Title: HCC Screening using DNA methylation Changes in ctDNA

ClinicalTrials.gov Identifier: NCT03483922
Date: January 11, 2022

Clinical study design

Study protocol was approved by IRB board of icddr,b (Dhaka, Bangladesh) study protocol PR-18025. 402 participants were recruited from the Dhaka area to the study included 49 healthy controls, 51 Chronic hepatitis B patients and 303 HCC patients from stages 0 to D (HCC O n=2, HCCA n=32, HCC B n=86, HCC C n=106, HCC D n=77 (See Table 2 for demographics). The age of the healthy control and the chronic Hepatitis B (CHB) groups were significantly younger 27 than the HCC group, however there was no significant effect of age on HCC prediction by DNA
methylation as determined by a logistic regression in either the HCC or control groups. However, in the CHB group age had a significant effect on HCC prediction by the DNA methylation test, consistent with the idea of a higher probability for older patients with chronic hepatitis to convert to HCC. There were no differences between the groups in the sex distribution. However, there was a somewhat significant lower alcohol use and higher fraction of smokers in the HCC groups (Table 2). HCC staging was diagnosed according to EASL–EORTC Clinical Practice Guidelines: Management of hepatocellular carcinoma. Hepatitis B diagnosis was confirmed using AASLD practice guideline for chronic Hepatitis B (https://www.aasld.org/publications/practice-guidelines). All participants were properly informed about the study and have signed the informed consent form approved by the icddr,b IRB. Inclusions criteria were participants of either sex 18 to 70 years of age, confirmed diagnosis of HCC using EASL-EORTC guidelines and chronic hepatitis B using AASLD guidelines, nonmetastatic
liver cancer, Hepatitis B surface antigen positive by ELISA and persistence of > 6
months. Exclusion criteria were unwilling or unable to provide informed consent, unwilling or unable to comply with requirements of protocol, participation in a different clinical trial investigating a vaccine, drug, medical device or medicinal procedure less than 4 weeks preceding the current study, planned participation in another clinical trial during present study period, known case of cirrhosis, any other known inflammatory disease (bacterial or viral infection with the exception of hepatitis B or C), known case of diabetes, asthma, autoimmune disease, any other diagnosed cancer, for healthy controls any known inflammatory or infectious disease including Hepatitis B and Hepatitis C and any diagnosis of chronic disease, cancer medication use or drugs of abuse. Patients were assigned an ID that was kept confidential according to

hospital regulations and identity was revealed only to approved hospital personnel. Participants provided consent for DNA methylation biomarker research. Blood sample collection ad plasma separation was performed at icddr,b in Dhaka Bangladesh and was then shipped to HKG epitherapeutics for further analysis. The HKG epitherapeutics lab team was blinded on the identity of the samples throughout the lab analytic procedures. Data was then analyzed in Montreal and shared with icddr,b who provided the results to the respective clinical personnel.

Preparation of CF plasma DNA

Blood was collected in 9-ml tubes containing K3-EDTA and processed within 1 h. Plasma and peripheral blood monocyte separation was performed according to GE Healthcare Cat No 71=7167-00 protocol. Plasma was frozen and shipped. Plasma samples (1 ml) were thawed, and DNA was extracted by previously described guanidine isothiocyanate method[43] and binding to silica magnetic beads followed by 80% ethanol washes and water elution.

Multiplexed Targeted DNA methylation Illumina amplicon sequencing

Bisulfate conversion was performed using EZ-96 DNA Methylation MagPrep (D5041, Zymo Research) followed by two rounds of polymerase chain reaction. For the first round we used primers that included an anchoring sequence and sequences targeting cg02012576 (CHFR), cg03768777 (VASH2), cg05739190 (CCNJ), cg24804544 (GRID2IP) and cg14126493 (F12) using Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad Laboratories, CA, USA) (the primers are available upon request). 5 microliters of the first PCR reaction were subjected to a second round of PCR amplification using primers containing indexes for barcoding the samples (the primers are available upon request). PCR products were pooled, and the pooled library was then purified twice using AMPure XP Beads (Beckman Coulter Life Sciences, CA, USA) and quantified by RealTime PCR using NEBNext® Library Quant Kit for Illumina (New England Biolabs, MA, USA).
Sequencing was performed on the Illumina platform using MiSeq Reagent Nano Kit V2 (Illumina, CA, USA).

Statistical Analysis Plan

Significant difference from the control group was tested by Kruskal-Wallis non-parametric one-way ANOVA and was adjusted by Dunn's multiple comparisons test. The two HCC stage O samples were included for statistics in the HCC A group. ROC was computed using the ROC test in GraphPad.