Combining Tissue and Circulating Tumor DNA Increases the Detection Rate of *CTNNB1* Mutation in Hepatocellular Carcinoma

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Abstract

Background and aims: Studies suggest that mutations in the *CTNNB1* gene are predictive of response to immunotherapy, an emerging therapy for advanced hepatocellular carcinoma (HCC). Analysis of circulating tumor DNA (ctDNA) offers the possibility of serial non-invasive mutational profiling of tumors. Combining tumor tissue and ctDNA analysis may increase the detection rate of mutations.

This study aimed to evaluate the frequency of the *CTNNB1* p.T41A mutation in ctDNA and tumor samples from HCC patients and to evaluate the concordance rates between plasma and tissue. We further evaluated changes in ctDNA after various HCC treatment modalities and the impact of the *CTNNB1* p.T41A mutation on the clinical course of HCC.

Methods: We used droplet digital PCR to analyze plasma from 95 patients and the corresponding tumor samples from 37 patients during three years of follow up.

Results: In tumor tissue samples, the mutation rate was 8.1% (3/37). In ctDNA from HCC patients, we found a *CTNNB1* mutation rate of 9.5% (9/95) in the pre-treatment samples. Adding results from plasma analysis to the subgroup of patients with available tissue samples, the mutation detection rate increased to 13.5% (5/37). There was no difference in overall survival according to *CTNNB1* mutational status. Serial testing of ctDNA indicated a clonal evolution of HCC or arising of multicentric tumors with separate genetic profiles.

Conclusion: Combining analysis of ctDNA and tumor tissue increased the detection rate of *CTNNB1* mutation in HCC patients. A liquid biopsy approach may be useful in a tailored therapy of HCC.

Introduction

Hepatocellular carcinoma (HCC) is a severe disease with significant morbidity and high mortality[1]. Treatment of HCC is guided by tumor stage, underlying liver disease and performance status. For the past decade, systemic treatment options were limited to sorafenib but recently, other tyrosine-kinase inhibitors demonstrated a survival benefit in advanced HCC[2, 3]. In addition, new treatment strategies with immunotherapy for advanced HCC are emerging[4]. As many treatments will be available for patients with advanced HCC, clinicians need methods to select the right treatment for the right patient.

Pronounced intratumor heterogeneity and the presence of multicentric tumors challenge a global tumor molecular profiling from a single biopsy[5, 6]. In addition, mutational profiling of tumor biopsies taken at an early stage of HCC do not necessarily provide optimal guidance for treatment decisions later in the disease course. Conversely, ctDNA potentially represents the whole tumor burden and may provide information on specific tumor mutations during a clonal evolution. ctDNA analysis is non-invasive and can be performed repeatedly during the course of disease and thereby provide an accurate picture of the tumor mutation profile before clinical decision-making.

CTNNB1 mutations belong to the most prevalent genetic alterations in HCC[7]. The p.T41A mutation is detected in 10–15% of tissue samples from primary tumors and leads to activation of the Wnt/ β -catenin signaling pathway[8]. *CTNNB1* mutations can appear at any time in tumor evolution[9], and Vilarinho et al. suggested a progressive clonal evolution in the *CTNNB1* gene during malignant transformation and development of metastasis[10]. Studies indicate that patients with mutations in the Wnt/ β -catenin pathway, including *CTNNB1* mutations, have an inferior response to regorafenib[11] and particularly to immunotherapy[12]. A murine HCC model supports these clinical findings showing resistance to anti-PD-1 therapy in tumors with activation of the Wnt/ β -catenin pathway[13].

In the present study we aimed to prospectively monitor and evaluate the concordance between the *CTNNB1* p.T41A mutation in ctDNA and tumor tissue in patients with HCC. For this purpose, droplet digital PCR (ddPCR) is a sensitive, feasible and affordable method for detecting specific low frequency mutated alleles in a background of abundant non-mutated alleles[14]. We hypothesized that the combined analysis of ctDNA and tumor tissue DNA may provide a more complete picture of the frequency of *CTNNB1* mutation. Further, we aimed to evaluate serial testing of ctDNA in a clinical setting and the correlation of the *CTNNB1* mutation to clinical outcome.

Patients And Methods

Ethical approval

The study was approved by the Central Denmark Region Committee on Biomedical Research Ethics (no. 1-10-72-240-16) and conducted in accordance with the Declaration of Helsinki.

Patients and sample preparation

The study included 95 patients with the following inclusion criteria; a clinical diagnosis of HCC; plans to initiate a new treatment modality; and age above 18 years and no synchronous malignancy (apart from non-melanoma skin cancer). All patients gave written informed consent. Patients were recruited from November 2016 through October 2018 at the Department of Hepatology and Gastroenterology or the Department of Oncology, Aarhus University Hospital.

Clinical data on disease stage, previous and new treatment(s), treatment response, and mortality were obtained from patient charts.

Blood samples were collected from each patient before commencing a new treatment, after one month and every six months thereafter for up to three years. Samples were collected in 9 mL EDTA tubes (Becton Dickinson, Plymouth, United Kingdom) and processed within 4 hours. Samples were centrifuged (1800 g at 4°C for 10 minutes), and plasma was carefully removed to avoid contamination and stored at -80°C. Tumor tissue was collected from 37 of the patients.

Cell-free DNA (cfDNA) and tumor tissue DNA analysis

Using QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) cfDNA was extracted from 4-5 mL of plasma and eluted in 100 µl of elution buffer. DNA extraction from tissue samples was performed with the DNA Mini Kit (Qiagen) on a QIAsymphony.

Droplet digital PCR (ddPCR) analyses were performed with the *CTNNB1* p.T41A and wild type (WT) assay from Bio-Rad using the QX200 AutoDG Droplet Digital PCR system (Bio-Rad, Hercules, CA, USA). Samples were run in triplicates. Each run included a nontemplate control, cell-free DNA (cfDNA) from a healthy donor and a mutation-positive control. QuantaSoft analysis software version v.1.7.4.0917 (Bio-Rad) was used in all data analyses.

The limit of detection (LoD) was determined as previously described by analyzing cfDNA from healthy donors[15]. See *Supplementary material* for a more detailed description of the methods used.

Statistical analysis

Statistical analyses were performed in STATA version 16.0 (Stata Corporation) and GraphPad Prism version 8.3.0. All tests were two-sided, and P values <0.05 were considered statistically significant. Survival analyses were performed using the Cox proportional hazards regression. Correlations between the mutational status and patient characteristics were evaluated by the chi-squared test. Concordance between the mutational status in plasma and tumor tissue was calculated by Cohen's kappa statistics. Data collection was managed using Research Electronic Data Capture (REDCap) hosted at Aarhus University[16, 17].

Results

Patient characteristics

Tabel 1 shows the characteristics of the 95 patients in the study. Median follow-up time was 439 days (IQ range 146-724 days) and 69 (71.5%) patients died within the follow-up period.

Females were more often *CTNNB1* mutation positive and there was a trend towards association with Hepatitis C virus infection etiology in plasma mutation positive patients.

Mutational status and clinical course of disease

A total of 37 (8 fresh frozen and 29 formalin-fixed, paraffin-embedded) tissue samples were analyzed and three samples were positive for the *CTNNB1* mutation (8.1%). In the inclusion plasma sample 9 patients out of 95 (9.5%) were positive for the *CTNNB1* p.T41A mutation.

The agreement between plasma and tissue mutational status was 91.7% (kappa value 0.52, P=0.0007). Adding results from plasma analyses increased the *CTNNB1* mutation detection rate to 13.5% (5/37) in the subgroup of patients with tumor tissue available.

Two patients were positive in plasma, but negative in tissue. In both cases, the biopsies were from large inhomogeneous tumors (6.2cm and 14.5 cm respectively). Another patient had a positive tissue sample but negative plasma sample at inclusion. Follow up revealed a negative plasma sample at week 4 and week 26 but at week 104 the mutation was detectable, coinciding with progression of disease.

There was no correlation between *CTNNB1* mutation status and mortality (HR 0.62 (0.25-1.54) P=0.30, adjusted for tumor, node, metastasis (TNM) stage, sex and age). Figure 1 shows the changes in concentration of mutated alleles during the course of follow up in relation to treatment and response in the five patients positive for *CTNNB1* mutation in plasma at inclusion with follow-up samples available.

The patient depicted in Figure 1A, had two separate intrahepatic tumors on a background of hepatitis C virus, and had a resection of one tumor and radiofrequency ablation of the other tumor. The resected tumor was positive for the *CTNNB1* mutation, while there was no tissue available from the ablated tumor. The patient later had recurrent disease at the ablation site, but without detectable *CTNNB1* mutation in plasma on subsequent follow up samples.

Discussion

In this study we applied ddPCR to detect the *CTNNB1* p.T41A hotspot mutation in plasma and tumor tissue from HCC patients at inclusion and during three years of follow-up. We showed that it is feasible to detect the *CTNNB1* p.T41A mutation in both plasma and tumor samples using ddPCR, and the serial testing of ctDNA indicated a clonal evolution of HCC or arising multicentric tumors with separate genetic features. We did not detect any difference in mortality depending on *CTNNB1* mutational status, however, this study was not designed to evaluate the efficacy of systemic treatment.

At inclusion, we detected the *CTNNB1* p.T41A mutation in plasma in 9.5% of patients. Mutation rate was 13.5% when combining results from plasma and tumor tissue analysis, leading to a more exhaustive characterization of *CTNNB1* mutational status with potential implications for future treatment strategy.

Our results are in line with the expected mutation rate, considering previous studies of *CTNNB1* p.T41A mutations in HCC patients[18, 9]. *CTNNB1* mutations are more frequent in alcohol-related HCC[19] and associated with old age and negatively associated with hepatitis B and elevated alpha-fetoprotein[9], thereby mirroring the etiologic profile of the northern European HCC population.

The majority of previous studies on molecular profiles of individual tumors were performed on early stage tumors and without real-time evaluation of impact on treatment effect. ddPCR is a non-invasive method for *CTNNB1* p.T41A mutation that is readily implementable in most larger HCC treatment facilities. ddPCR may supplement next generation sequencing of tumor tissue samples, as it requires a relatively low amount of input ctDNA, results are readily interpretable, and ctDNA reflects the genetic background of the entire tumor burden. Importantly, in this study we detected the *CTNNB1* mutation in plasma in two patients in whom biopsy was mutation negative. ddPCR is useful for serial testing of tumor mutational

status, as demonstrated by one patient with a positive tissue biopsy who became ctDNA positive during follow-up.

Concordance between plasma and tissue mutational status was high. Discrepancy between mutational status in tissue and plasma may be due to tumor heterogeneity, tumor clonal evolution or multicentric tumors. In one case of discrepancy, with *CTNNB1* mutation in ctDNA while not present in the index biopsy, we observed progression in satellite tumors during follow-up. The results from the patient in Figure 1A could be explained by the presence of multicentric tumors which often occurs in HCC especially on a background of viral hepatitis.

The *CTNNB1* p.T41A mutation activates the Wnt pathway and is associated with poor outcome in patients treated with immunotherapy[12]. This underlines the importance of knowing the mutational status in the individual patient. Furthermore, adenomas with mutations in the Wnt/ β -catenin pathway have higher risk of malignant transformation[20], therefore this noninvasive strategy might also guide decisions regarding treatment or surveillance, although the sensitivity of the assay for small tumors was not evaluated in this study.

The strength of the present study is the number of patients with HCC and the follow-up of patients during different treatment scenarios. A weakness is the number of tumor tissue available for analysis caused by international guidelines where the HCC diagnosis in cirrhosis patients can be based exclusively on imaging. This study focused on only the most frequent mutation known in *CTNNB1* gene. Further studies could apply the multiplex ddPCR method and hereby increase the number of different mutations investigated. Further studies are needed to investigate whether ctDNA can be used to predict and monitor treatment response. This study points at using a combination of tumor mutation profiling in tissue and ctDNA for the most complete characterization. We therefore propose that future studies of HCC therapies should include profiling of ctDNA.

Abbreviations

cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; ddPCR, droplet digital polymerase chain reaction; FFPE, formalin-fixed paraffin-embedded; HCC, hepatocellular carcinoma; LoD, limit of detection; WT, wild-type.

Declarations

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Ethics approval: The study was approved by the Central Denmark Region Committee on Biomedical Research Ethics (no. 1-10-72-240-16).

Consent to participate: All patients gave written informed consent to participate in the study.

Consent for publication: All patients gave written informed consent to publication of the study results.

Availability of data: The data for the study is available upon request to the corresponding author.

Code availability: Not applicable

Authors contribution: SKO, JK, HG, BW and BSS designed the study. SKO authored the first draft of the manuscript and performed the statistical analysis. MSC and BSS performed the analysis for *CTNNB1* mutation and validated the results. SJHD secured data from tissue samples including extraction of DNA and revision of samples. All authors were involved in revising and editing the final manuscript.

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Tables

Table 1
Patient characteristics

Plasma			
	CTNNB1 mutation positive	CTNNB1 mutation negative	P value
All	(n = 9)	(n = 86)	
Age	68 (64–82)	72 (66–78)	0.992
Sex			
Female	5 (55.6%)	19 (22.1%)	0.028
Male	4 (44.4%)	67 (77.9%)	
Cirrhosis			
Yes	6 (66.7%)	54 (62.8%)	0.918
No	3 (33.3%)	25 (29.1%)	
Unknown	-	7 (8.1%)	
Child Pugh stage			
Non-cirrhosis	3 (33.3%)	25 (29.1%)	0.527
A	4 (44.4%)	52 (60.5%)	
B	5 (55.6%)	28 (32.6%)	
C	-	5 (5.8%)	
Cirrhosis etiology			
Alcohol	2 (22.2%)	29 (33.7%)	0.802
Hepatitis C	3 (33.3%)	10 (11.6%)	0.071
Non-alcoholic steatohepatitis	-	4 (4.7%)	0.509
Other	-	7 (8.1%)	0.374
Unknown/no cirrhosis	4 (44.4%)	36 (41.9%)	0.881
TNM stage			0.914
I	2 (22.2%)	29 (33.7%)	
II	2 (22.2%)	18 (20.9%)	
III	2 (22.2%)	16 (18.6%)	
IV	3 (33.3%)	23 (26.7%)	

Plasma			
Alpha-fetoprotein			
<20 ng/mL	5 (55.6%)	36 (41.9%)	0.642
≥20 ng/mL	4 (44.4%)	40 (46.5%)	
Not available	-	10 (11.6%)	

Figures

Patient underwent resection and simultaneously radiofrequency ablation. Relapse on CT scans after 1 year.

Patient received sorafenib. Terminated treatment after 8 weeks because of progression and declining performance status.

Patient underwent radiofrequency ablation with complete response.

Patient received Trans Arterial Chemo Embolization with mixed response.

Patient received sorafenib, terminated treatment because of declining performance status. Deceased after 6 months.

Figure 1

Correlation between amount of CTNNB1-mutated alleles during follow up and clinical course of disease in five patients.

Supplementary Files

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- [Supplementaryfile1.docx](#)