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The association between vitamin D receptor gene polymorphisms and hepato-cellular carcinoma in Egyptian patients with chronic liver disease

Yasmine Moemen[a], Fatma Khalil[b], Ashraf Khalil[c,*]

[a] Department of Clinical Pathology, National Liver Institute, Menoufia University, Egypt
[b] Department of Microbiology, National Liver Institute, Menoufia University, Egypt
[c] Department of Biochemistry, National Liver Institute, Menoufia University, Egypt

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ABSTRACT

Aim: To evaluate the possible association between vitamin D receptor single nucleotide polymorphisms and hepato cellular carcinoma in Egyptian patients with chronic liver disease.
Method: Specific primer sequences of the vitamin D receptor gene rs1544410, rs7975232, and rs731236 were used to detect vitamin D receptor polymorphisms in blood sample by polymerase chain reaction restriction fragment polymorphism. Allele specific restriction enzymes *Bsm*I, *Apa*I and *Taq*I were used to digested PCR amplified products and the bands of specific PCR-RFLP fragments were resolved by 2% Ethidium bromide agrose gel electrophoresis. Genotypes and alleles were identified by their specific banding pattern on the gel electrophoresis via ultraviolet illumination.
Result: The frequencies of the 3 single nucleotide polymorphisms *Bsm*I, *Apa*I, and *Taq*I genotype and the haplotype distribution were similar in patients with chronic liver disease either with or without hepato-cellular carcinoma. Neither of vitamin D receptor gene polymorphisms were a risk or protective factor for HCC; nor did these polymorphisms alter the risk for HCC development by coexisting HCC with chronic hepatitis C virus infection. TaqI polymorphism was associated with increased risk of progression of chronic liver disease to liver cell failure and decompensated liver disease.
Conclusion: vitamin D receptor single nucleotide polymorphisms at *Bsm*I, *Apa*I, and *Taq*I did not provide significant association with HCC cancer risk or protection in the overall analysis in Egyptian population with chronic liver disease.

1. Introduction

Hepatocellular carcinoma (HCC) is a severe form of malignant liver tumor with a brisk high mortality rate (Mohd Hanafiah et al., 2013). In Egypt HCC is a growing health hazard due to high incidence of hepatitis C virus (HCV), a major risk factor for the disease (Lehman and Wilson, 2009; Khalil et al., 2013). Genetic factors particularly gene polymorphisms of inflammatory cytokines and growth factor ligands and receptors have been emerged as important biological factors in the development of HCC (Bataller et al., 2003).

Vitamin D receptor (VDR) is an intracellular receptor which binds to its specific ligand, the metabolically active form of vitamin D which stimulates specific nucleotide sequences of target genes responsible for vitamin D biological effects including cell growth and differentiation, embryonic development, and metabolic homeostasis. VDR is also crucial for cell signaling pathways that influence the development of many cancers and the role of VDR in cancer has recently attracted much attention (Guy et al., 2004; Slattery, 2007; Yee et al., 2005).

The VDR gene is located on chromosome 12q12–q14 and comprises eleven exons that span 75–100 kb. The 5′ non-coding end of the gene includes regions of exons 1a, 1b, and 1c (Al-Eisa and Haider, 2016; Springer et al., 2000). The translated VDR protein is encoded by exons II to IX. Exons VII to IX play a critical role in binding of VDR to its ligand vitamin D (Li et al., 2008; Zhou et al., 2009). VDR gene is highly polymorphic with many single nucleotide polymorphisms (SNPs) have been identified (Zhou et al., 2009). VDR polymorphisms *Bsm*I, *Apa*I, and *Taq*I are located at the 3′ untranslated region of the VDR gene (Morrison et al., 1992). The BsmI is located in intron VIII, *Apa*I is located between exon VIII and Exon IX and TaqI is located in exon IX. Variation in DNA sequence of the coding and non-coding region by a

Abbreviations: HCC, Hepatocellular carcinoma; HCV, hepatitis C virus; VDR, Vitamin D receptor; AFP, alpha feto protein; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism

* Corresponding author at: Department of Biochemistry, National Liver Institute, Menoufia University, Egypt.
E-mail address: ashraf.khalil@liver.org.eg (A. Khalil).

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Table 1
VDR gene allele type, SNPs reference numbers, base changes, primer sequence and fragments size.

SNP	Ref.#	Base change	Primer	Sequence	Fragment size
ApaI	*rs7975232*	a allele T > G transition in intron 8	Forward	5′-CAG AGC ATG GAC AGG GAG CAA-3′	740 bp uncleaved
			Reverse	3′-GCA ACT CCT CAT GGC TGA GGT CTC-5′	530 bp, 210 bp
BsmI	*rs1544410*	b allele G > A transition	Forward	5′-CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA-3′	825 bp uncleaved
			Reverse	3′-AAC CAG CGG GAA GAG GTC AAG GG-5′	650 bp, 175 bp
TaqI	*rs731236*	t allele silent transition T > C in exon 9	Forward	5′-CAG AGC ATG GAC AGG GAG CAA-30	740 bp uncleaved
			Reverse	3′-GCA ACT CCT CAT GGC TGA GGT CTC-5′	495 bp, 245 bp or 290 bp, 245 bp, 205 bp

SNP may influence stability, quantity and activity of the VDR protein and rate of VDR mRNA synthesis (Ogunkolade et al., 2002). Genetic variations in the VDR gene may also leads to changes in the physiological process involving vitamin D including its synthesis in the skin, hydroxylation in the liver and kidney, transport, metabolism and degradation. VDR SNPs have been associated with the increased risk of tumor development as breast (Dalessandri et al., 2012), prostate (Shui et al., 2012), colon (Rasool et al., 2014), renal cell carcinoma, and malignant melanoma (Denzer et al., 2011; Murtaugh et al., 2006; Zhou et al., 2006).

In this study, the frequency of VDR gene polymorphisms at the restriction site *Bsm*I, *Apa*I, and *Taq*I was explored in Egyptian patients diagnosed with different stages of chronic liver disease including HCC.

The aim was to investigate the possible association between VDR gene polymorphisms and the existence of HCC in patients with chronic liver disease, and to examine the effect of these polymorphisms on the progress of the chronic liver disease into liver cell failure. The selection of these particular SNPs based on their frequency in many ethnic population and the apparent functional or regulatory influence on cancer cell. Additionally previous studies had shown an association of these SNPs with increased risk for many cancers including HCC (Egan et al., 2010).

2. Patients and methods

A total of 65 chronic liver disease (CLD) patients were enrolled at National Liver Institute (NLI) hospital between January 2016 and March 2016. The study was approved by the Institutional Review Board of NLI and consent for the study was obtained from all participants.

2.1. Inclusion criteria

Patients initially presented with chronic liver cirrhosis were subjected to diagnostic work-up including clinical evaluation, imaging study (abdominal US, CT and MRI), laboratory tests including liver function test, alpha feto protein (AFP), complete blood count (CBC), and anti HCV antibodies. EASL criteria were used to indicates the stage of chronic liver disease and the presence of liver cell failure or decompensation liver cirrhosis (DLC). HCC was diagnosed when one or more liver masses ≥2 cm in diameter was/were detected by imaging study and an AFP ≥400 ng/ml, or the presence of early arterial phase contrast enhancement and early venous phase contrast washout irrespective of AFP level (Schraml et al., 2015). The presence of portal vein invasion, lymph node metastasis and the clinical stage of the disease were also determined and the patients were grouped as CLD with and without HCC.

2.2. Exclusion criteria

Patients with concomitant chronic heart diseases, chronic renal diseases, tumor suggestive of cholangio-carcinoma and liver metastasis from primary tumors other than HCC.

2.3. Blood sample collection and DNA extraction

2 ml of peripheral blood collected from each patient under strict sterile condition, and DNA was extracted using QIAamp DNA blood mini kits (Qiagen, CA, USA) following the manufacturer's instructions. The extracted DNA was quantified using the nano-drop and stored at −20 till the time of the assay.

3. VDR gene amplification

VDR SNPs, allele types and base substitution, reference numbers; forward and revere primers sequence used for PCR amplification of the SNPs as well as fragments size of the digested PCR products are presented in (Table 1). The gene fragments containing VDR polymorphic sites *Bsm*I (rs1544410) with a base changes G > A, *Apa*I (rs7975232) with T > G, and *Taq*I (rs731236) with T > C were amplified by PCR. The PCR reaction was prepared by a mixture of 50 ng genomic DNA, 1.5 mmol/l MgCl2, 0.2 mmol/l primers, 0.08 mmol/l dNTPs, 16 µl buffer and 1 unit DNA Taq polymerase (Thermo Fisher Scientific, MA, USA). PCR thermal cycles were run using the following setting: a) initial heating at 94 °C for 5 min b) 30 cycles of denaturing at 94 °C for 45 s, followed by annealing for 45 s at the same temperature, and extension for 45 s at 72 °C. A final extension cycle for 5 min at 72 °C was permitted to ensure completeness of the reaction. The amplified PCR products were resolved by electrophoresis running for 30 min at 120 V in 2% agarose gel. Ethidium bromide was used to stain the bands and then identified under ultraviolet light.

3.1.1. VDR restriction fragments length polymorphism assays

For the detection of *Bsm*I polymorphisms a forward primer (5′CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA3′) and a reverse primer (3′AAC CAG CGG GAA GAG GTC AAG GG5′) were used. For the detection of *Apa*I or *Taq*I polymorphisms, the forward primer (5′CAG AGC ATG GAC AGG GAG CAA3′) and the reverse primer (3′GCA ACT CCT CAT GGC TGA GGT CTC5′) were used. The previously amplified PCR products were digested by allele specific restriction enzymes *Bsm*I, *Apa*I, and *Taq*I (Thermo Fisher Scientific, MA, USA; Catalog #; BsmI ER0961, ApaI ER1411, and TaqI ER0671). The reaction mixture was prepared by adding 0.5 µg of DNA in 18 µl nuclease-free water, 2 µl of 10× Buffer and 2 µl of the restriction enzyme at 37 °C for 18 h. Cleaved DNA fragments were resolved in ethidium bromide stained 2% agarose gel electrophoresis and identified under ultraviolet light. Gel images were capture using Bio-Rad Molecular GEL Universal Hood Imaging System.

4. PCR RFLPs analysis and genotypes determination

The genotype and allelic type was determined by analyzing the banding pattern of the PCR RFLPs images as presented in (Fig. 1a–c). Digestion of the amplified DNA by the specific restriction enzymes was used to determine *Bsm*I/*Apa*I/*Taq*I polymorphisms. The lower case letter for an allele represents the presence of the restriction site (b, a, t) and the uppercase letter for an allele represents the absence of the