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Apa1 (rs7975232) SNP in the vitamin D receptor is linked to hepatocellular carcinoma in hepatitis C virus cirrhosis

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ABSTRACT

Background: As hepatocellular carcinoma (HCC) arising from chronic hepatitis C virus (HCV) infection in liver cirrhosis is a major problem in public health, early and rapid prediction of HCC is urgent. We hypothesized that a single nucleotide polymorphism in the *Apa1* SNP in the vitamin D receptor may help diagnosis.

Methods: We recruited 3 groups: 80 HCC patients with HCV cirrhosis, 80 HCV cirrhotic patients free of HCC and 80 healthy controls. *Apa1* rs7975232 SNP was detected by PCR- RFLP technique. Routine laboratory markers were determined by standard methods.

Results: The Apa1 CC genotype was more frequent (75%) in HCC than in the cirrhosis (35%) and control (20%) groups ($P<0.0001$). CC patients were more likely to have a more severe Child-Pugh score ($P=0.027$) and MELD score ($P<0.05$). In multivariate analysis, the CC genotype outperformed AFP is determining HCC.

Conclusion: Apa1 CC genotype is linked to HCC in HCV C cirrhotic patients, and so has the potential to be an independent biomarker predictor for HCC occurrence in HCV cirrhosis.

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Apa1 polymorphism; hepatic cirrhosis; hepatitis C virus; hepatocellular carcinoma

Introduction

Hepatitis C virus (HCV) is a major public health issue worldwide, bringing fibrosis, cirrhosis and hepatocellular carcinoma (HCC) [1–3]. Early rapid prediction of HCC in post-hepatitis C cirrhosis is an urgent aim in starting effective treatment such as surgical removal of small tumours [4,5]. Although vitamin D has a cardinal role in the skeletal metabolism, it also can be involved in the development of cancer via its receptor [6,7]. Numerous vitamin D receptor (VDR) single nucleotide gene polymorphism (SNP) variants are linked with liver, breast, prostate, skin, colon, bladder, and kidney cancers [8]. Additionally, some VDR gene variants may be linked to chronic states of liver diseases, as chronic hepatitis B, primary biliary cirrhosis and autoimmune hepatitis [7,9].

Certain SNPs in the VDR gene, present at 12q12-14, may be molecular biomarkers to foresee the risk and to analyse the severity of the disease in HCC patients with chronic hepatitis B [10]. Most studies of VDR and cancer have focused on six SNPs: rs 10,735,810/Fok1, rs 1,544,410/Bsm 1, rs 731,236/Taq 1, rs 7,975,232/Apa 1, rs 757,343/Tru 91, and a poly (A) mononucleotide repeat [11].

We hypothesized that the Apa1 (rs7975232) A\C SNP is associated with HCC in chronic hepatitis C virus-infected cirrhotic patients.

Subject and methods

To test our hypothesis, we recruited 80 hepatitis C cirrhotic patients with HCC, 80 hepatitis C cirrhotic patients without HCC, and 80 healthy patients' relatives' volunteers as controls. Patients were recruited from the Tropical Medicine Department (either admitted or outpatient clinics attendees). Inclusion criteria were hepatitis C cirrhosis patients with or without HCC. Exclusion criteria were malignancies other than HCC, co-infection with HBV or HIV, and cirrhosis due to any other causes other than chronic HCV. Comprehensive history taking with full clinical examination was done for all subjects. The protocol was in accordance with Declaration of Helsinki II principles and authorized by Tanta University Local Research Ethics Committee. Radiological investigations in the form of abdominal ultrasonography and triphasic computed tomography (CT) were used for diagnosis of cases with HCC and ascites.

Venous blood samples (10 ml) were taken from all individuals after 10 hours of overnight fasting into EDTA, sodium citrate or plain plastic tubes. The latter was allowed to clot, and centrifuged at 2000 rpm for 10 minutes to separate the serum. The collected sera were stored in aliquots at – 80°C until used. Unless stated otherwise, reagents were from Sigma-Aldrich (St. Louis, MO, USA). A full blood count was done on

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an ERMA PCE-210N cell counter, liver function tests were performed on Kone lab bio thermo scientific analyser. Prothrombin time was assayed in citrated plasma by Neoptimal Kit #Cat NO. 253,643 (Stago) and reported as international normalizing ratio (INR). Viral markers included HBs antigen and anti-HCV ab, detected by ELISAs (Innotest Fmjirebo # Cat NOs. 404,265 and 404,475 respectively). Alpha-fetoprotein (AFP) levels were measured via (ST AIA- Pack AFP Kits #Cat NO. IY 11,451 (Tosch autoanalyser).

For Apa1 A/C rs7975232 SNP Genotyping, QIAamp whole blood genomic DNA extraction Mini kits (QIAGEN, Germany) were used for genomic DNA extraction from EDTA-whole blood cells. Yield and purity of the extracted total DNA were evaluated by UV-spectrophotometer at 260 and 280 nm respectively. DNA was diluted with nuclease free water until reaching a final concentration of 50 ng/µl. Until analysis, the extracted DNA was stored at −20°C. For the detection of ApaI A/C rs7975232 SNP, a forward primer (5′-CAGAGCATGGACAGGGAGCAA-3′) and a reverse primer (5′- GCAACTCCTCAGGCTGAGGTCTC-3′) were used. The PCR mix contained 5µL of each primer (10 Pmol), 5µL buffer, 1.5µLMgCl2 (50 mM), 5µL template DNA (50–100 ng), 5 µL dNTPs (2 mmol/L, Tag Polymerase (MBI) 25µL, H_2O 21.5µL. The DNA template was denatured at 95°C for 2 minutes. A total of 40 cycles of PCR were performed, consisting of a denaturation step for 45 sec at 95°C, an annealing step for 45 sec at the optimum temperature (67°C), and an extension reaction for 1 min at 72°C. A final step of extension at 72°C for 2 min was done after the last PCR cycle. After amplification, The PCR products of length 745 bps were digested with ApaI restriction endonucleases at 25°C for 15 minutes. Then, ApaI rs7975232 genotyping was demonstrated by ethidium bromide-UVB illumination of the DNA fragments separated 2% agarose gels. ApaI restriction endonuclease digestion products showed two fragments of 531 bp and 214 bps. Polymorphism banding patterns, shown in Figure 1, were: one single fragment of 745-bps length indicating the wild-type homozygous AA genotype, and two fragments of 531- and 214-bp indicating the variant homozygous CC genotype. Heterozygous (AC) showed a combination of 214-, 513- and 745-bp three fragments.

Clinical assessment for evaluation of the liver cirrhosis severity was done by a modified Child-Pugh score in all cirrhotic patients. This scoring depends on clinical and laboratory evaluation of ascites, grade of encephalopathy, serum albumin, bilirubin, INR, and prothrombin time [12]. The Model for liver disease end-stage (MELD) score was used for assessing the degree of patient liver cirrhosis [13], based on laboratory evaluation of serum bilirubin, creatinine and INR [14].

Statistical analysis was by SPSS 25 (IBM, Armonk, NY; U.S.). Categorical variables are shown as numbers and percentage, continuous variables as mean with SD or median (1R). Chi-square test was applied to test links between categorical variables. Kruskal Wallis and ANOVA followed by Tukey's post-hoc test were used for multiple comparisons between groups. Logistic regression was performed to detect the independent predictors of HCC. Adjusted odd's ratios and 95% CI were calculated. The adopted level of significance was p-value <0.05. The Apa1 (rs7975232) SNP obeyed Hardy Weinberg equilibrium.

Results

Clinical and demographic characteristics of the groups (matched for age and sex) are shown in Table 1. Table 2 shows Child-Pugh scoring, genotyping, haematology, liver function tests and AFP. Unsurprisingly, haemoglobin, bilirubin, albumin, AST, ALT and INR all reflected disease severity, whilst low platelet count did not differ between patient groups, and AFP was raised only in HCC. There was no difference in white blood cell

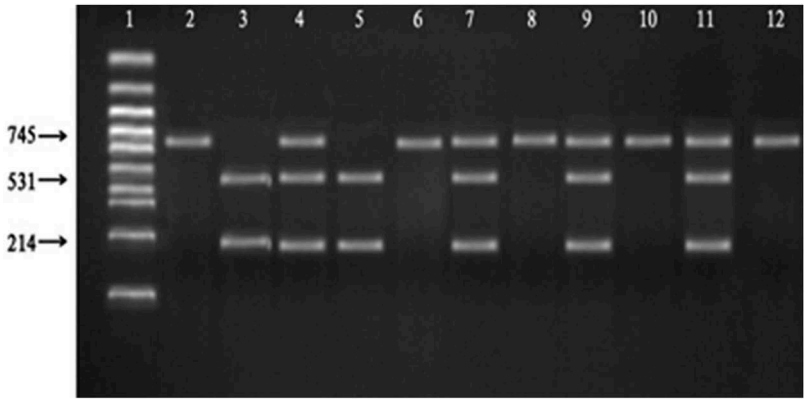

Figure 1. Electrophoresis of 2% agarose gel with Apa1 endonuclease digestion: heterozygous and homozygous genotyping.

Figure 1: A photograph of PCR-RFLP products of Apa1 A/C SNP rs7975232 run on EtBr-stained 2% agarose gel. Lane 1 represents a marker ladder served as reference for DNA fragment size. Lanes 1, 6, 8, 10 and 12 show one band at 745 bp and it is homozygous for the absence of restriction site (AA). Lanes 3 and 5 show 2 bands at 214 bp and 531 bp and they are homozygous for the presence of restriction site (CC). Lanes 4, 7, 9 and 11 show 3 bands at 214 bp, 531 bp and 745 bp and they are heterozygous for the absence and presence of restriction site (AC).

Table 1. Socio-demographic and clinical characteristics.

		HCC patients with hepatitis C virus cirrhosis (n = 80)		Hepatitis C virus cirrhotic patients without HCC (n = 80)		Controls (n = 80)		
		No	%	No	%	No	%	P
Gender	Male	65	81.3	60	75.0	55	68.8	0.189
	Female	15	18.8	20	25.0	25	31.3	
Age (years)	mean ± SD	57.6 ± 13.5		58.6 ± 14.5		58.1 ± 13.0		0.674
Smoking	Yes	24	30.0	18	22.5	0	0.0	0.001
	No	56	70.0	62	77.5	80	100.0	
Abdominal pain	Present	62	77.5	37	46.3	-	-	0.001
	Absent	18	22.5	43	53.8	-	-	
Haematemesis	Present	11	13.8	6	7.5	-	-	0.201
	Absent	69	86.3	74	92.5	-	-	
Jaundice	Present	27	33.8	37	46.3	-	-	0.107
	Absent	53	66.3	43	53.8	-	-	
Lower limb oedema	Present	48	60.0	80	100.0	-	-	0.001
	Absent	32	40.0	0	0.0	-	-	
Encephalopathy	Present	0	0.0	34	42.5	-	-	0.001
	Absent	80	100.0	46	57.5	-	-	
Ascites	Present	49	61.3	74	92.5	-	-	0.001
	Absent	31	38.8	6	7.5	-	-	
Previous Blood transfusion	Present	12	15.0	16	20.0	-	-	0.405
	Absent	68	85.0	64	80.0	-	-	

Table 2. Child-Pugh score, Apa1 genotype and laboratory indices.

		HCC patients (n = 80)		Cirrhotic patients (n = 80)		Controls (n = 80)		
		No	%	No	%	No	%	p
Child-Pugh score								
	A	0	0	26	32.5	–	–	0.001
	B	34	42.5	21	26.3	–	–	
	C	46	57.5	33	41.3	–	–	
Apa1 genotypes								
	CC	60	75	28	35	16	20	<0.0001
	CA	16	20	40	50	30	37.5	
	AA	4	5	12	15	34	42.5	
	C vs A	136:24		96:64		62:98		
Haemoglobin (g/L)	Median (IQR)	103 (48–148)		115 (55–153) P1: 0.030, P2: 0.001, P3: 0.001		130 (86–195)		0.001
White blood cell count (cells/10^9)	Mean ± SD	7.1 ± 3.2		7.2 ± 2.8		7.4 ± 2.3		0.187
Platelets (platelets/10^9)	Median (IQR)	150 (100–235)		131 (89–240) P1: 0.176, P2: 0.001, P3: 0.001		250 (195–300)		0.001
Erythrocyte sedimentation rate	Median (IQR)	74 (11–87)		72 (10–85) P1: 0.144, P2: 0.001, P3: 0.001		20 (12–31)		0.001
Total Bilirubin (μmol/dL)	Median (IQR)	1220 (950–1640)		530 (370–860) P1: 0.001, P2: 0.001, P3: 0.001		270 (190–320)		0.001
Albumin (g/L)	Mean ± SD	25 ± 4		31 ± 5 P1: 0.001, P2: 0.001, P3: 0.001		41 ± 3		0.001
ALT (U/L)	Median (IQR)	60 (28–105)		57 (25–100) P1: 0.473, P2: 0.001, P3: 0.001		32 (24–38)		0.001
AST (U/L)	Median (IQR)	80 (25–135)		61 (18–92) P1: 0.012, P2: 0.001, P3: 0.001		33 (28–39)		0.001
INR	Mean ± SD	1.8 ± 0.5		1.3 ± 0.2 P1: 0.001, P2: 0.001, P3: 0.001		1.1 ± 0.1		0.001
AFP (μg/L)	Median (IQR)	126 (54–298)		4 (4–7) P1: 0.001, P2: 0.254, P3: 0.001		5 (3–7)		0.001

P1: HCC group vs Hepatitis C cirrhotic patients without HCC, P2: HCC group vs Controls, P3: Hepatitis C cirrhotic patients without HCC vs Controls.

counts. The CC Apa1 genotype frequency also reflected disease severity, i.e. HCC (75%) > cirrhosis (35%) > controls (20%).

Table 3 shows clinical, platelet and AFP data according to genotype in all 160 patients. There were links with several indices, and AFP was highest in those with the CC variant, whilst platelet count was low in both CC and CA groups. Child-Pugh class C was more frequent in the CC group. The MELD score in the HCC group was 12.4 ± 4.8, and in HCV cirrhotic group without HCC was 20.0 ± 5.2 ($P<0.05$).

Our univariate/multivariate analysis sought to determine those factors linked to HCC. The odds ratio (95% confidence interval) for age was 1.81 (0.37–1.60) ($P=0.606$), gender was 2.01 (0.99–8.03)($P=0.457$), smoking was 1.99 (0.88–6.13)($P=0.943$), low platelets was

Table 3. Association of Apa1 genotypes with clinical characteristics, AFP and platelet count in the combined patient group.

		CC (n = 88)		CA (n = 56)		AA (n = 16)		
		No	%	No	%	No	%	p
Haematemesis	Yes	56	63.6	40	71.4	2	12.5	0.0005
	No	32	36.4	26	46.4	14	87.5	
Ascites	Yes	63	71.6	41	73.2	5	31.3	0.003
	No	25	28.4	15	26.8	11	68.7	
Encephalopathy	Yes	14	15.9	8	14.3	4	25.0	0.587
	No	74	84.1	48	85.7	12	75.0	
Jaundice	Yes	62	70.5	34	60.7	5	31.3	0.007
	No	24	29.5	22	39.3	11	68.8	
Lower limb edema	Yes	61	69.3	40	71.4	6	37.5	0.029
	No	27	30.7	16	28.6	10	62.5	
Child score	A	15	17	12	21.4	8	50	0.027
	B	27	30.7	21	37.5	5	31.3	
	C	46	52.3	23	41.1	3	18.7	
AFP (µg/L)	Median (IQR)	209 (55–267)		20 (5–32)		18 (4–30)		0.015
		CC v CA = 0.010, CC v AA = 0.008, CA v AA = 0.323						
Platelets count (platelets/10^9)	Median (IQR)	130 (65–198)		120 (58–192)		168 (84–235)		0.012
		CC v CA = 0.512, CC v AA = 0.006, CA v AA = 0.003						

0.61 (0.28–1.17)(*P*=0.852), Apa1 CC genotype was 2.55 (1.16–9.94)(*P*=0.012), and AFP was 3.52 (1.75–6.32) (*P*=0.027). In multivariate analysis of Apa1 CC and AFP, Apa1CC had an odds ratio (95% confidence interval) of 8.99 (3.88–17.13)(*P*=0.024), for AFP the result was 1.33 (0.65–3.71)(*P*=0.145).

Discussion

HCC pathogenesis is complex and multifactorial related to many genetic and non-genetic factors. Studying genetic factors associated with the occurrence of HCC may improve our studying of different biological pathways involved in hepatocarcinogenesis and as well improve the scientific basis for preventive intervention [15,16]. We hypothesized a link between Apa1 (rs7975232) gene SNPs with the development of HCC in hepatitis C virus cirrhotic patients, finding an increase in the CC genotype in HCC and in cirrhosis compared to age and sex matched healthy controls. Furthermore, the presence of this genotype was a better determinant of HCC than levels of serum AFP, and was more closely linked to clinical scores of disease severity (Child-Pugh, MELD). These results part-confirm and extend similar observations in this group of patients [17–21]. Baur et al. [22] showed that Apa1 CC genotype is associated considerably with a rapid fibrosis progression rate in HCV-cirrhotic patients. Also, Falleti et al. [23] showed a link between Apa1 genetics and occurrence of HCC in cirrhotic patients who were undergoing liver transplantation for alcoholism.

We recognize a number of limitations to our study, principally the small numbers. Accordingly, our data needs to be confirmed in a larger population. Furthermore, we look forward to data from other ethnic and racial groups. We also acknowledge that our cross sectional design cannot confirm if the SNP predicts those individuals who are at risk of HCC. This can only be determined in a longitudinal study by assessing Apa1 genotype in patients with a simple HCV infection, and then observing which, over a long follow-up period, become cirrhotic and/or develop HCC. This work represents an advance in biomedical science because it shows Apa1 (rs7975232) CC genotype can be used as an independent molecular biomarker predictor for the HCC risk in chronic HCV cirrhosis.

Summary table

What is known about this subject:

- HCC is a common complication in chronic HCV cirrhosis, even in those received anti-HCV treatment.
- As HCC is aggressive type of cancer that rapidly metastases to numerous sites, early management is essential

What this paper adds:

- Apa1 (rs7975232) CC genotype has a greater frequency in HCC derived from HCV cirrhosis
- Apa1 (rs7975232) CC genotype is an independent molecular biomarker predictor for the HCC risk in chronic HCV cirrhosis

Disclosure statement

No potential conflict of interest was reported by the authors.

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