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Vitamin D receptor gene polymorphism and hepatocellular carcinoma in chronic hepatitis C patients

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Abstract

Background: Hepatocellular carcinoma (HCC) is a prevalent malignancy worldwide. Vitamin D receptor (VDR) gene polymorphisms were linked to different cancers. This study was carried out to assess the possible relation between VDR gene polymorphism and the occurrence of HCC in chronic hepatitis C patients. This study included 102 subjects classified into three groups. Group A included 34 healthy subjects as control. Group B included 34 chronic hepatitis C patients with HCC. Group C included 34 chronic hepatitis C patients without HCC. Estimation of Apa-1 VDR gene polymorphism was performed by restriction fragment length polymorphism-Polymerase chain reaction (RFLP-PCR).

Results: In HCC group, C allele was more frequent than A allele (80.88% and 19.12%), respectively. In chronic hepatitis group, C allele was more frequent than A allele (64.71% and 35.29%), respectively. In control group, A allele was more frequent than C allele (73.53% and 26.47%), respectively. Genotype CC + CA was dominant in HCC group (91.18%) and chronic hepatitis group (79.41%). In the control group, the dominant genotype was AA (58.82%). Moreover, there was a significant relation between Apa-1 VDR genotype CC and tumor size.

Conclusions: There is an association between VDR Apa-1 polymorphism and the occurrence of HCC in chronic hepatitis C patients.

Keywords: Vitamin D receptor gene polymorphism, Hepatocellular carcinoma, Chronic hepatitis C

Background

Hepatitis C virus (HCV) infection is a common health problem worldwide. Chronic HCV commonly proceeds to cirrhosis which may predispose to hepatocellular carcinoma (HCC). Hepatocarcinogenesis is affected by environmental and genetic features [1].

Risk factors for HCC include alcohol use, viral hepatitis, and metabolic diseases. Host genetics may play an important role. Improving our recognition of molecular factors may help us in early detection, stratification, individual treatment, and predicting the prognosis [2].

The importance of vitamin D has widened, from being only involved in bone metabolism to a broad scope of other physiological and pathological processes. It has an effect on immunity, intellectual and fetal development, insulin secretion, cancer, cell proliferation, and differentiation, and the cardiovascular system via the vitamin D receptor (VDR) which is a nuclear receptor. Vitamin D binds to its receptor to form a complex that enters the nucleus and binds to DNA regulating gene expression [3].

The VDR gene is located in chromosome 12q and has multiple allelic variants. Some of these variants may cause changes in the VDR function and may lead to immune-mediated disorders and cancer development. The most common of these variants are single nucleotide polymorphisms (SNPs). Four polymorphisms, Fok1, Bsm1, APa1, and Taq1, are being most investigated [4].

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Several studies were performed and proved the association between VDR gene polymorphism and different types of cancer as cancer breast, prostate, bladder, and kidney. In our study, we clarified the effect of VDR gene polymorphism Apa1 in the occurrence of HCC among chronic hepatitis C patients.

Methods

This is a case-control study, and it was conducted in the Medical Biochemistry & Molecular Biology and Internal medicine Departments, Faculty of Medicine, Zagazig University, Egypt. Our study included 102 subjects classified into three groups. Group A comprised of 34 healthy subjects as control. Group B comprised of 34 chronic hepatitis C patients with HCC. Group C comprised of 34 chronic hepatitis C patients without HCC. Written informed consents from the participants were obtained before being involved in the study.

The patients participated in the study were chronic hepatitis C infection diagnosed by HCV-Ab and quantitative real-time polymerase chain reaction (PCR) and HCC was diagnosed by a triphasic CT scan. Patients excluded from the study were those with chronic hepatitis B infection, patients with other chronic liver diseases, patients having any other malignancy and patients who had diabetes mellitus, chronic renal failure or bone disorders.

All the participants were completely evaluated by complete history, examination, and full investigations in the form of laboratory studies as complete blood count (CBC) and complete renal and liver functions. DNA extraction was performed from whole blood from all cases. Serum levels of alpha-fetoprotein (AFP) were assayed using enzyme linked immunosorbent assay (ELISA).

Restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) was done to recognize the distribution of allele and genotype frequencies of the Apa-1 C/A polymorphism in the VDR gene. Genomic DNA extraction was performed from whole blood using GeneJET whole Blood Genomic DNA Extraction Mini Kit (Molecular Biology, Thermo Fisher Scientific, USA). The following primers: F: 5'-CAGAGCATGGACAGGG AGCAA-3' and R: 5'-GCAACTCCTCATGGCTGA GGTCTC-3' [3] were used.

Cycling conditions were carried out using the Thermal Cycler. An initial denaturation at 95 °C for 2 min was performed, followed by 40 cycles; each cycle consisted of denaturation at 94 °C for 45 s then annealing at 67 °C for 45 s, elongation at 72 °C for 60 s. The final elongation at 72 °C for 2 min was carried out after the last PCR cycle.

The PCR products were digested with Apa-I restriction enzyme. The following reaction components were mixed gently at room temperature: 7 µl nuclease-free water, 2 µl 10 × fast digest green buffer, 1 µl Apa-I restriction enzyme (Thermo Fisher Scientific), and 10 µl of

DNA amplification product. The mixture was incubated at 65 °C for 5 min. The reaction mixture was loaded directly and electrophoresed on 2% agarose gel containing ethidium bromide and visualized under ultraviolet illumination. PCR products with C at the polymorphic site were digested into two fragments, 531 and 214 bp, where those with A were not because of the absence of Apa-I enzyme restriction site. Samples yielding 531 bp and 214 bp fragments were scored as C/C, those with single 745 bp fragment as A/A, and 745-bp, 531-bp, and 214-bp fragments as A/C. The amount of size marker to load on the gel is 10 µl per lane and the result was photographed (Fig. 1).

Statistical analysis

Data were checked, entered, and analyzed by using (SPSS version 17 for windows, SPSS Inc., Chicago, IL, USA) and MedCalc 13 for windows (MedCalc Software bvba, Ostend, Belgium). Data were expressed as mean ± SD for quantitative variables or number and percentage for qualitative variables. Chi-squared (χ^2), *t* test, and analysis of variance (ANOVA) tests were performed. Unpaired Student's *t* test was used to compare between two groups in quantitative data.

Results

In our study, there were significant differences between the three groups regarding direct bilirubin, total bilirubin, serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), serum albumin, serum alkaline phosphatase (ALP), serum AFP, serum creatinine, serum urea, platelet count, INR, and PT. But, there were no significant differences between the three studied groups regarding hemoglobin and body mass index (BMI) (Table 1).

According to genotypes distribution, in control group, CC genotype represents (11.76%), CA genotype (29.41%), and AA genotype (58.82%). In HCC group, CC genotype represents (70.59%), CA genotype (20.59%), and AA genotype (8.82%). Chronic hepatitis C group represents CC genotype (50%), CA genotype (29.41%), and AA genotype (20.59%). There was a significant difference between the groups regarding genotype distribution ($P < 0.001$) (Table 2).

According to allele frequency, the control group shows that A allele more frequent than the C allele (73.53%, 26.47%), respectively. In the HCC group, the C allele was more frequent than A allele (80.88%, 19.12%), respectively. In the chronic hepatitis C group, the C allele was more frequent than A allele (64.71%, 35.29%) respectively. There was a significant difference between the groups regarding allele frequency (Table 3).

Our study showed a significant relationship between VDR genotypes in HCC and both AFP ($P = 0.02$) and

Table 1 Different biochemical data in the studied groups

	Group A Control (n = 34)	Group B HCC (n = 34)	Group C chronic hepatitis (n = 34)	P value
Age	49.3 ± 6.5	53.8 ± 8.3 ^a	50.9 ± 6.9	0.06*
Gender (M/F)	16/18	23/11	17/17	0.182
BMI (kg/ m ²)	23.09 ± 0.45	23.19 ± 0.47	23.16 ± 0.49	0.668
Total bilirubin (mg/dl)	0.85 ± 0.17	2.32 ± 1.33 ^a	1.87 ± 1.14 ^b	0.005*
Direct bilirubin (mg/dl)	0.39 ± 0.11	0.81 ± 0.58 ^a	0.97 ± 0.67 ^b	0.032*
ALT (IU/L)	25 ± 3.55	97.79 ± 25.32 ^a	91.41 ± 60.27 ^b	< 0.001**
AST (IU/L)	22.67 ± 4.28	77.71 ± 21.53 ^a	93.94 ± 85.15 ^b	< 0.001**
Albumin (g/dl)	4.22 ± 0.44	2.82 ± 0.71 ^a	3.06 ± 0.47 ^b	< 0.001**
ALP (IU/L)	64.35 ± 11.39	108.97 ± 37.7 ^a	112.38 ± 57.31 ^b	< 0.001**
AFP (ng/mL)	6.45 ± 1.63	3599.09 ± 4450.75 ^a	26.13 ± 23.87 ^c	< 0.001**
Creatinine (mg/dL)	0.87 ± 0.12	1.04 ± 0.27 ^a	0.94 ± 0.19	0.004*
Urea (mg/dL)	33.85 ± 3.74	31.59 ± 3.89 ^a	33.15 ± 2.87	0.029*
Hemoglobin (g/dL)	12.71 ± 0.76	12.28 ± 1.61 ^a	12.03 ± 1.61 ^b	0.125
Platelets count (× 10 ³ mm ³)	222 ± 49.14	134.97 ± 7.13 ^a	130.77 ± 73.72 ^b	< 0.001**
INR	1.02 ± 0.04	1.38 ± 0.39 ^a	1.49 ± 0.43 ^b	< 0.001**
PT (s)	12.27 ± 0.46	16.68 ± 4.91 ^a	17.89 ± 5.17 ^b	< 0.001**

*P < 0.05 is significant

**P < 0.001 is highly significant

^aSignificant difference between group A (control) and B(HCC)^bSignificant difference between group A (control) and C(chronic hepatitis)^cSignificant difference between group B (HCC) and C(chronic hepatitis)

tumor size ($P = 0.008$). There was a significant relationship between genotype CC and tumor size (Table 4).

Discussion

HCC is resistant to radiotherapy and chemotherapy, and long-term survival of patients occurred only with small asymptomatic HCC [5]. Early detection of HCC is

helpful as it improves the prognosis [6]. And, it allows using several modalities as resection, radiofrequency ablation, and transplantation. These modalities improve the outcome in these patients [7].

It is important to recognize HCV-infected patients who are at a higher risk of developing HCC [8]. The difference in the incidences of HCC among different

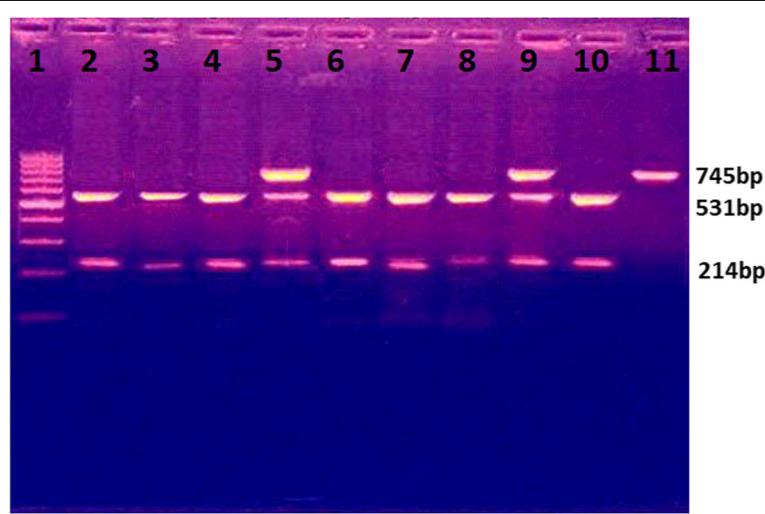


Fig. 1 2% Agarose gel stained with ethidium bromide of APa1 PCR genotyping reaction. CC homogenous genotype show two bands of 531 and 214 bp, The CA heterogenous genotype show three bands of 745, 531, and 214 bp, AA homogenous genotype show single bands of 745 bp

Table 2 Comparison between the three studied groups according genotypes distribution

Genotype	Group						χ^2	P		
	Group A Control (n = 34)		Group B HCC (n = 34)		Group C chronic hepatitis (n = 34)					
	N	%	N	%	N	%				
AA	20	58.82	3	8.82	7	20.59				
CA	10	29.41	7	20.59	10	29.41				
CC	4	11.76	24	70.59	17	50	30.200	< 0.001**		

**P value < 0.001 (highly significant)

populations may be explained by genetic background [9]. Besides, multiple susceptible genetic loci of HCC were also recognized and validated [10]. Thus, the study of genetics can be added to the tools of risk prediction, allowing better stratification and personalized assessment of optimal long-term management, thereby increasing the efficacy of surveillance programs [2].

The mechanism of development of HCC among chronic hepatitis C patients, including host- and viral-related factors, is still unknown. The differences in the prevalence and the strong gender distribution in HCC are due to differences in the exposure to the causative agents as well as genetic factors, particularly gene polymorphisms of inflammatory cytokines and growth factor ligands and receptors [11].

Vitamin D is not only involved in bone metabolism as a hormone but it also has immunomodulatory, anti-inflammatory, and antifibrotic properties. It also plays an important role in the regulation of cell proliferation, differentiation, and carcinogenesis via VDR [12]. VDR is a member of the nuclear receptor superfamily of ligand-inducible transcription factors, which are involved in many physiological processes, including, cell growth and differentiation, embryonic development, and metabolic homeostasis [13]. VDR performs heterodimerization with auxiliary proteins for effective DNA interaction as the retinoid-X receptors (RXRs). Vitamin D response elements have been recognized in many genes responsible for cellular growth, differentiation, apoptosis, invasion, and metastasis of tumor cells as cell cycle regulators. Therefore, it can be assumed that VDR-mediated

signaling pathways and VDR gene polymorphisms are related to carcinogenesis. Furthermore, VDR gene variants can modulate vitamin D effect without affecting serum vitamin D levels [14].

VDR polymorphisms have been studied in chronic liver diseases [15]. Yao et al. (2013) and Hoan et al. (2019) found that VDR polymorphism was a risk predictor and a prognostic molecular marker of HCC in patients with chronic hepatitis B [3, 16].

Polymorphisms are defined as variations in DNA sequence which occur in at least 1% of a certain population and have real biological effects. They have been studied with the aim of explaining association with the risk for common diseases [17]. According to Shastry (2002) and Li et al. (2001), humans have a huge number of polymorphisms that lead to different cellular effects due to different mechanisms such as transcription level modification, posttranscriptional, or posttranslational activity or changes in the tertiary structure of the gene product [17, 18]. Bai et al. (2012) stated that several SNPs have been described in the VDR gene, and some polymorphisms are associated with tumor occurrence in the breast, prostate, skin, colon-rectum, bladder, and kidney [19]. The most common allelic variants studied included a start codon polymorphism Fok1 (T/C) in exon II, Bsm1 (A/G), and APa1 (C/A) polymorphisms in the intron between exon VII and IX and a Taq1 (T/C) variant in exon IX [20]. The Apa-I is a silent SNP. No replacement of the amino acid occurs in the protein. However, it affects mRNA stability [21].

In this study, there was no statistically significant difference between the three studied groups regarding

Table 3 Comparison between the three studied groups according to allele frequency

Genotype	Group						χ^2	P		
	Group A Control (n = 34)		Group B HCC (n = 34)		Group C chronic hepatitis (n = 34)					
	N	%	N	%	N	%				
A allele	50	73.53	13	19.2	24	35.29	43.409	< 0.001**		
C allele	18	26.47	55	80.88	44	64.71				

**P value < 0.001 (highly significant)

Table 4 Relation between Apa-1vitamin D receptor genotypes and different parameters in the hepatocellular carcinoma (HCC) group

Group B (HCC)		Genotype			ANOVA	
		AA	CA	CC	F	P
Age (years)	Range	44–66	39–60	33–68	0.339	0.715
	Mean ± SD	54.000 ± 11.136	51.429 ± 7.161	51.429 ± 7.161		
T. bilirubin (mg/dl)	Range	0.9–3.1	1.11–4.6	0.9–8.2	0.523	0.598
	Mean ± SD	1.723 ± 1.121	1.919 ± 1.222	2.456 ± 1.685		
D. bilirubin (mg/dl)	Range	0.35–0.61	0.42–1.4	0.087–1.4	1.588	0.221
	Mean ± SD	0.520 ± 0.147	0.836 ± 0.299	0.787 ± 0.264		
ALT (IU/L)	Range	77–133	65–180	68–134	0.125	0.883
	Mean ± SD	98.667 ± 30.072	102.000 ± 39.770	96.458 ± 20.532		
AST (IU/L)	Range	49–112	62–116	53–119	0.117	0.890
	Mean ± SD	72.000 ± 34.771	79.143 ± 17.995	78.000 ± 21.685		
S. albumin (g/dl)	Range	2.3–3.8	1.8–3.2	1.5–3.9	1.598	0.218
	Mean ± SD	3.033 ± 0.751	2.400 ± 0.432	2.908 ± 0.748		
ALP (IU/L)	Range	94–122	80–156	59–240	0.491	0.616
	Mean ± SD	104.000 ± 15.620	121.714 ± 24.074	105.875 ± 42.470		
S. Creatinine (mg/dL)	Range	0.65–1.3	0.65–1.8	0.75–1.42	1.625	0.213
	Mean ± SD	0.950 ± 0.328	1.199 ± 0.385	1.004 ± 0.219		
AFP (ng/mL)	Range	70–430	86–1214	430–20834	4.455	0.020*
	Mean ± SD	256.667 ± 180.370	447.571 ± 369.296	4936.083 ± 4694.481		
PLT (×103)	Range	134–215	76–200	61–334	1.100	0.346
	Mean ± SD	181.333 ± 42.194	133.143 ± 48.746	129.708 ± 59.979		
HGB (g/dL)	Range	12–13.3	10.8–14.6	8.5–17.5	0.137	0.873
	Mean ± SD	12.633 ± 0.651	12.443 ± 1.218	12.192 ± 1.806		
INR	Range	1.02–1.26	1.18–2.5	1.02–2.7	1.234	0.305
	Mean ± SD	1.123 ± 0.123	1.534 ± 0.442	1.369 ± 0.385		
PT (sec)	Range	12.2–15	14.3–30	12.2–33	1.141	0.332
	Mean ± SD	13.400 ± 1.442	18.457 ± 5.283	16.575 ± 4.981		
BMI (kg/m2)	Range	22.7–23.5	22.2–23.9	22.1–23.8	0.556	0.579
	Mean ± SD	23.133 ± 0.404	23.029 ± 0.582	23.242 ± 0.456		
S.UREA range (ng/l)	Range	27–30	26–43	26–40	1.259	0.298
	Mean ± SD	29.000 ± 1.732	33.143 ± 5.581	31.458 ± 3.413		
Tumor size	Range	2.5–3	2.5–4.5	2.5–5.5	5.672	0.008*
	Mean ± SD	2.667 ± 0.289	2.943 ± 0.714	3.917 ± 0.903		

*P-value<0.05 (significant)

sex distribution. There was a significant difference between HCC and control regarding age but not significantly higher than chronic hepatitis patients. These results were similar to those found by Barooah et al. (2019) [22]. In contrary to our results, Yang and Roberts (2010) reported that the risk of HCC is 2–7 times higher in men than in women, although this ratio varies across the world. Their explanation for this might be due to higher rates of environmental exposure to liver carcinogens (such as smoking or alcohol) and hepatitis virus

infections. Also, estrogen might suppress interleukin IL-6 mediated inflammation in women, reducing both liver injury and compensatory proliferation. Moreover, testosterone effects could increase androgen receptor signaling in men, promoting liver cell proliferation [23]. Hammad et al. (2013) reported that HCC is significantly higher in men than women (77.7 and 22.3%, respectively) [24].

In our study, there were significant differences between the three groups regarding direct bilirubin, total bilirubin, serum ALT, serum AST, serum albumin,

serum ALP, serum AFP, serum creatinine, serum urea, platelets, INR, and PT. But, there were no significant differences between the three studied groups regarding hemoglobin and BMI. No significant difference was found between HCC and chronic hepatitis groups regarding studied parameters except AFP. Moreover, a significant difference between control and chronic hepatitis groups regarding all parameters except AFP, serum creatinine, and urea.

Similar results were found by Barooah et al. (2019) and Raafat Rowida et al. (2020). They reported that serum levels of ALT, AST, and bilirubin were higher among patients of HCC than those of chronic liver disease [22, 25]. Also, Bruix and Sherman (2011) showed that the serum level of ALT and AST were elevated in HCC especially the advanced cases, and the difference becomes greater as the disease progresses [26]. On the other hand, AFP levels may be normal in up to 40% of patients with HCC, particularly during the early stages (low sensitivity). Elevated AFP levels may be seen in patients with cirrhosis or exacerbations of chronic hepatitis (low specificity) [27].

Our study revealed that patients with HCC had a higher frequency of Apa-I CC genotype compared to those with chronic hepatitis C or control. These results are similar to those found by Barooah et al. (2019), Raafat Rowida et al. (2020), and Hung et al. (2014) [22, 25, 28]. Hung et al. (2014) revealed that patients carrying the corresponding APa-1 CC genotype had a higher prevalence of HCC than those with CA or AA type. They revealed that age, male gender, lower platelet count ($< 15 \times 10^9/\mu\text{L}$), and Apa-1 CC genotype were independent predictors for developing HCC [28]. Barooah et al. (2019) found that the frequency of the Apa-I CC genotype and ApaI C allele of the VDR gene was significantly higher in HCC and cirrhotic patients than controls. After adjusting for other covariates (age, gender, platelet count, AST, ALT, serum albumin, and viral load), logistic regression analysis showed that the Apa-I CC genotype was independent predictor of HCC development [22]. Baur et al. showed that Apa1 CC genotype was associated with a rapid fibrosis progression in cirrhotic HCV patients [29].

Our study showed a significant relation between Apa-1 VDR genotypes and AFP levels. This result was similar to that found by Raafat Rowida et al. (2020). Similar to our findings, they found that AFP was highest in those with the CC variant and platelet count was low in both CC and CA groups. However, in our study, platelet count did not reach a significant level as detected by them. They also found that Child-Pugh class C was more frequent in the CC group [25].

Conclusions

Apa-1 polymorphism is a possible predictor and prognostic marker in HCV patients that develop HCC.

Abbreviations

HCC: Hepatocellular carcinoma; VDR: Vitamin D receptor; HCV: Hepatitis C virus; SNPs: Single nucleotide polymorphisms; PCR: Polymerase chain reaction; CBC: Complete blood count; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; AFP: Alpha-fetoprotein; RFLP-PCR: Restriction fragment length polymorphism-polymerase chain reaction; ELISA: Enzyme-linked immunosorbent assay

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Authors' contributions

EE and EH designed and directed the project. HM, EAE, and SH performed the experiments and analyzed the data. HM and SH wrote the manuscript. All authors have read and approved the manuscript.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Written informed consent was obtained from each participant in the study. The study was approved by Institution Review Board, Faculty of Medicine, Zagazig University. Approval number: ZU-IRB#3753-16-5-2017.

Consent for publication

Not applicable

Competing interests

Not applicable

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