Yigit B, Boyle M, Ozler O, Erden N, Tutucu F, Hardy T, Bergmann C, Distler JHW, Adali G, Dayangac M, Mann DA, Zeybel M, Mann J. Plasma cell-free DNA methylation: a liquid biomarker of hepatic fibrosis. *Gut* 2018

DOI link to article:

https://doi.org/10.1136/gutjnl-2017-315668

Date deposited:

06/02/2018

Gut Online First, published on January 20, 2018 as 10.1136/gutjnl-2017-315668

LETTER

Plasma cell-free DNA methylation: a liquid biomarker of hepatic fibrosis

We recently reported dynamic epigenetic markers of fibrosis detectable in patients' plasma that may have utility in non-invasive diagnosis and staging of fibrosis in patients with chronic liver disease.[1] Specifically, we uncovered DNA methylation markers at the human PPARγ promoter detectable in circulating cell-free DNA (ccfDNA) that display differential methylation densities. Remarkably, PPARγ hypermethylation correlated with progression to cirrhosis in alcoholic liver disease (ALD) and with specific stages of liver fibrosis in non-alcoholic fatty liver disease (NAFLD). Furthermore, ccfDNA signatures were traced back to the molecular pathology in fibrotic liver tissue, providing a biomarker of the underlying pathological process and defining hepatocytes as the source of hypermethylated DNA found in plasma.[1]

The original study posed several important outstanding questions: (1) Can ccfDNA methylation be used as a biomarker of fibrosis in liver diseases of other aetiologies? (2) Does the presence of hepatocellular carcinoma (HCC) alter the biomarker in plasma? (3) Does presence of fibrosis in other organs generate similar biomarker profiles?

In the present letter, we answer these questions and demonstrate the broader utility of DNA methylation at three CpG dinucleotides within PPARγ promoter in several new patient cohorts (figure 1A and table 1). Employing pyrosequencing we detect hypermethylation at all three CpGs in ccfDNA from a cohort of patients suffering from cirrhosis caused by chronic HBV infection (figure 1B–D). The level of hypermethylation resembled that found in patients with cirrhotic NAFLD and ALD in our original study. However, since the HBV cohort was of another ethnicity to our original UK-based patients with NAFLD and ALD, we also measured methylation density in a Turkish NAFLD cohort, which was mirroring those detected in the HBV cohort. Our new data also demonstrate that presence of HCC with chronic liver disease does not alter the specificity of the DNA methylation markers for detection of liver fibrosis (figure 1B–D). As we had access to explant liver tissue from patients with NAFLD, HBV and HCC, we determined methylation densities in the liver (figure 1E–G). A high similarity was observed between the degree of DNA methylation at PPARγ gene promoter in ccfDNA and in the patient-matched liver tissues. We found a significant spread of values for DNA methylation in the healthy control ccfDNA, this being in contrast with our original UK-based study in which low-level methylation density was consistent across individuals within the control

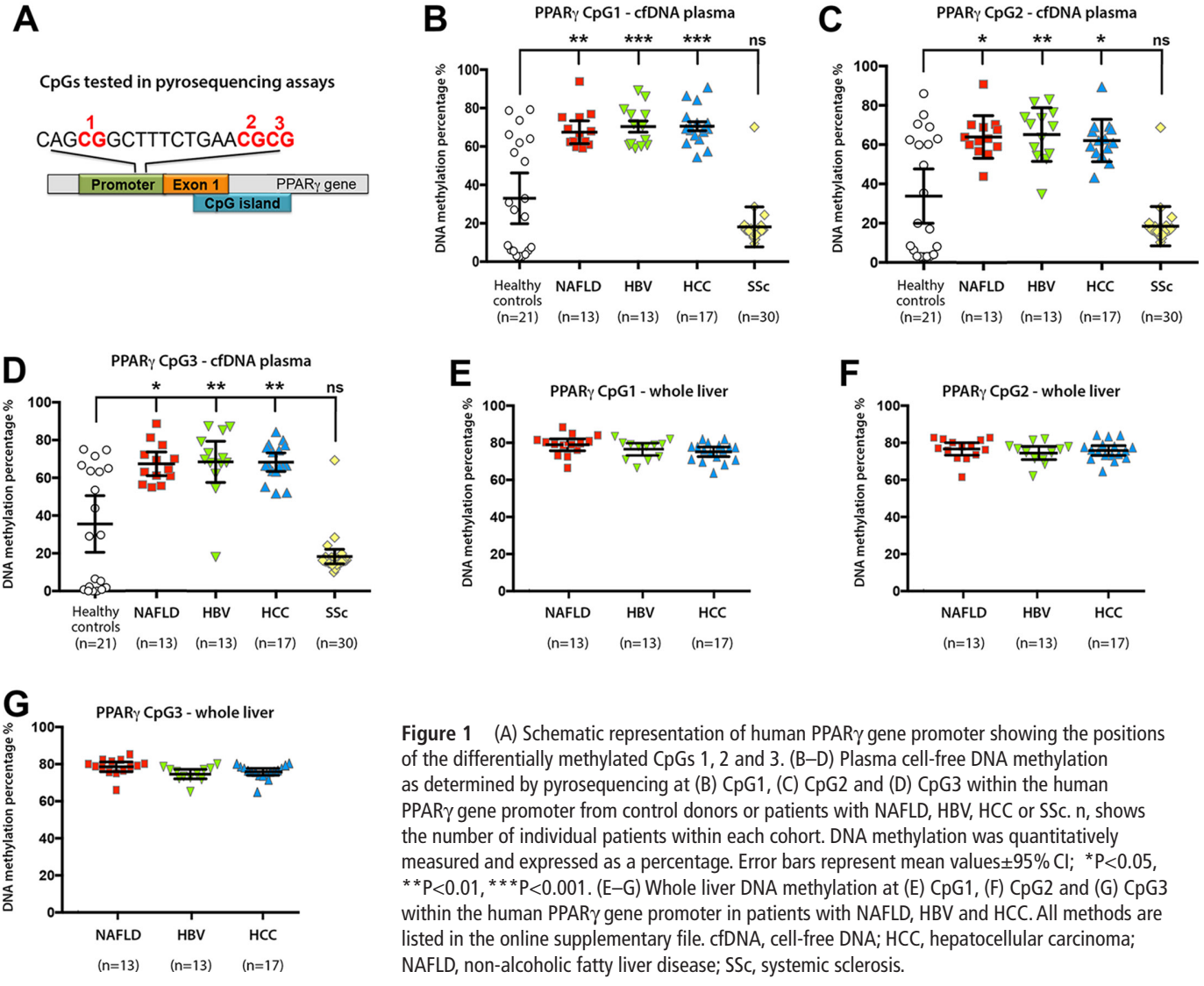

Figure 1 (A) Schematic representation of human PPARγ gene promoter showing the positions of the differentially methylated CpGs 1, 2 and 3. (B–D) Plasma cell-free DNA methylation as determined by pyrosequencing at (B) CpG1, (C) CpG2 and (D) CpG3 within the human PPARγ gene promoter from control donors or patients with NAFLD, HBV, HCC or SSc. n, shows the number of individual patients within each cohort. DNA methylation was quantitatively measured and expressed as a percentage. Error bars represent mean values±95% CI; *P<0.05, **P<0.01, ***P<0.001. (E–G) Whole liver DNA methylation at (E) CpG1, (F) CpG2 and (G) CpG3 within the human PPARγ gene promoter in patients with NAFLD, HBV and HCC. All methods are listed in the online supplementary file. cfDNA, cell-free DNA; HCC, hepatocellular carcinoma; NAFLD, non-alcoholic fatty liver disease; SSc, systemic sclerosis.

Table 1 Characteristics of patient cohorts used in the study

	Age (years)	Gender (male/female)	BMI (kg/m^2)	Diabetes (%)	ALT (IU/L)	AST (IU/L)
NAFLD cohort	56 ± 7	10/3	29.8±3.2	69	33±23	54±36
Hepatitis B cohort	51±7	10/3	26.5±2.4	38	47±50	80±64
HCC cohort	57±7	16/1	27.5±4.2	29	55±36	65±53

Systemic sclerosis cohort (n=30)	Age (years)	Gender (male/female)	BMI (kg/m^2)	Diffuse cutaneous limited SSc	Disease duration (years)	Heart involvement	Lung involvement	DLCO (%)	Antinuclear antibody-positive	Anticentromere antibody-positive	Antitopoisomerase I antibody-positive
	55±14	10/20	26±3.8	12 (40%)	7.5±4	2 (7%)	11 (37%)	71.7±17	30 (100%)	11 (37%)	12 (40%)

Notes: Viral hepatitis in HCC cohort: HBV-positive, n=8; HCV-negative, n=2; HBV-positive and HCV-positive, n=3.
Data expressed as mean±SD or median (range).
BMI, body mass index; HCC, hepatocellular carcinoma; NAFLD, non-alcoholic fatty liver disease; SSc, systemic sclerosis.

group. We are unable to explain this wider spread of methylation densities in the Turkish cohort, but cannot rule out an undetected liver disease in the apparently 'healthy' controls that display elevated ccfDNA methylation.

We next determined if hypermethylation is specific to fibrosis of liver origin. To this end, we quantified ccfDNA methylation in a cohort of patients with limited and diffuse systemic sclerosis (SSc) who have various combinations of skin, lung and kidney fibrosis, but no hepatic fibrosis.[2] All three CpG sites in SSc were relatively hypomethylated (figure 1B–D), with similar methylation densities between individual patients with SSc. All methods relating to the study are listed in 'online supplementary materials and methods 1'.

This important validation study supports our original hypothesis that hypermethylation at the PPARγ gene promoter is a marker for fibrotic progression of chronic liver disease and holds true for viral, alcoholic and metabolic disease aetiologies. As fibrosis in other organs does not generate a similar epigenetic signature, it is likely that the PPARγ hypermethylation specifically reflects a liver pathology. The ability to detect and quantify hypermethylation at the promoter of the PPARγ in ccfDNA as a new liquid biomarker that specifically reports the fibrotic progression of liver diseases of multiple aetiologies offers the potential for a cost-effective blood-based liquid biomarker of liver fibrosis.

Buket Yiğit,[1] Marie Boyle,[2] Oğuz Özler,[1] Nihan Erden,[1] Faik Tutucu,[1] Timothy Hardy,[2] Christina Bergmann,[3] Joerg H W Distler,[3] Gupse Adalı,[4] Murat Dayangaç,[4] Derek A Mann,[2] Mujdat Zeybel,[1] Jelena Mann[2]

[1]Department of Gastroenterology and Hepatology, School of Medicine, Koç University, Istanbul, Turkey
[2]Faculty of Medical Sciences, Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, UK
[3]Department of Internal Medicine, University of Erlangen-Nuremberg, Erlangen, Germany
[4]Liver Transplantation Unit, Istanbul Bilim University, Florence Nightingale Hospital, Istanbul, Turkey

Correspondence to Dr Jelena Mann, Institute of Cellular Medicine, Faculty of Medical Sciences, Newcastle University, Newcastle upon Tyne, NE2 4HH, UK; jelena.mann@ncl.ac.uk

Acknowledgements The authors gratefully acknowledge use of the services and facilities of the Koç University Research Center for Translational Medicine (KUTTAM), equally funded by the Republic of Turkey Ministry of Development Research Infrastructure Support Program. Findings, opinions or points of view expressed on this article do not necessarily represent the official position or policies of the Ministry of Development.

Contributors BY: acquisition, analysis and interpretation of majority of the data for the manuscript. MB, OÖ, CB, NE, FT, TH, GA, MD, JHWD: acquisition of sample or data for the manuscript. MZ: acquisition, analysis and interpretation of data for the manuscript; drafting the manuscript and revising it critically for important intellectual content. JM, DAM: conception and design of the manuscript, acquisition of data for the manuscript, drafting the manuscript and revising it critically for important intellectual content.

Funding Marie Curie Fellowship, European Commission and TUBITAK 2232 Fellowship, National Institute on Alcohol Abuse and Alcoholism (AA018663), and Medical Research Council (MR/K001949/1, MR/L002094/1).

Competing interests None declared.

Ethics approval Koç University Ethics Committee for Clinical Research, 04.02.2016-2016.024.IRB2.005, 18.9.2015-2015.215.IRB1.020.

Provenance and peer review Not commissioned; internally peer reviewed.

► Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/gutjnl-2017-315668).

MZ and JM contributed equally.

To cite Yiğit B, Boyle M, Özler O, *et al*. *Gut* Epub ahead of print: [*please include* Day Month Year]. doi:10.1136/gutjnl-2017-315668

Received 14 November 2017
Revised 24 November 2017
Accepted 30 November 2017

Gut 2018;**0**:1–2. doi:10.1136/gutjnl-2017-315668

REFERENCES

1 Hardy T, Zeybel M, Day CP, *et al*. Plasma DNA methylation: a potential biomarker for stratification of liver fibrosis in non-alcoholic fatty liver disease. *Gut* 2017;66:1321–8.
2 Selmi C, De Santis M, Gershwin ME. Liver involvement in subjects with rheumatic disease. *Arthritis Res Ther* 2011;13:226.

Plasma cell-free DNA methylation: a liquid biomarker of hepatic fibrosis

Buket Yigit, Marie Boyle, Oguz Özler, Nihan Erden, Faik Tutucu, Timothy Hardy, Christina Bergmann, Joerg H W Distler, Gupse Adali, Murat Dayangaç, Derek A Mann, Mujdat Zeybel and Jelena Mann

Gut published online January 20, 2018

Supplementary materials and methods to the letter "Plasma cell free DNA methylation: a liquid biomarker of hepatic fibrosis"

Clinical Cohorts

Use of human tissue was approved by Koç University Ethics Committee for Clinical Research (04.02.2016 - 2016.024.IRB2.005). Informed consent was obtained from all participants. All liver samples were collected and used subject to patient's written consent prior to the day of liver transplantation. Blood samples and liver tissues were collected during transplantation. Clinical and laboratory data were collected at the time of surgery.

NAFLD cohort- The NAFLD diagnosis was based on imaging findings and histologic examination of the explanted liver. Patients with alternative diagnosis (chronic viral hepatitis, viral autoimmune liver disease, drug-induced liver injury, haemochromatosis, Wilson's disease, alpha-1-antitrypsin deficiency) were excluded. Patients who consumed more than 20 g of alcohol per day for males or more than 10 g per day for females were excluded.

HBV and HCC cohort- For the HBV cohort, patients were selected based on the medical records with a positivity for hepatitis B surface antigen; patients with alternate liver diagnoses or evidence of coexistent liver disease were excluded. Diagnosis of HCC was made if typical features were detected on computed tomography/magnetic resonance and by pathologic examination. Severity of fibrosis was assessed on explanted liver tissues.

Clinical details such as gender, age, weight, height were obtained from all patients at the time of transplantation. The body mass index (BMI) was calculated by the

formula: weight (kg)/height^2 (m^2). Patients were identified as having type 2 diabetes if they were receiving dietary, oral hypoglycaemic drug or insulin treatment, or had fasting blood glucose >7.0 mmol/L. For the control cohort, use of human tissue was approved by Koç University Ethics Committee for Clinical Research (18.9.2015-2015.215.IRB1.020). Subjects had no signs or symptoms of liver disease, and no history of chronic illnesses.

Scleroderma Cohort - Systemic sclerosis (SSc) patients were recruited from a study site managed by Professor Jörg Distler, Professor for translational matrix biology, University of Erlangen-Nuremberg. Blood samples from patients were collected subject to patients' written consent. Recruited patients fulfilled the American College of Rheumatology (ACR)/ European League against Rheumatology (EULAR) criteria for the diagnosis of systemic sclerosis. SSc was classified according to the conventional criteria defined by LeRoy et al [1]. "Diffuse SSc" was diagnosed if the skin thickening extends proximal to the elbows and knees or includes the trunk, while "Limited SSc" was diagnosed if the skin thickening was confined to the elbows and knees, or to the face. Thirty SSc patients were recruited in total; eighteen had limited cutaneous SSc and twelve had diffuse cutaneous SSc. Information collected at time of blood sample collection involved clinical details (gender, age, weight, height, disease duration, organ involvement) and laboratory data (including Scleroderma related antibodies). The body mass index (BMI) was calculated by the formula: weight (kg)/height2 (m2). Lung involvement was considered present if there was evidence of pulmonary fibrosis or pulmonary arterial hypertension. Heart involvement was defined by a past/current diagnosis of congestive heart failure, cardiac arrhythmia, pericarditis, a pericardial effusion, or cardiomegaly.

Cell free circulating and liver DNA extraction

Whole blood was collected into EDTA and the plasma was separated by centrifugation for 10 min at 3000rpm followed by transfer to new tubes and re-centrifugation. For the chronic liver disease and hepatocellular carcinoma cohort, liver tissues were selected 3 cm away from tumour margin. Genomic DNA was extracted from plasma and liver specimens using QIAamp DNA Blood Mini or Micro Kit (Qiagen, Germany, catalogue no: 51106 - 56304). Plasma and liver tissues were lysed at 56°C for 10 minutes and overnight respectively. The lysate was processed and transferred to spin columns as per manufacturer's instructions.

Bisulfite modification

EZ DNA Methylation Gold TM Kit (Zymo Research, Irvine, CA, USA) was used for bisulfite conversion of genomic DNA. Cell free circulating and liver tissue DNA were bisulfite modified by incubating at 98°C for 10 min and 64°C for 2 h and 30 min. Product was transferred into columns; desulphonated and washed according to manufacturer's protocol and eluted in elution buffer. A 5µl of bisulphite modified cell free DNA was amplified in a PCR mix containing 2µl of forward and reverse primer, 12.5µl of HotStarTaq Master Mix Kit (Qiagen, Germany, catalogue no: 203445) or Pyromark PCR kit (Qiagen, Germany, catalogue no: 978703) and 5.5µl of water. 2.5µl Q solution and 1.5µl $MgCl_2$ (25mM/ml) were added. Amplification of DNA was performed in a thermocycler according to the following PCR conditions: one cycle at 95°C for 6 min, followed by 50 cycles of 95°C for 30 s, annealing temperature of 55°C for 30 s and 72°C for 30 s, followed by one cycle at 72°C for 30 s.

Pyrosequencing analysis

Methylation of specific cytosines within CpG dinucleotides was quantified by pyrosequencing using a Pyromark Q96 ID (Qiagen) instrument. PCR and sequencing primers were obtained from a custom designed assay for PPARγ as previously described [2]. 10µl of biotin-labelled PCR product was used in each well and combined by streptavidin- coated sepharose beads, washed in 70% ethanol, denatured in denaturation buffer (Qiagen, PyroMark Denaturation Buffer, 979007) and washed in a wash buffer (Qiagen, PyroMark Wash Buffer, 979008). Sequencing primers were annealed to DNA product at 80°C. The samples were analyzed in duplicate, and the mean of the two measurements was used as the final value. Assay efficiency was validated by fully unmethylated as well as fully methylated DNA (Qiagen, EpiTect PCR Control DNA Set, 59695). CpG methylation data was analysed by Pyro Q-CpG software 1.0.6.

Statistical analysis

All statistical analyses and graphs were made using GraphPad Prism Software. Continuous normally distributed variables were represented as mean ± standard deviation (SD). To determine differences between groups for continuous non-normally distributed variables, means were compared using the Mann-Whitney U test.

References

[1] LeRoy EC, Black C, Fleischmajer R, Jablonska S, Krieg T, Medsger TA, Jr., et al. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. The Journal of rheumatology 1988;15:202-205.

[2] Zeybel M, Hardy T, Wong YK, Mathers JC, Fox CR, Gackowska A, et al. Multigenerational epigenetic adaptation of the hepatic wound-healing response. Nature medicine 2012;18:1369-1377.