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Methylation of tumour suppressor genes *RUNX3, RASSF1A* and *E-Cadherin* in HCV-related liver cirrhosis and hepatocellular carcinoma

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ABSTRACT

Background: HCV infection is related to aberrant methylation of several genes. *RASSF1A, E-Cadherin* and *RUNX3* are tumour suppressor genes that may be inactivated by hypermethylation in many tumours including hepatocellular carcinoma (HCC). We hypothesized that methylation is a diagnostic biomarker for HCC in patients with HCV-related liver cirrhosis.

Methods: We recruited 207 cases of HCV-related liver cirrhosis, 193 HCC patients and 53 healthy controls. Methylation-specific polymerase chain reaction for detection of circulating hypermethylated *RASSF1A, E-Cadherin*and *RUNX3*. Alpha fetoprotein (AFP) was measured by commercial immunoassay.

Results: Significant hypermethylation of the three genes was found in the HCC group compared to both cirrhosis and healthy groups ($P < 0.001$), whereas no significant difference in hypermethylation was found between cirrhosis and healthy groups ($P = 0.17$, 0.50 and 0.14, respectively). No significant links were found between hypermethylated *RASSF1A, E-Cadherin* and *RUNX3* and stages of Barcelona Clinic of Liver Cancer score ($P = 0.21$, 0.63 and 0.98, respectively). No significant associations were found between AFP value and hypermethylated genes in cirrhosis and HCC groups ($P = 0.82$) except with *E-Cadherin* in HCC ($P = 0.02$). In multiple regression analysis, *RASSF1A* and *E-Cadherin* were predictors of HCC within cirrhosis cases, but only *E-Cadherin* was an independent risk factor for prediction of HCC in cases with low AFP ($P = 0.01$).

Conclusions: The presence of hypermethylated serum *RASSF1A, E-Cadherin* and *RUNX3* is linked to HCC in patients with HCV-related cirrhosis. Only *E-Cadherin* is an independent risk factor for prediction of HCC with low AFP. These findings may be of diagnostic value.

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Introduction

Hepatocellular carcinoma (HCC) is a common and rapidly fatal human malignancy, with chronic hepatitis C virus (HCV) infection recognized as a well-documented risk factor for the development of this aggressive cancer [1]. Several hosts-related factors, including genetic and epigenetic alterations, are involved in carcinogenesis [1–4]. Methylation of DNA at promoter regions plays an important established role during embryonic evolution and the development of human diseases, including malignancies. DNA methylation my undergo alteration in the form of hypermethylation, hypomethylation, and loss of imprinting [5]. Epigenetic malfunction may result in oncogene activation as a consequence of hypomethylation and either dampening of tumour suppressor genes or remodelling of chromatic and RNA-related silencing due to hypermethylation [6]. Methylation of several genes has been shown to occur not only in HCC and its precursor lesions but also in chronic hepatitis and liver cirrhosis, suggesting that these changes are early events in the development of this aggressive cancer [7]. The role of methylation in the pathogenesis of HCC is not fully understood, but several studies have proposed a possible role of HCV viral proteins in epigenetic alterations, including the inactivation of tumour suppressor genes, seen in a significant proportion of patients with HCV-related HCC [6].

An important mechanism of tumour suppressor gene inactivation is aberrant methylation in the promoter region of several tumour suppressor genes [8]. Ras association domain family 1 isoform A (RASSF1A) gene belongs to the Ras association gene family, located in 3p21.3, and which encodes regulatory proteins with tumour suppressor functions [9]. Hypermethylation of this gene may be related to hepatocarcinogenesis [10]. Runt-related transcription factor 3 (*RUNX3)* is located at 1p36–35, a chromosomal region frequently deleted in various cancers, including colon, bladder, breast, lung and gastric cancers, as well as HCC [11]. *E-Cadherin* codes for a transmembrane glycoprotein expressed on the surface of epithelial cells and mediates haemophilia [12]. It is located on the long arm of chromosome 16, a location frequently deleted in different types of cancers [12].

Although methylation of tumour suppressor genes has been previously studied in numerous cancers,

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limited data exit on the role of methylation in HCC. Therefore, we hypothesized that the methylation status of candidate genes *RUNX3, RASSF1A* and *E-Cadherin* is linked to HCC in patients with HCV-related liver cirrhosis.

Materials and methods

This cross-sectional case–control study was conducted on patients attending the clinics of Tropical Medicine Department at Mansoura University during the period from May 2015 to December 2017 and included 207 HCV-related liver cirrhosis patients, 193 HCV-related HCC cases and 53 apparently healthy individuals forming a healthy control group. Laboratory investigations were conducted in the Molecular Genetic Unit of Endemic Hepatogastroenterology and Infectious Diseases of Mansoura Faculty of Medicine. Patients were HCV antibody positive with polymerase chain reaction (PCR) for hepatitis C RNA positivity for more than 6 months. HCC was diagnosed by serum alpha fetoprotein (AFP) and imaging by abdominal ultrasound and spiral computed tomography or dynamic contrast-enhanced magnetic resonance imaging. Diagnostic imaging standards with high specificity for HCC ≥1 cm were established according to national and international guidelines and included hyperenhancement in the arterial phase combined with washout appearance and/or capsule appearance. With an AFP ≥200 ng/ml, one typical dynamic imaging technique is needed, but with an AFP <200 ng/ml, two typical dynamic imaging techniques were needed to confirm the diagnosis [13,14]. Patients with lesions that did not meet these guidelines or were smaller than 1 cm were excluded. All cases of HCC were further classified according to Barcelona Clinic of Liver Cancer (BCLC) criteria [15]. Liver cirrhosis was diagnosed on the basis of combined clinical, laboratory and radiological findings. Selected patients had no history of any other causes of hepatitis, e.g. hepatitis B virus (HBV) or HCV co-infection with HBV, as well as absence of other liver disorders, e.g. alcoholic liver disease or drug-related liver disease, and other causes of liver cirrhosis, e.g. autoimmune hepatitis, haemochromatosis or Wilson's disease. All groups provided a full medical history and clinical examination. Written consent was obtained for all participants, local research ethics committee approval was obtained and the study protocol complied with the ethical guidelines of the 1975 Declaration of Helsinki.

Eight millilitres of blood sample were taken from each patient. Four millilitres were delivered to k2EDTA vacationer tubes for DNA analysis. The EDTA blood was then aliquoted and stored at −70°C for epigenetic analysis. The residual blood sample was delivered into plain tubes centrifuged at 4000 rpm for 10 min. Serum was separated and aliquoted into 250 µl volumes and kept at −50°C until used for biochemical testing. Anti-HCV Abs were assessed by ELISA (Abbott Laboratories, Abbott Park, IL, USA). Serum AFP concentration was measured by microparticle enzyme immunoassay (Abbott Laboratories, AXSYM, USA).

DNA was extracted using QIAamp® DNA Blood Mini kit (Qiagen) and quantified using Nanodrop spectrophotometer (Thermoscientific 2000) to calculate the purity and concentration. Sodium bisulphite conversion of genomic DNA extracted from peripheral blood was carried out using EpiTect Bisulphite kit (Qiagen). After incubation of the mixture briefly centrifuge the PCR tubes containing the bisulfite reactions, reagents and transferred to 1.5 ml microcentrifuge tubes. Then, 560 µl freshly prepared Buffer BL containing 10 µg/ml carrier RNA was added to each sample. The mixture was mixed by vortexing and then centrifuged briefly. Subsequently, the entire mixture from each tube was transferred into the corresponding EpiTect spin column which was centrifuged at 13,000 rpm for 1 min and the flow-through discarded. The spin columns were placed back into the collection tubes and 500 µl Buffer BW was added to each spin column and centrifuged at 13,000 rpm for 1 min. The flow-through was discarded, and the spin column was placed the back into the collection tubes. Subsequently, 500 µl Buffer BD was added to each spin column and incubated for 15 min at room temperature (15–25°C). The spin column was centrifuged at 13,000 rpm for 1 min and the flow-through discarded. The spin column was placed back into the collection tubes and 500 µl Buffer BW was added to each column and centrifuged at 13,000 rpm for 1 min. The spin column was placed into new 2 ml collection tubes and centrifuged at maximum speed for 1 min to remove any residual liquid. The spin columns were then incubated for 5 min at 56°C in a heating block to evaporate any remaining liquid and then placed into clean 1.5 ml microcentrifuge tubes. Finally, 20 µl Buffer EB was dispensed onto the centre of each membrane. The purified DNA was eluted by centrifugation for 1 min at 13,000 rpm. Purified bisulphite-converted DNA was stored at −20°C until use. Primers and TaqMan probes were described previously [16–18]. All primers and fluorogenic probes were custom-synthesized by Perkin-Elmer Applied Biosystems (Foster City, CA, USA).

Standard curve establishment and evaluation of quantitative analysis of DNA methylation was performed as previously described [18]. The relative amount of methylation (%) was calculated in each sample according to the formula [M/(U + M)] ×100. Concentrations of methylated (M) and unmethylated (U) portions were determined from simultaneously amplified standard curves for each gene. Methylation levels up to 0.5% were considered to be the background of this sensitive quantitative method. The cumulative methylation index (CMI) was calculated as the sum of percentage methylation for all evaluated genes. For all genes, CMI of 500 was the maximum value of methylation.

Statistical analysis was run on SPSS 17.0 (Chicago, IL, USA). Quantitative parametric data were presented as mean with SD, while non-parametric data were presented as median and range. Differences between two groups were tested using Mann–Whitney test, while differences between several groups were tested using Kruskal–Wallis with Mann–Whitney tests when appropriate. Categorical data are presented as number (per cent) and compared using a 2 × 2 contingency table for odds ratio (OR) with 95% confidence interval (95% CI) and Fisher exact *P* values using the Medcalc software. *P* values were adjusted using Bonferroni formulae. Correlation between different variables was tested using Pearson or Spearman correlation tests when appropriate. Receiver operator characteristic (ROC) curves were constructed for the studied genes and the determination of sensitivity, specificity, the positive predictive value, negative predictive value and accuracy at different cut-off levels. All tests were statistically significant at a *P* value of <0.05.

Results

Table 1 shows clinical, demographic and laboratory data. The three groups were matched for age and sex, but not smoking. Patients were matched for ascites, history of haematemesis and melaena, but encephalopathy was more common in cirrhosis, and jaundice was more common in HCC. Levels of AFP reflected clinical severity: controls < cirrhosis < HCC. Table 2 shows the degree of methylation of the different genes in controls, cirrhosis and HCC. All were significantly different between HCC and cirrhosis, between HCC and controls, but not between cirrhosis and controls. When HCC patients were classified by BCLC score (merging A + B versus C + D), there was no difference in hypo/hypermethylation of *RASSF1A, E-Cadherin* or *RUNX3* (*P* =0.21, *P* = 0.63 and *P* = 0.98, respectively) (Table 3).

Table 1. Socio-demographic and clinical data distribution within studied groups.

	Control (*n* = 53)	Cirrhosis (*n* = 207)	HCC (*n* = 193)	*P* value
Age (years)	54.4 [9.8]	55.1 [7.1]	56.4 [6.2]	0.085
Female	6 (11.3%)	28 (13.5%)	24 (12.4%)	0.894
Male	47 (88.7%)	179 (86.5%)	169 (87.6%)	
Ascites		14 (7.4%)	19 (9.8%)	0.263
History of HE		115 (60.8%)	45 (23.3%)	<0.001
Jaundice		93 (49.2%)	155 (80.3%)	<0.001
History of H&M		179 (94.7%)	175 (90.7%)	0.188
Smoking	12 (22.6%)*	82 (43.4%)*	115 (59.6%)	<0.001**
AFP (ng/ml)	1.8 [0.84–9.2]	24.5 [0.70–254]	55 [0.10–20,248]	<0.001**

Data are *n* (%), mean [SD] or median [IQR]. HCC: hepatocellular carcinoma; HE: hepatic encephalopathy; H&M: haematemesis and melaena. **$P < 0.001$ between each pair of groups, except *$P = 0.022$ between controls and cirrhosis.

Table 2. Risk of methylation of different genes on the development of HCC.

	HCC vs. cirrhosis	HCC vs. controls	Cirrhosis vs. controls
RASSF1A	9.75 (5.74–16.57) *P* = 0.001	4.9 (2.39–10.18) *P* < 0.001	0.51 (0.23–1.13) *P*= 0.09
RUNX3	5.32 (3.21–8.81) *P* < 0.001	4.79 (2.14–10.72) *P* < 0.001	0.90 (0.38–2.13) *P* = 0.81
E-Cadherin	2.68 (1.65–4.37) *P* < 0.001	5.15 (1.96–13.57) *P* = 0.003	1.9 (0.71–5.21) *P* = 0.20

Data are odds ratios (95% CI) with *P* value. HCC: hepatocellular carcinoma.

Table 3. Association between the degree of methylation of candidate genes in HCC in relation to stages of BCLC score.

		BCLC stage			
		A + B *N* = 137		C + D *N* = 427	
		N	%	*N*	%
RASSF1a	Hypo *N* = 81	23	16.8	58	13.8
	Hyper *N* = 107	22	16.1	85	19.9
E-Cadherin	Hypo *N* = 100	26	19.0	74	17.3
	Hyper *N* = 87	20	14.6	67	15.7
RUNX3	Hypo *N* = 123	30	21.9	93	21.8
	Hyper *N* = 66	16	11.7	50	11.7

BCLC: Barcelona Clinic of Liver Cancer; HCC: hepatocellular carcinoma. Failure of methylation occurred in five cases for *RASSF1a*, six cases for *E-Cadherin* and four cases for *RUNX*3. All differences not significant (*P* > 0.05).

Table 4 shows a comparison of AFP levels according to the degree of methylation of the three genes. The only significant difference was that AFP was higher in HCC patients with hypermethylation of *E-Cadherin*. Table 5 shows the performance of the hypermethylated *RASSF1A, E-Cadherin* and *RUNX3* as biomarkers for distinguishing HCC from cirrhosis.

Logistic regression analysis was performed (Table 6) for the prediction of HCC within cirrhotic cases with smoking, AFP and candidate genes as covariates. In multivariate analysis, *RASSF1A* and *E-Cadherin* predicted HCC within cirrhosis cases, but in patients with AFP <200 ng/ml, only *E-Cadherin* predicted HCC.

Discussion

In areas where hepatitis C infection is endemic, screening for early detection of HCC is a common clinical practice, but its effectiveness remains controversial [1]. While several non-invasive methods and scoring systems have been proposed for prediction of cirrhosis and determination of stages of fibrosis, there remains an unmet need for establishing predictors for the development of HCC in HCV cirrhotic patients [19–25].

Methylation of several genes has been shown to occur not only in HCC and its precursor lesions but also in different liver diseases during the process of cirrhosis, suggesting that these changes are early events during HCC development [7]. It has been suggested that there are actively induced HCC-

Table 4. Association between methylation status of candidate genes and AFP value within studied groups.

Gene methylation		Control		Cirrhosis		HCC	
		n	AFP	*n*	AFP	*n*	AFP
RASSF1a	**Hypo-**	43	1.8 (0.8–9.2)	180	24 (0.7–254)	80	36.4 (0.1–20248
	Hyper-	10	2.1 (1.1–4.1) $P = 0.48$	22	25.5 (4.9–222) $P = 0.43$	108	60 (1–5065) $P = 0.54$
E-Cadherin	**Hypo-**	44	1.8 (0.8–9.2)	175	25 (0.7–254)	100	102 (1–20248)
	Hyper-	8	2.15 (1.0–3.5) $P = 0.53$	26	12.9 (0.8–222) $P = 0.31$	87	39.7 (0.1–5065) $P = 0.02$
RUNX3	**Hypo-**	49	1.8 (0.8–9.2)	170	25 (1–254)	122	55 (0.1–20248)
	Hyper-	4	2.1 (1.6–3.5) $P = 0.32$	33	23.5 (0.7–222) $P = 0.36$	67	65 (1–9854) $P = 0.91$

Mann–Whitney test. AFP: alfa fetoprotein. Data median (range). Failure of methylation was occurred in some cases (five for *RASSF1a*, six for *E-Cadherin* and four for *RUNX3*).

Table 5. Diagnostic performance of methylation and AFP in discrimination between HCC and cirrhosis.

Variable (cut-off)	AUC	Sens.	Spec.	PPV	NPV
RASSF1A (>0.4)	0.77 (0.64–0.84)	64.4	86.7	82.9	70.9
E-*Cadherin* (>0.1)	0.78 (0.72–0.82)	83.9	59.5	67.7	78.6
RUNX3 (>0.2)	0.70 (0.64–0.75)	76.2	58.6	65.2	70.8
AFP (>124)	0.64 (0.57–0.69)	33.3	91.3	78.8	59.6

AUC: area under ROC curve; PPV: positive predictive value; NPV: negative predictive value; AFP: alpha fetoprotein; Sens.: sensitivity; Spec.: specificity.

Table 6. Regression analysis for prediction of HCC in cirrhosis.

	Univariate		Multivariate	
All patients	OR (95% CI)	*P*	OR (95% CI)	*P*
Smoking	1.57 (0.81–3.06)	0.18		
AFP	1.0 (0.1–1.01)	0.40		
RASSF1A	2.95 (1.18–7.41)	0.02	3.98 (1.43–11.1)	0.01
E-Cadherin	2.96 (1.53–13.76)	0.001	2.61 (1.04–14.39)	<0.001
RUNX 3	2.85 (0.84–9.68)	0.09		
Patients with AFP <200 ng/mL				
Smoking	1.88 (0.69–5.09)	0.214		
RASSF1A	2.77 (1.71–10.73)	0.041	1.93 (0.45–9.04)	0.108
E-Cadherin	2.18 (1.91–19.14)	0.003	5.29 (1.90–22.91)	0.01
RUNX 3	3.82 (1.45–18.46)	0.021	3.32 (0.53–14.32)	0.141

OR: odds ratio; CI: confidence interval; AFP: alpha fetoprotein.

specific epigenetic changes induced by HCV viral proteins, so identification of host epigenetic alterations may provide new possible biomarkers for early detection, as well as therapeutic targets for HCV-related HCC [26]. Many studies have been published on the prognostic value of DNA methylation in HCC [26–29]: the current study hypothesized epigenetic methylation changes in tumour suppressor genes *RASSF1A, E-Cadherin* and *RUNX3*, in patients with HCV-related liver cirrhosis and HCC that are linked to AFP and BCLC stage.

One of the more important tumour suppressor genes is *RASSF1A*, of which inactivation by DNA methylation is known to drive hepatocarcinogenesis [30]. Up-regulation of the methylation status of the *RASSF1A* promoter may be due to HCV [31]. In the current study, a significant elevation of methylation of *RASSF1A* was detected in HCC patients when compared to a control group, a finding that conforms with the results of Zhang et al. showing similar association of *RASSF1A* hypermethylation and positivity of HCV infection [10]. Similar results were reported in another cohort prospective study in Brazilian patients that was conducted to determine whether aberrant methylation of *RASSF1A* promoters was associated with the progression of liver disease [32]. These results reinforce the hypothesis that hypermethylation of *RASSF1A* contributes to hepatocarcinogenesis and is associated with clinicopathological characteristics. In addition, *RASSF1A* promoter hypermethylation may be a valuable biomarker for early diagnosis of HCC and a potential molecular target for epigenetic-based therapy [32]. Mansour et al. found that hypermethylated *RASSF1A* level was significantly higher in HCC on top of HCV compared to patients with chronic HCV infection without malignancy [29].

We report a significant elevation of hypermethylated gene of *E-Cadherin* in our HCC patients while Wei et al. observed significant hypermethylation of CpG islands in the promoter region of *E-Cadherin* in HCC [33], representing the most common cause of inactivation of *E-Cadherin* detected in many malignancies, including HCC [34,35]. Additionally, another study demonstrated methylation positivity of *E-Cadherin* in HCC tissues to be significantly higher than non-tumour tissues [34]. On a

similar note, methylation analysis studied in HBV-related HCC in Chinese patients revealed that significantly more *E-Cadherin* promoter methylation was detected in HCC tissue than adjacent non-tumour tissues [36]. Furthermore, Qiu et al. observed that repressed *E-Cadherin* expression by HBX (hepatitis B virus X protein) transfection due to the DNA methylation, which suggests a plan for restoring *E-Cadherin* by targeting its epigenetic mechanism [37].

Regarding the relationship between AFP and *RASSF1A* hypermethylation, findings of this study are in agreement with those by Mansour et al. reporting no significant link between the serum concentrations of AFP and *RASSF1A* [29]. However, when the regression analysis was conducted, *E-Cadherin* was found to be an independent risk factor for prediction of HCC with low AFP. As *E-Cadherin* is a major adherence junction protein, it is considered a new HCV host factor that not only improves the understanding of the first steps of HCV infection but also identifies tight junctions as a pathogenic relation between viral cell entry and HCC [38]. In addition, *E-Cadherin* regulates the localization of tight junction proteins CLDN1 and OCLN, facilitating HCV entry at a post-binding stage. *E-Cadherin* expression is downregulated during HCV infection, which alters the subcellular localization and expression of CLDN1 and OCLN. HCV infection activates *TGF-β*, which contributes to the induction of epithelial-to-mesenchymal transition (EMT) and an increase in EMT marker expression, including VIM, FN1 and N-Cad [39]. Reduced CLDN1 expression, which follows *E-Cadherin* downregulation, has been associated with HCC development [40].

We recognized several limitations, one being a cross-sectional study in a single centre and including a relatively small number of patients. The use of DNA methylation profiles as an alternate biomarker remains an active area of clinical cancer research. Nevertheless, our work represents an advance in medical science because it shows detection of hypermethylated *RASSF1A, E-Cadherin* and *RUNX3* may be a tool used in early detection of HCC.

Summary table

What is known about this subject:

- Chronic HCV infection recognized is a well-documented risk factor for the development of HCC.
- Methylation of several genes has been shown to occur not only in HCC and its precursor lesions but also in chronic hepatitis and liver cirrhosis.
- Several studies suggested a possible role of HCV viral proteins in epigenetic alterations, including the inactivation of tumour suppressor genes.

What this paper adds:

- The presence of hypermethylated *RASSF1A, RUNX3* and *E-Cadherin* may be useful biomarkers for early prediction of HCC HCV-related liver cirrhosis.
- Methylated *RASSF1A* and *E-Cadherin* are predictors of HCC within cirrhosis, but only *E-Cadherin* is a predictor of HCC in cases with low AFP.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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