
Findings: We included 289 patients with cirrhosis (initial: 186; replication: 103), among whom 98 had HCC (initial: 51; replication: 47). The m*SEPT9* test exhibited high diagnostic accuracy for HCC diagnosis, with an area under the receiver operating characteristic curve (AUROC) of 0.944 (0.900–0.970, $p < 0.0001$) in the initial study (replication: 0.930 [0.862–0.971, $p < 0.0001$]; meta-analysis: AUROC = 0.940 [0.910–0.970, $p < 0.0001$], no heterogeneity: $I^2 = 0\%$, $p = 0.67$; and no publication bias). In multivariate logistic regression analysis, the number of positive m*SEPT9* triplicates was the only independent variable significantly associated with HCC diagnosis (initial: OR = 6.30, for each m*SEPT9* positive triplicate [2.92–13.61, $p < 0.0001$]; replication: OR = 6.07 [3.25–11.35, $p < 0.0001$]; meta-analysis: OR = 6.15 [2.93–9.38, $p < 0.0001$], no heterogeneity: $I^2 = 0\%$, $p = 0.95$; no publication bias). AUROC associated with the discrimination of the logistic regression models in initial and validation studies were 0.969 (0.930–0.989) and 0.942 (0.878–0.978), respectively, with a pooled AUROC of 0.962 ([0.937–0.987, $p < 0.0001$], no heterogeneity: $I^2 = 0\%$, $p = 0.36$; and no publication bias).
Interpretation: Among patients with cirrhosis, the m*SEPT9* test constitutes a promising circulating epigenetic biomarker for HCC diagnosis at the individual patient level. Future prospective studies should assess the m*SEPT9* test

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in the screening algorithm for cirrhotic patients to improve risk prediction and personalized therapeutic management of HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignant tumor of the liver (El-Serag, 2011; Bruix et al., 2016; Oussalah et al., 2016). It is the fifth most common cancer in men and the seventh in women, and ranks second in annual cancer mortality rates worldwide, with liver cancer diagnosed in >700,000 people annually (El-Serag, 2011; Bruix et al., 2016; World Health Organization, 2017). Major risk factors for HCC include cirrhosis, infection with hepatitis B (HBV) or C virus (HCV), alcoholic liver disease, and non-alcoholic fatty liver disease. The 5-year cumulative risk for HCC development in patients with cirrhosis ranges between 5 and 30%, depending on the cause, with the highest risk among those infected with HCV (El-Serag, 2011).

Alpha-fetoprotein (AFP) has been widely used as a diagnostic marker for HCC. However, according to the American Association for the Study of Liver Diseases (AASLD) guidelines, AFP is insufficiently sensitive or specific for use in a screening assay (Bruix & Sherman, 2011). Moreover, according to the European Association For The Study Of The Liver-European Organization for Research and Treatment of Cancer (EASL-EORTC) clinical practice guidelines on HCC management, AFP is suboptimal for routine clinical practice (J. Hepatol., 2012). Furthermore, AFP has a lower diagnostic accuracy for detecting HCC in patients with HCV-related cirrhosis (Gopal et al., 2014).

Epigenetic alterations are a common hallmark of human cancer (Moran et al., 2017; Hao et al., 2017). Several lines of evidence suggest that the septin 9 gene (*SEPT9*) is a key regulator of cell division and a tumor suppressor whose hypermethylation is associated with liver carcinogenesis (Scott et al., 2005; Kakehashi et al., 2011; Villanueva et al., 2015). *SEPT9* expression is ubiquitous in healthy tissues but is decreased or silenced by aberrant promoter hypermethylation in liver cancer (Uhlen et al., 2015; Wasserkort et al., 2013). An epigenome-wide association study of 304 HCC tissue samples showed that *SEPT9* is a significant epi-driver gene in liver carcinogenesis, *via* *SEPT9*-promoter hypermethylation (Villanueva et al., 2015).

Aberrantly methylated DNA sequences originating from tumors are detectable in the circulation of patients with cancer using polymerase chain reaction (PCR) (Esteller et al., 1999; Wong et al., 1999; Xu et al., 2017). Several circulating epigenetic markers are under evaluation in HCC, notably those identified using "omics" approaches (Xu et al., 2017). Nevertheless, to date, no Food and Drug Administration (FDA)-approved circulating epigenetic biomarker has been shown to be useful to diagnose HCC at the individual patient level. Given the high incident risk of HCC in patients with cirrhosis, we conducted initial and replication studies to investigate whether measurement of *SEPT9* promoter methylation in circulating cell-free DNA (m*SEPT9* test) would be useful to diagnose HCC among patients with cirrhosis.

1.1. Specific Objectives

The primary aim was to evaluate the diagnostic accuracy of the m*SEPT9* test to diagnose HCC among patients with cirrhosis. The secondary aims were: 1) to identify variables that are independently associated with HCC diagnosis to account for potential confounders for the diagnostic performance of the m*SEPT9* test; 2) to evaluate the accuracy of the m*SEPT9* test to diagnose Barcelona Clinic Liver Cancer (BCLC) stage A HCC; 3) to evaluate the diagnostic accuracy of the m*SEPT9* test among patients with HCV- and alcohol-related cirrhosis; 4) to compare the diagnostic performance of the m*SEPT9* test with that of AFP; and 5) to calculate the categorical net reclassification improvement (NRI) of a m*SEPT9*-based strategy to diagnose HCC compared with an AFP-based strategy.

2. Methods

2.1. Study Design

We report two phase II biomarker studies that included patients with cirrhosis with or without HCC from France (initial study: the SEPT9 study) and Germany (replication study) (Fig. 1). According to Sackett's classification, phase II biomarker studies aim to assess the magnitude of the association between the results of a biomarker and the disease status (Sackett & Haynes, 2002). Phase II biomarker studies are concerned with reproducibility and aim to assess the sensitivity and specificity of the biomarker, together with its ability to "rule out" (high sensitivity) or "rule in" (high specificity) the disease (Sackett & Haynes, 2002; Akiyama et al., 2014).

2.2. Initial Study: The SEPT9 Study (Nancy, France)

The SEPT9 study was a standardized observational study that recruited patients with cirrhosis with or without HCC with a high degree of ascertainment, including a multidisciplinary consultation meeting (gastroenterologists, radiologists, and surgeons) on a weekly basis at the University Hospital of Nancy. Patients were recruited between June 2012 and April 2014. The HCC study population included: 1) patients with cirrhosis enrolled in an HCC screening program and for whom a diagnosis of HCC was made and ascertained by an abdominal contrast-enhanced computed tomography (CT) scan, and 2) patients with cirrhosis who were directly referred for HCC. HCC-free patients with cirrhosis were recruited if the screening abdominal ultrasound showed no evidence of HCC at the time of blood sampling for the m*SEPT9* test and on the next visit six months later. Patients were excluded from the analysis if they had missing clinical and/or radiological data. All patients received clinical and biological evaluations and liver imaging, according to international recommendations (Bruix & Sherman, 2011). Biochemical data were collected in an electronic database and extracted for this study using the general laboratory information management system (v8.11.6; MIPS France S.a.r.l., Paris, France). Clinical data were retrieved through electronic chart review using DxCare software (MEDASYS, Clamart, France). All patients with a diagnosis of HCC were discussed in the weekly multidisciplinary gastrointestinal oncology meeting dedicated to HCC (gastroenterologists, radiologists, and surgeons). The clinical data and treatment decisions related to HCC were recorded in the e-RCP SOLSTIS platform (https://www.sante-lorraine.fr/), which is the official secured online service for managing and producing multidisciplinary consultation meeting reports for patients with a diagnosis of cancer. The following data were available in the electronic database: 1) demographic data, including age and gender; 2) clinical data, including the etiology of cirrhosis (alcohol, nonalcoholic steatohepatitis [NASH], HCV, HBV, autoimmunity, or hemochromatosis), and Child-Pugh score; 3) for patients with a diagnosis of HCC: the number of HCC nodules, the size of the largest HCC nodule, and the BCLC stage; and 4) blood biomarkers, including albumin, total bilirubin, and prothrombin activity (percentage). All patients gave their informed consent to participate in the study. The Institutional Review Board of the University Hospital of Nancy approved the study. The Nancy Biochemical Database was reported to the French National

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