Online ISSN: 2682-2628
Print ISSN: 2682-261X

IJCBR

International Journal of Cancer and Biomedical Research

https://jcbr.journals.ekb.eg

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Since 2014

INTERNATIONAL JOURNAL OF CANCER AND BIOMEDICAL RESEARCH (IJCBR)

RESEARCH ARTICLE

Correlating DNMT1, PCNA, and RB1 genes as new prognostic biomarkers in HCC patients with HCV

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ABSTRACT

Background: Hepatocellular carcinoma (HCC) can be successfully treated, and long-term survival rates can be significantly improved if diagnosed early enough. Understanding the molecular mechanisms underlying the onset and progression of HCC is critical for developing early detection methods and cutting-edge treatments. **Aim:** To establish DNMT1 as a noninvasive biomarker for the diagnosis and prognosis of HCV-induced HCC, we investigated how DNA methyltransferase 1 (DNMT1 (which controls DNA methylation patterns)) is associated with proliferating cell nuclear antigen (PCNA) expression levels and retinoblastoma susceptibility gene (RB1) expression levels in blood samples collected from HCV-induced HCC. **Methods:** A total of 109 HCV patients were included in this study. DNMT1 levels were measured by ELISA. PCNA and RB1 gene expression were measured using qRT-PCR. **Results**: DNMT1 levels increased consistently and significantly in all patients as the disease progressed toward the stage of HCC. As the disease progresses, PCNA and RB1 decrease gradually. DNMT1, PCNA, and RB1 were also able to discriminate between the studied groups in terms of their diagnostic abilities. As well, the DNMT1 gene may be a prognostic or predictive factor for cirrhosis and hepatocellular carcinoma. PCNA and RB1 can also be used as prognostic and predictive markers for HCC. **Conclusions:** The diagnostic and prognostic results demonstrated that DNMT1, RB1, and PCNA could be useful biomarkers for detecting and predicting HCC in patients with HCV infection**.**

Keywords: HCC, CLD, DNMT1, RB1, PCNA, Non-invasive biomarkers

Editor-in-Chief: Prof. M.L. Salem, PhD - Article DOI: 10.21608/JCBR.2024.252496.1325

ARTICLE INFO

Article history
Received: December 01, 2023
Revised: January 16, 2024
Accepted: February 21, 2024

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INTRODUCTION

Hepatocellular carcinoma (HCC) predominantly represents the major histological type (about 80-90%) of liver cancer. It is also a leading cause of cancer-related deaths around the world. The incidence of HCC was shown to be highest in Eastern Asia and Africa (McGlynn *et al*., 2021; Tang *et al*., 2018). HCC has been increasing in the U.S. and countries in the West over the last forty years (McGlynn *et al*., 2021; Fenton *et al*., 2021). Several underlying risk factors contribute to the wide prevalence of HCC. Consequently, this leads to chronic liver cirrhosis and, ultimately, liver cancer (McGlynn *et al*., 2021; Tang *et al*., 2018; Mak *et al*., 2018).

Especially in developing countries, HCC is commonly associated with chronic hepatitis C virus infection (HCV). About 40% of HCV patients develop HCC as a long-term complication after 10-15 years (Tanaka *et al*., 2008). According to the WHO, approximately 3% of the world's population is infected with HCV (Hoshida *et al*., 2014). Lovet *et al*. (2021) and their group report that the link between HCV infection and HCC has begun to clear (Llovet *et al*., 2021). Some data suggested that the expression of structural and non-structural viral proteins such as nonstructural proteins 3 (NS3) and Core, E1, and E2 as structural proteins, may enhance tumorigenesis and transformation of hepatic cells. The inflammatory response and viral replication cause oxidative stress, which has been identified as a key contributor to cancer development. Overproduction of free radicals causes DNA damage, which in turn creates a series of transcriptional changes in genes controlling cell proliferation, tumor suppression, cell survival, and angiogenesis (Llovet *et al*., 2021; Fu *et al*., 2017).

Epigenetic modifications, including DNA hypermethylation, are well recognized as a hallmark of cancer and significantly promote genomic instability inside cancer cells (Arzumanyan *et al*., 2013). DNA hypermethylation, a chemical modification of the 5' cytosine-phosphate-guanine 3' islands (CpG), is accompanied by transcriptional repression that may irreversibly lead to the silencing of tumor suppressor genes (Hanahan D and Weinberg, 2011; Bird, 2002). Interestingly, HCV was found to upregulate DNA methyltransferases (DNMTs) in HCV core protein expressing cells (Esteller *et al*, 2008); which in turn abrogated the expression of genes responsible for the DNA repair system, resulting in increased genomic instability and mutagenesis. In fact, elevated expression of both DNMT1 and DNMT3b was clinically correlated with a bleak HCC prognosis (Benegiamo *et al*., 2012; Toyota and Suzuki, 2010; Oh *et al*., 2007).

The first tumor suppressor gene to be discovered was RB1. It negatively controls cell growth and proliferation. Notably, *RB1* gene mutations contribute to various forms of retinoblastoma and several types of cancer (Dyson, 2016). Moreover, the non-structural HCV proteins trigger the ubiquitination of the RB1 protein (Sheng *et al*., 2021).

PCNA has been introduced as a molecular marker for cell proliferation. It helps in the recruitment of proteins required for the formation of the replication fork. Targeting PCNA appears to be an appealing approach for cancer therapy because PCNA is essential for cancer cell proliferation. As a tumor marker, PCNA expression level is currently routinely employed (Wang, 2014; Shen *et al*., 2021). Previously, meta-analysis identified a correlation between the aberrant methylation patterns of several genes and HCC incidence; suggesting that aberrant DNA methylation patterns can provide an efficient tool for HCC prediction and diagnosis (Zhang *et al*., 2016).

In this research, we aimed to elucidate the association between DNMT1 as a major regulator of the methylation pattern and PCNA and RB1 gene expression levels in blood samples collected from HCV-induced HCC. They can be used as non-invasive indicators for prognosis and diagnosis.

METHODS

An investigation of chronic liver disease (CLD) patients without HCC, including HCV patients without cirrhosis and HCV patients with cirrhosis, as well as CLD patients with HCC who were admitted to the Department of Gastroenterology and Hepatology in Giza, Egypt between November 2018 and August 2020 has been approved by the Theodor Bilharz Research Institute Research Ethics Committee (TBRI-REC). Data from the previous study suggest that the standard deviation of control will be 0.7 and that the standard deviation for regression errors will be 1.8. If the true slope of the line obtained by regressing patients against control is 1.7, we will need to study 20 subjects for each group to be able to reject the null hypothesis that this slope equals zero with a probability (power) of 90%. As a control, another twenty healthy individuals were recruited. All patients and controls signed informed consent forms in accordance with Helsinki Declaration in 1975. A local ethical committee issued the approval number (TBRI-REC number PT 674).

Criteria for inclusion: It should be noted that all patients had chronic hepatitis C and had not been treated in the past six months. Cirrhosis was diagnosed in all patient groups using AST Platelet Ratio Index (APRI) scores and Fibrosis-4 (FIB 4), as well as laboratory findings of hypoalbuminemia and hypoprothrombinemia. Cirrhosis was also diagnosed using ultrasonographic characteristics like (coarse echo pattern, surface irregularity, portal vein diameter, existence or absence of ascites, and splenic size). Abdominal ultrasound and CT and/or MRI reported the existence of focal hepatic lesions consistent with the European Association for the Study of the Liver (EASL) and American Association for the Study of Liver Diseases (AASLD) (Marrero *et al*., 2018; EASL, 2019) guidelines for the diagnosis of hepatocellular carcinoma (EASL, 2019).

Criteria for exclusion: Exclusion criteria included patients who have had schistosomiasis in the past or other long-term viral diseases than hepatitis C, Co-infection with hepatitis B virus (HBV) "HCV dual infection", nonalcoholic steato-hepatitis (NASH), disorders of the bile ducts, autoimmune hepatitis, Other malignant neoplasms than HCC, Hepatotoxic drugs regularly used, diabetes, alcoholism, and patients with hepatitis C virus infection who had received or were receiving direct-acting antivirals (DAAs) treatment.

A full clinical examination, physical examination, abdominal ultrasound, and laboratory assessment were performed on all patients and control subjects. Tests included CBCs, liver function tests (LFTs), kidney function tests (KFTs), alpha-fetoprotein tests (AFPs), coagulation testing, prothrombin times (PTs), prothrombin concentration tests (PCs), and international normalized ratios (INRs). According to the inclusion/exclusion criteria, the study included 109 patients. A total of 89 chronic hepatitis C patients were divided into those without HCC (n = 66) and those with HCC (n = 23). In Group I CLD without HCC, there were 34 non-cirrhotic and cirrhotic subgroups (32). As a second check, the researchers used a healthy control group of twenty volunteers in good physical health of the same gender and age.

DNMT1 Protein level: Serum protein level; Human DNMT1 ELISA kit Antibodies-online Inc. USA, Cat. No: (PRS-02439hu) was used to detect the level of DNMT1. https://www.antibodies-online.com.

Target gene expression: The RNA extraction was carried out using a high-purity RNA isolation kit (version 12, 2011) (Cat. No: #11828665001). For the detection of gene expression, the HERA SYBR Green RT-qPCR kit was used (version 08hp421191), Cat. No: (WF10303001). The primer sequences are depicted in (Table 1). The results of the Light Cycler HERA SYBR Green I master SYBR green I filter combination (465–510) were analyzed. Comparative threshold (CT) was used to analyze the data. Housekeeping gene B-actin (used as an endogenous control to normalize the total mRNA levels in each sample) of PCNA and RB1 between different samples. When calculating gene expression, this was done using the formula $2^{-\Delta\Delta CT}$ (www.bitesizebio.com).

Statistical analysis: SPSS 24.0 for Windows was used to conduct the statistical evaluation of the data (SPSS IBM., Chicago, IL). In the case of continuous normally distributed variables, means and standard deviations (SD) were presented along with 95% confidence intervals; categorical variables were presented as frequencies and percentages; a p-value of 0.05 was considered statistically significant. When comparing groups of non-normally distributed variables, Mann–Whitney tests and Kruskal–Wallis H tests were applied, when comparing groups with normally distributed variables, ANOVA tests were applied, and Fisher's exact tests were applied to identify group differences in categorical variables. By using receiver operating characteristic (ROC) curves, we assessed DNMT1, PCNA, and RB1 diagnostic performance. An area under the receiver operating characteristic curve (AUC) was used to determine a test's prognostic accuracy. In one group of the study, maximum sensitivity and specificity were taken as the cutoffs for diagnosis. The correlation coefficient (r) was calculated using Spearman's rank. To identify genes associated with an increased risk of developing HCC, a logistic regression analysis was used.

RESULTS

Group-specific demographic and routine lab data were presented in table format (Table 2). There is a significant (p-value = 0.001) difference between all diseased groups and control groups in terms of DNMT1 protein levels. The cirrhotic and HCC groups, however, had significantly higher DNMT1 protein levels than the HCV group (p-values = 0.004, 0.001, respectively). In addition, DNMT1 protein levels were significantly higher in the HCC group than in the cirrhotic group (p-value 0.001), as well as in CLD cases overall (p-value 0.001). (Table 3, Figure 1a).

The expression of RB1 and PCNA genes was significantly downregulated (p-values 0.001, 0.001, respectively) in all diseased groups compared to the control group. In comparison with the HCV group, the cirrhotic (p = 0.006, 0.01) and HCC (p = 0.001, 0.001) groups showed substantially lower expression of RB1 and PCNA. The HCC group had significantly lower levels of RB1 and PCNA expression (p-values = 0.01, 0.04, respectively) compared to the cirrhotic group, as well as in comparison to CLD cases in general (p-values = 0.001, 0.001, respectively) (Table 3, Figures 1b, 1c).

Diagnostic efficiency has been assessed using the ROC curve for gene expression as an indicator of cirrhosis and HCC at various cutoff points. In Table 4, the cut-off points and the calculated sensitivity, and specificity of the studied biomarkers for identifying research groups are presented. For discrimination of HCV versus the control group, regarding DNMT1, the AUC is 0.825 (95% CI 0.697–0.915, p-value <0.0001), while for RB1, the AUC is 0.809 (95% CI 0.679–0.903, p-value <0.0001), and for PCNA, the AUC is 0.841 (95% CI 0.716–0.926, p-value <0.0001). However, the overall combined ROC for all three studied candidates (DNMTI, RB1, PCNA) showed that the AUC is 0.941 (95% CI 0.862 - 1.00, p-value <0.0001), but the AFP did not show a significant difference for discrimination of HCV versus the control group (Figure 2a). While in the cirrhotic group versus the HCV group, DNMT1 showed an AUC of 0.669 (95% CI 0.542–0.780, p-value = 0.0112), whereas for RB1, the AUC was 0.697 (95% CI 0.571–0.804, p-value = 0.0037), and for PCNA, the AUC was 0.686 (95% CI 0.560–0.794, p-value = 0.0052). Interestingly, the overall combined ROC for all three studied candidates (DNMTI, RB1, PCNA) showed that the AUC was 0.682 (95% CI 0.554 - 0.810, p-value = 0.005), while the AFP showed that the AUC was 0.878 (95% CI 0.792 - 0.963, p-value <0.0001) (Figure 2b).

With regards to the distinction between the HCC and the cirrhotic groups, the results indicated that the AUC of DNMT1 was 0.784 (95% CI 0.652–0.884, p-value 0.0001), while for RB1, the AUC was 0.704 (95% CI 0.565–0.819, p-value = 0.0053), and for PCNA, 0.663 was the AUC (95% CI 0.523–0.785, p-value = 0.0315). Remarkably, the overall combined ROC for all three studied candidates (DNMTI, RB1, PCNA) showed that the AUC was 0.913 (95% CI 0.840 - 0.986, p-value <0.0001), while the AFP showed that the AUC was 0.840 (95% CI 0.723 - 0.956, p-value <0.0001) (Figure 2c). Also, on discrimination between HCC groups versus CLD cases in general, the results indicated that the AUC of DNMT1 was 0.825 (95% CI 0.730–0.898, p-value 0.0001), while for RB1, the AUC was 0.803 (95% CI 0.705–0.880, p-value 0.0001), and for PCNA, the AUC was 0.751 (95% CI 0.648–0.837, p-value 0.0001). Interestingly, the overall combined ROC for all three studied candidates (DNMTI, RB1, PCNA) showed that the AUC was 0.758 (95% CI 0.611 – 0.906, p-value <0.0001), while the AFP showed that the AUC was 0.919 (95% CI 0.856 – 0.983, p-value <0.0001) (Figure 2d). This finding indicates that DNMT1, RB1, and PCNA are close to the gold standard method "AFP" and can be used as significant diagnostic parameters for identifying cirrhotic patients from HCV patients, HCC patients from cirrhotic patients, and HCC cases from CLD patients.

DNMT1, RB1, and PCNA were characterized as predictors and/or prognostic parameters for cirrhosis progression using a univariate logistic regression analysis. The odds of developing cirrhosis increased by 1.811 with an increase of 1 degree of DNMT1, with a p-value of 0.008. PCNA and RB1 cannot predict cirrhosis progression. In terms of the prognostic value of DNMT1, RB1, and PCNA for HCC progression, DNMT1 showed an increase of 1 degree. This was coupled with a p-value of 0.001, which enhanced the HCC odds by 2.093.

Table 1. Oligonucleotides seq. used in RT-PCR

	Sequence (5'->3')	Template strand	Tm	Product length	References
PCNA					
Forward primer	CAGTTCCCTTAGCAGCCCAG	Plus	57.0	310	(https://www.ncbi.nlm.nih.gov)
Reverse primer	AATCGCACACTGAAACGCAC	Minus	57.0		
RB1					
Forward primer	AGGTGGTGATGGTGATGCTAC	Plus	57.0	250	(https://www.ncbi.nlm.nih.gov)
Reverse primer	TTCTAGCTGAGCAGGGAACA	Minus	57.0		
B-actin					
Forward primer	GCACCACACCTTCTACAATG	Plus	57.0	-	(http://hgsv.washington.edu)
Reverse primer	TGCTTGCTGATCCACATCTG	Minus	57.0		

Table 2. Demographic characteristics and laboratory investigations of the studied groups

		CLD N= 66		HCC N= 23	*P*. value
		HCV N=34	Cirrhosis N=32		
Age		48.1±9.8	58.5±9.2	57.5±6.8	0.001**
Gender	**Female**	21(61.8%)	16(50.0%)	6(26.1%)	0.03*
	Male	13(38.2%)	16(50.0%)	17(73.9%)	
Albumin		4.4±0.6	2.7±0.8	2.2±1.0	0.001**
T. Bilirubin		0.9(0.6- 1)	1.5(0.8- 3.1)	1.9(1.6- 5.4)	0.001**
D. Bilirubin		0.3(0.2- 0.3)	0.5(0.3- 1.5)	1.2(0.5- 2.3)	0.001**
ALT		38.5(15- 55)	45(22.8- 84)	59(38- 87)	0.003**
AST		34.5(17.8- 44.3)	51(32.5- 67.5)	58(24- 108)	0.002**
AFP		4.4(3.3- 6)	16.9(12.8- 19.7)	156(30.2- 320)	0.001**
UREA		36.5(29- 41)	52(45- 55)	52(48- 57.7)	0.001**
CREAT		0.7(0.7- 0.8)	1.3(1.1- 1.5)	1.1(0.9- 1.3)	0.001**
PT		14.3(12.4- 18.8)	15.1(14.2- 18.1)	17(14.8- 19)	0.09
PC		49.6(45- 59.2)	71.6(50- 79.5)	71.5(60- 78)	0.07
INR		1.1(1- 1.5)	1.3(1.1- 1.6)	1.4(1.2- 1.6)	0.14
HB		12.2±2.0	11.0±2.1	10.8±2.1	0.02*
WBCs		7.4±2.1	6.5±2.6	7.9±4.1	0.2
Platelets		212.8±60.2	107.8±65.2	100.1±35.9	0.001**
APRI Score		0.4±0.2	1.7±0.8	2.1±1.0	0.001**
FIB.4 Value		1.3±0.6	5.6±3.6	7.1±4.8	0.001**
FIB.4 score	**F0 = <1.45**	22(64.7%)	0(0.0%)	0(0.0%)	0.001**
	F 1-2 = (1.45 - 3.25)	12(35.3%)	12(37.5%)	0(0.0%)	0.001**
	F 3-4 = ≥3.25	0(0.0%)	20(62.5%)	23(100.0%)	0.001**

Age, Albumin, HB, WBCs, Platelets, APRI score and FIB.4 Value are represented as mean ± SD; the data were analyzed by ANOVA Test. But gender and FIB.4 scores are represented as frequency and percent; the data were analyzed by X2 Test. While total bilirubin, direct bilirubin, ALT, AST, AFP, Urea, Creat., PT, PC and INR are represented as the median and interquartile range (25–75%); the data were analyzed by Kruskal–Wallis Test. APRI score calculated regarding AST to Platelet Ratio Index (APRI) = [AST Level (IU/L)/ AST (Upper Limit of Normal) (IU/L)]/ Platelet Count (109/L) X100. (Normal < 0.05, CLD without cirrhosis 0.5–1.5 and Cirrhosis ≤ 1.5). while FIB.4 calculated regarding Fibrosis-4 (FIB-4) score = Age (years) x AST Level (U/L) / Platelet Count (10^9/L) x vALT (U/L). (F0 = <1.45, F 1-2 = (1.45 - 3.25), F 3-4 = ≥3.25). *P value < 0.05 is significant, ***P* value < 0.01 is highly significant

Table 3. Descriptive of the studied biomarkers in the studied groups

	Control N=20	CLD N= 66		HCC N= 23
		HCV N=34	Cirrhosis N=32	
DNMT1 "Protein level"	1.21±0.37	2.82±1.50 [aa]	3.79±1.06 [aa, b]	6.11±2.44 [aa, bb, cc, **]
RB1 "Gene expression"	1	0.022(0.012 - 0.249) [aa]	0.01(0.003 - 0.019) [aa, b]	0.005(0.002 - 0.006) [aa, bb, c, **]
PCNA "Gene expression"	1	0.094(0.014 - 0.31) [aa]	0.05(0.009 - 0.09) [aa, b]	0.013 (0.006 - 0.021) [aa, bb, c, **]

Gene expressions of RBI and PCNA genes are calculated depending on the fold-change law: Fold-Change ($2^{-\Delta\Delta CT}$) is the normalized gene expression ($2^{-\Delta\Delta CT}$) in the Test Sample divided the normalized gene expression ($2^{-\Delta\Delta CT}$) in the Control Sample. (Fold-change values less than 1 indicate a negative or downregulation). DNMT1 is represented as mean ± SD; the data were analyzed by t-Test. While RB1 and PCNA are represented as Median with Interquartile range (25–75%) of the fold-change of the studied groups, the data were analyzed by Mann–Whitney U test. *[a]P value* is significantly different compared with the control group. *[b]P value* is significantly different compared with HCV group.*[c]P value* is significantly different compared with Cirrhosis group.*[*]P value* is significantly different compared with CLD cases.
[One Initial] p value < 0.05 is significant. [Two Initial] *p* value < 0.01 is significant.

Table 4. Diagnostic performance of the studied genes to discriminate between the studied groups.

		Cut-off	Sn.	Sp.	AUC	95% C. I	*P*. value
HCV Vs Control	DNMT1	>1.75	79.41	100.00	0.825	0.697 - 0.915	<0.0001**
	RB1	>0.0145	70.59	90.00	0.809	0.679 - 0.903	<0.0001**
	PCNA	>0.0433	67.65	100.00	0.841	0.716 - 0.926	<0.0001**
	Combined (DNMTI, RB1, PCNA)	--	94.00	100.00	0.941	0.862 - 1.00	<0.0001**
	AFP	>4.4	50.00	70.00	0.626	0.472 - 0.779	0.108
Cirrhosis Vs HCV	DNMT1	>2.1	100.00	29.41	0.669	0.542 - 0.780	0.0112*
	RB1	≤0.0112	71.87	76.47	0.697	0.571 - 0.804	0.0037**
	PCNA	≤0.00908	37.50	100.00	0.686	0.560 - 0.794	0.0052**
	Combined (DNMTI, RB1, PCNA)	--	61.00	60.00	0.682	0.554 - 0.810	0.005**
	AFP	>13.65	59.00	89.00	0.878	0.792 - 0.963	<0.0001**
HCC Vs Cirrhosis	DNMT1	>4.9	69.57	90.62	0.784	0.652 - 0.884	<0.0001**
	RB1	≤0.00899	100.00	56.25	0.704	0.565 - 0.819	0.0053**
	PCNA	≤0.0269	95.65	56.25	0.663	0.523 - 0.785	0.0315*
	Combined (DNMTI, RB1, PCNA)	--	83.00	72.00	0.913	0.840 - 0.986	<0.0001**
	AFP	>87.9	65.20	83.6	0.840	0.723 - 0.956	<0.0001**
HCC Vs CLD	DNMT1	>4.9	69.57	95.45	0.825	0.730 - 0.898	<0.0001**
	RB1	≤0.00899	100.00	66.67	0.803	0.705 - 0.880	<0.0001**
	PCNA	≤0.0269	95.65	62.12	0.751	0.648 - 0.837	<0.0001**
	Combined (DNMTI, RB1, PCNA)	--	74.00	70.00	0.758	0.611 – 0.906	<0.0001**
	AFP	>87.9	65.20	92.00	0.919	0.856 – 0.983	<0.0001**

Sn: Sensitivity, Sp: Specificity, PPV: Positive predictive value, NPV: negative predictive value, AUC Area under the curve and C.I: 95% Confidence Interval. * *p* value <0.05 is significant, ** *p* value <0.01 is highly significant.

Table 5. Univariate analysis showing the predictive power of different biomarkers between the studied groups

		OR	95% C. I		*P*. value
			Lower	Upper	
HCV Vs Control	DNMT1	3.200	1.621	6.317	0.001**
	RB1	4.62	0.001	30.55	0.208
	PCNA	2.305	0.449	11.809	0.013*
Cirrhosis Vs HCV	DNMT1	1.811	1.168	2.807	0.008*
	RB1	0.605	0.201	1.823	0.372
	PCNA	0.811	0.552	1.192	0.287
HCC Vs Cirrhosis	DNMT1	2.093	1.363	3.214	0.001**
	RB1	2.087	0.786	4.781	0.038*
	PCNA	2.586	1.247	5.024	0.01*
HCC Vs CLD	DNMT1	2.487	1.612	3.837	0.001**
	RB1	3.021	1.564	4.854	0.002**
	PCNA	3.245	1.873	6.357	0.01*

OR: Odd Ratio, C.I; Confidence Interval, p value of Prognostic viability is calculated depending on logistic regression analysis. * *p*. value <0.05 is significant, ** *p*. value <0.01 is highly significant.

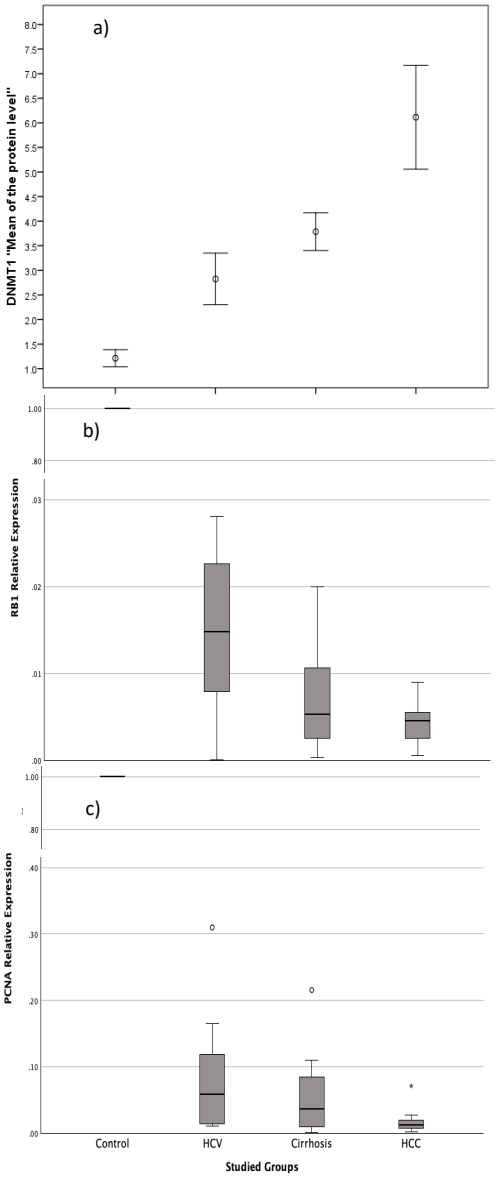

Figure 1. Mean of DNMT1 and Box plot of RB1 and PCNA in the studied groups. (a) DNMT1 Protein level. (b) RB1 Gene expression. (c) PCNA Gene expression

Increases in RB1 expression by 1 degree increased the odds of being HCC by 2.087; the p-value for this was 0.038. With an increase of 1 degree in PCNA expression level, the odds of developing HCC increased by 2.586, with a p-value of 0.01.

The CLD group had an increase in 1 degree of DNMT1, resulting in a 2.487-fold increase in the odds of HCC. By increasing 1 degree of expression of RB1, the odds of being HCC increased by a factor of 3.021 with a p-value of 0.002. While increasing 1 degree of PCNA expression level increased the odds by 3.245 with a p-value of 0.01 (Table 5).

According to the correlation analysis, DNMT1 and RB1 are significantly inversely correlated (r=-0.233 and p = 0.028) (Figure 3a). However, there was no correlation between DNMT1 and PCNA. While there is a significant direct correlation between RB1 and PCNA (r=0.487 and p-value = 0.001) (Figure 3b).

DISCUSSION

There are many causes of HCC, which is the most common type of liver cancer (Villanueva, 2019; McGlynn *et al*., 2021). HCC induction has been reported as a long-term complication in HCV-infected patients (Tanaka *et al*., 2008; Hoshida *et al*., 2014). Accordingly, several studies were performed to identify the main driver of genetic alterations that emerge from the heterogeneous molecular landscape involved in HCC initiation and progression. Epigenetic reprogramming of several genes has been recognized as a prominent contributor to multistep carcinogenesis (Fernández-Barrena *et al*., 2020). A variety of chromatin modifications, including methylation, hypomethylation, acetylation, and deacetylation, are used to accomplish this reprogramming. Methylation of DNA is the major epigenetic modification that affects histone dynamically, leading to the formation of closed chromatin structures and thus the silencing of targeted genes (Jones, 2012; Skvortsova *et al*., 2019). The DNA methyltransferase family, including DNMT1, DNMT3A, and DNMT3B canonical enzymes, plays a traditional role in maintaining DNA methylation patterns (Lyko, 2018).

Therefore, the present study aims to determine if DNMT1 as a chromatin modeler, RB1 as a tumor suppressor, and PCNA as a proliferation marker, are related to HCV-induced HCC. As compared to the control group, we found significant increases in the levels of DNMT1 protein expression in the diseased groups (HCV without cirrhosis, HCV with cirrhosis, and HCC). The evidence suggests that progressive upregulation of DNMT1 results in a hypermethylation epigenetic pattern, resulting in a perplexed transcriptome, ultimately leading to HCC development, as previously reported (El-Araby *et al*., 2020; Arora *et al*., 2008; Nagai *et al*., 2003).

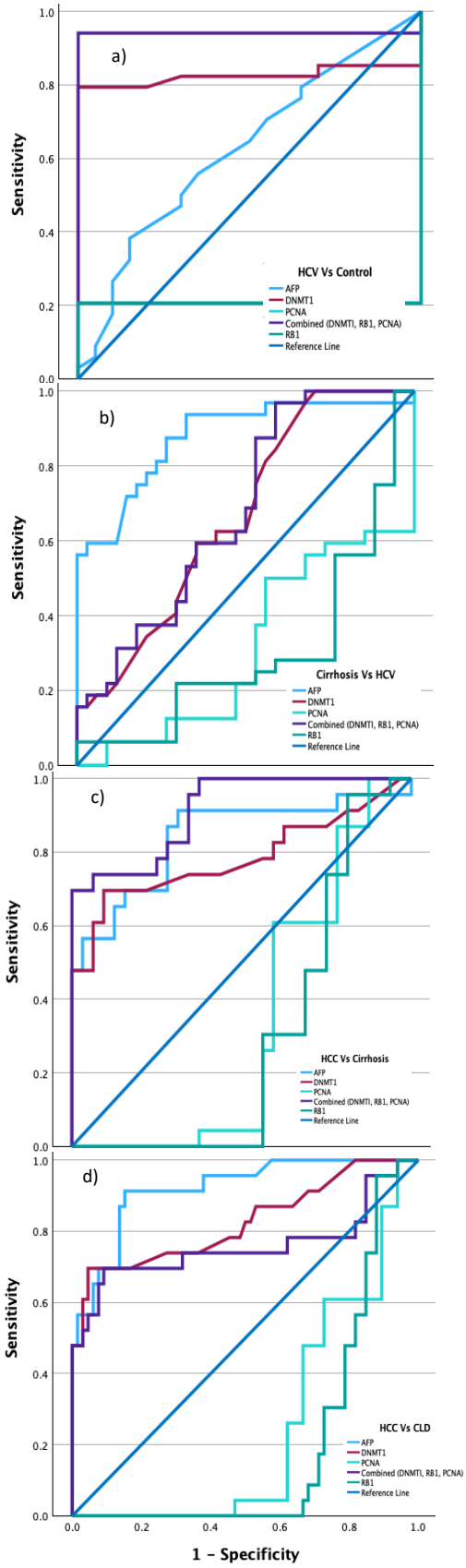

Figure 2. ROC Curve for the studied genes in the studied groups. a) HCV group vs. Control group, b) Cirrhosis group vs. HCV group, c) HCC group vs. Cirrhosis group, d) HCC group vs. CLD group.

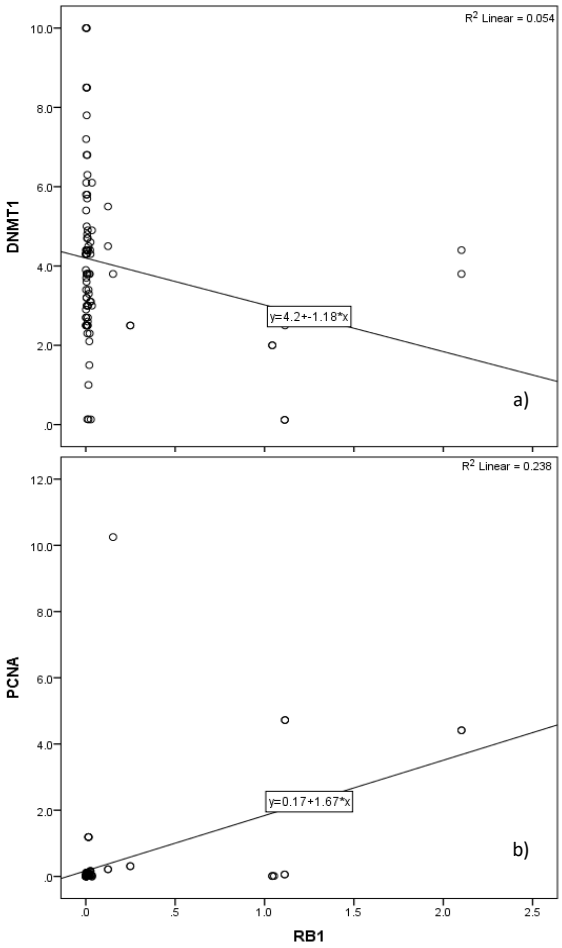

Figure 3. The correlation between the studied biomarkers. a) Correlation between DNMT1 and RB1, b) Correlation between RB1 and PCNA.

When compared to the control group, the diseased groups' expression of the RB1 gene was much lower, also accompanied by an overexpression of DNMT1. It was previously shown that miR-106b, which is generated by the hepatitis B antigen (HBeAg), encourages cell proliferation by suppressing the expression of the RB gene (Samal *et al*., 2017). As well, HCV-1a core+1/ARFP promotes cell proliferation and carcinogenesis by phosphorylating RB (Moustafa *et al*., 2018). In this context, our data suggest that silencing of the RB1 tumor suppressor may induce HCC in HCV-infected patients.

Unexpectedly, we found that PCNA gene expression was significantly decreased in all diseased groups compared to the control group. PCNA is well recognized as a cell cycle marker involved in many biological processes, including DNA replication, metabolism, repair, chromatin assembly, and cell cycle control (Juríková *et al*.,

2016). In addition, PCNA expression was previously reported to be increased in hepatocellular carcinomas but inversely correlated with tumor size (Francalanci *et al*., 2020). However, we can explain the difference in our findings by saying that the sample used in the present study is blood, not a tissue biopsy. So, our data does not reflect the actual transcriptional events occurring in the liver during cirrhosis or HCC development. On the contrary, it may shed light on the para-neoplastic transcriptional machinery in blood cells of HCV-induced HCC.

Moreover, we also found a significant difference in DMNT, RB1, and PCNA expression levels among the diseased groups themselves. So, we examined whether the expression levels of these genes can be used as a diagnostic tool for discrimination among normal, HCV-without-cirrhosis, HCV-cirrhotic, and HCV-induced HCC patients. In this situation, the ROC curve was used to examine the diagnostic implementation of the studied genes expressed as biomarkers in cirrhotic cases. This was done at various cutoff values, as shown in Table 4. According to our interpretation of the ROC curve results, we found that the levels of DMNT protein rise in lockstep with the progression of the disease, starting with HCV infection without cirrhosis, HCV with cirrhosis, and finally HCC groups in a consistent process (1.75, p-value <0.0001), (>2.1, p-value 0.0112), and (> 4.9, p-value 0.0001), respectively.

On the contrary, RB1 and PCNA expression levels were abrogated progressively with the progression of the disease. As regards RB, the discrimination values of (HCV versus control group), (cirrhotic group versus HCV group), and (HCC group versus cirrhotic group) were (0.903, p-value 0.0001), (0.804, p-value = 0.0037), and (0.819, p-value = 0.0053), respectively. In the same way, for PCNA, the discrimination values of HCV versus control group, cirrhotic group versus HCV group, and HCC group versus cirrhotic group were found to be (0.926, p-value 0.0001), (0.794, p-value = 0.0052), and (0.837, p-value 0.0001), respectively.

Interestingly, there was significant discrimination between the studied groups according to the combined ROC analysis for DNMT1, RB1, and PCNA. Accordingly, after comparing with the gold standard methods (AFP) our data strongly suggest that DNMT1, RB1, and PCNA can be used as delicate and sensitive parameters not only for the diagnosis of HCV-induced HCC but also for the categorization of HCV patients according to their current clinical status. However, the findings of this study must be confirmed by carrying out the same study with a larger sample size.

Next, our regression analysis found that the expression levels of our genes of interest were suitable for use as a significant prognostic and predictive indicator of complications associated with HCV-chronic liver disease. Elevated DNMT1 levels significantly increased the odds of cirrhosis and HCC development in HCV patients. On the other hand, diminished RB1 and PCNA levels were strongly significant and increased the risk of HCC development. However, they were not indicative of cirrhosis in HCV patients.

We expanded our investigation to see whether DNMT1 hypermethylation is the root cause of abrogated RB1 and PCNA amounts. Our correlation analysis revealed that DNMT1 and RB were persistent among all diseased groups. We found that RB expression level is inversely proportional to DNMT1 protein expression level. Also, we found that both RB and PCNA expression levels were directly proportional among all diseased groups. However, there was no correlation between DNMT1 and PCNA expression levels. This suggests that a cumulative rise in the production of DNMT1 protein is concurrent with progressive hypermethylation of the downstream target RB1. This ultimately results in RB1 silence. These results are in line with a prior investigation that found that inhibiting DNA methyltransferases causes a concurrent rise in RB gene expression levels (Selvakumar *et al*., 2012). In addition to the association between the DNMT1 gene and the PCNA and RB1 genes, bioinformatics gene network analysis using GeneMANIA (http://www.genemania.org) revealed a large number of functionally related genes, including CDKNA, FEN1, and CCND1, as well as many functionally related genes associated with DNMT1, PCNA, and RB1.

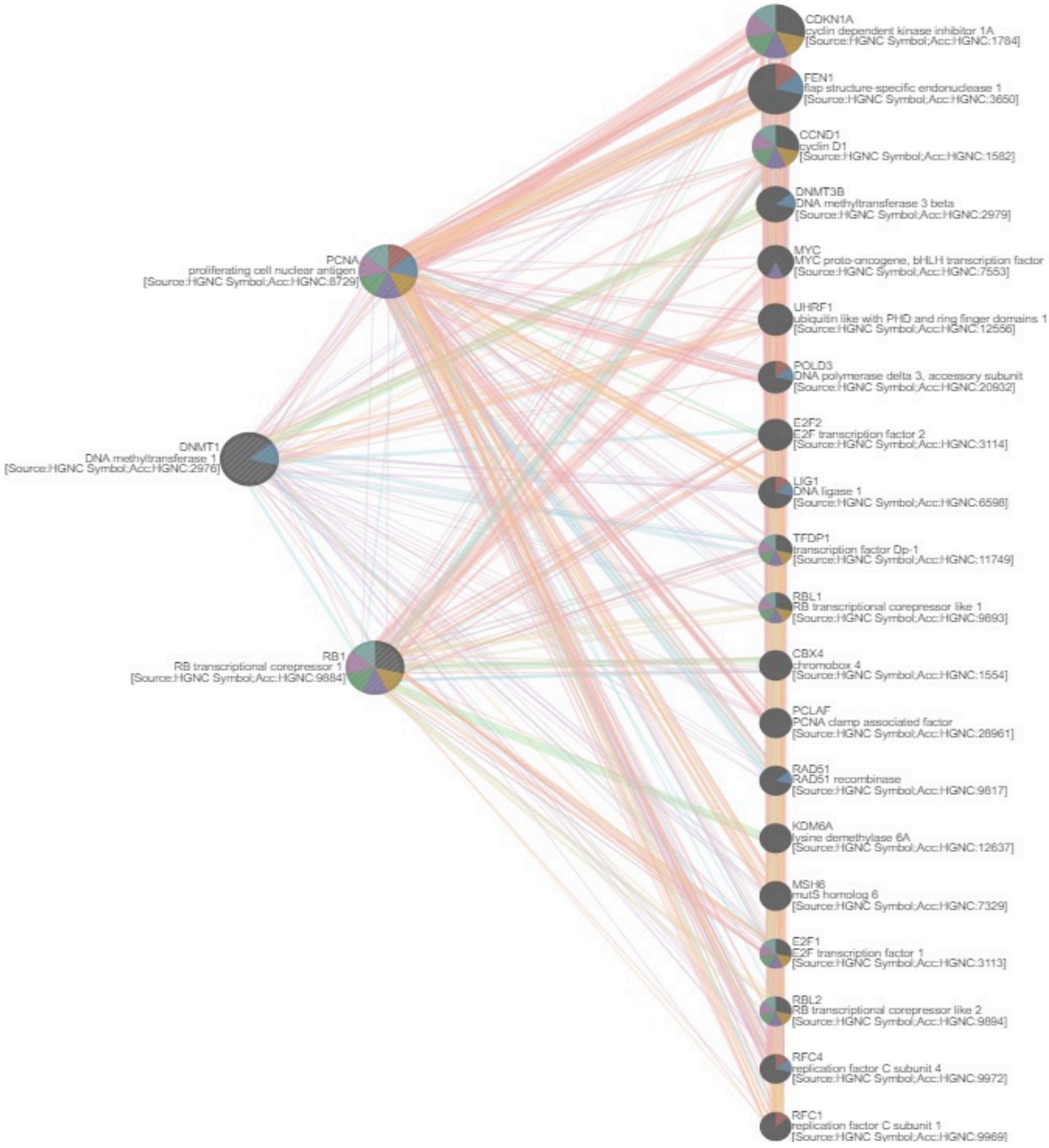

Figure 4. GeneMANIA: showing possible functional related genes to DNMT1, PCNA and RB1 in HCC disease. In addition to their interaction between them.

This could point to an emerging connection between DNMT1, PCNA, RB1, and HCC development (Figure 4). Additionally, they interact with each other.

CONCLUSION

Finally, the present study strongly suggests that DNMT1, RB1, and PCNA are powerful biomarkers for diagnosis and can predict HCC in HCV-infected individuals. As using blood samples for HCC diagnosis is beneficial for chronic hepatic patients who are mostly ineligible for biopsy. Acquisition of tissue samples from malignant hepatic HCC focal lesions is not always feasible due to several problems inherent in biopsy, like hemorrhage, needle tracking seedlings, and patient acceptance (Di Tommaso L *et al*., 2019). A larger group of patients is needed for further studies. As well as following up on and tracking the responsiveness of HCC patients, these biomarkers may also be useful.

AUTHOR CONTRIBUTION STATEMENTS

The key hypotheses of this study were established by 1st and 2nd authors. The molecular biology practical section was done by the following authors: 1st, 2nd, 9th, 10th, 11th, and 12th. The major manuscript texts were written by the 1st, 2nd, 3rd, and 13th. The results were prepared by the 1st, 2nd, and 3rd authors. The clinical laboratory investigations with the interpretations were prepared by the 4th, 5th, 6th, 7th, and 8th authors. The manuscript was reviewed by all the authors who participated.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

FUNDING

This work did not receive any funds.

AVAILABILITY OF DATA

Our data will be available upon reasonable request.

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