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RESEARCH ARTICLE

Study of the association of Vitamin D Receptor Gene- FOK1 single nucleotide polymorphism with chronic HCV related hepatocellular carcinoma in sample of Egyptian patients and its relation to insulin resistance state.

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Abstract

Background and aim of the work : Hepatocellular carcinoma (HCC) is the fifth common cause cancer - related death worldwide. The vitamin D receptor (VDR) is an intracellular hormone receptor that mediates vitamin D action and interacts with specific nucleotide sequences of target genes to produce a variety of biologic effects .The VDR gene polymorphisms have been identified that may influence cancer development including HCC .This study aims to evaluate the possible association between VDR gene polymorphism with (HCC) in top of chronic hepatitis C (HCV) patients and correlate it with insulin resistance (IR) .

Subjects and Methods: 103 HCV related HCC, 44 non-viral HCC ,100 control subjects were analyzed for serum 1,25 (OH)2D, fasting insulin level using and VDR-FOK1 gene polymorphism.

Results : showed that individuals with F/F homozygote have a lower risk in HCC development ,while others with f allele have a higher risk (with OR =2.3) and we found that vitamin D level was significant lower in HCC with IR in HCC cases than normal control**.**

Conclusion: VDR genetic polymorphisms are significantly associated with the occurrence of HCV related HCC specially f allele carriers. Moreover, IR is associated with the course of chronic HCV infection and considered to be an additional predisposing factor for progression to HCC in such patients**.**

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INTRODUCTION

Hepatocellular carcinoma is the most common primary malignant liver tumor and one of the most common causes of cancer mortality in the world. It usually arises on top of cirrhotic liver. It has high incidence rate above 40 years with male to female ratio about 8:1 with more than 90% mortality rate. It is estimated that HCC is responsible for more than 600,000 deaths annually worldwide **[1]**.The burden of HCC has been increasing in Egypt with doubling in the incidence rate in the past 10 years **[2]**. Many risk factors predispose to HCC, these risk factors may present individually or collectively depending on the environmental situations such as hepatitis B virus (HBV),

hepatitis C virus (HCV), alcoholism, aflatoxin (AF), schistosomiasis and some hereditary diseases as haemochromatosis and haemophilia **[3]**.

Obesity and the metabolic syndrome (MS) are growing epidemics associated with an increasing risk for many types of cancer. In the liver, inflammatory and angiogenic changes due to insulin resistance and fatty liver disease are associated with an increasing incidence of liver cancer. Regardless of underlying liver disease, cirrhosis remains the most important risk factor for hepatocellular carcinoma (HCC) although rare cases of HCC arising without cirrhosis raise the possibility of a direct carcinogenesis secondary to nonalcoholic fatty liver disease (NAFLD). Moreover, MS and its different features may also increase the risk of HCC in the setting of chronic liver diseases of other causes such as viral hepatitis or alcohol abuse. Taking into account all these data, it is necessary to better determine the risk of developing HCC in patients with MS to improve the screening guidelines and develop prophylactic treatments in this setting **[4].**

Numerous population studies have revealed strong links between obesity and the development of liver cancer. Obesity can alter hepatic pathology, metabolism and promote inflammation, leading to nonalcoholic fatty liver disease (NAFLD) and the progression to the more severe form, non-alcoholic steatohepatitis (NASH). NASH is characterized by prominent steatosis and inflammation, and can lead to HCC **[5]**.

Non-alcoholic steatohepatitis (NASH) is the liver manifestation of metabolic syndrome, which constellates obesity, insulin resistance and dyslipidemia. It is correlated significantly in many studies with HCC. Recent evidence also indicates the significant role of genetic factors in contributing to the pathogenesis of NASH and induced hepatic malignancy **[6]**.

Vitamin D is involved in the metabolism of skeleton as asystemic hormone but also has important roles in the regulation of host immune responses, fibrogenesis and development of cancer through vitamin receptor (VDR) **[7]**. Previous data have suggested that vitamin level may influence cancer development. In particular ,several single nucleotide polymorphisms have been described in the VDR gene ,and some polymorphisms are associated with tumor occurrence **[8]**.For instance ,VDR polymorphisms have been related to cancers of the breast ,prostate ,skin ,colon-rectum ,bladder and kidney ,although with conflicting observations**[9]**.

VDR polymorphisms have also been investigated in the context of some chronic liver disease,such as chronic hepatitis B ,primary biliary cirrhosis and auto immune hepatitis **[10]**.In recent published study, VDR polymorphism may be used as molecular marker to predict the risk and to evaluate the disease severity of HCC in patients with chronic hepatitis B **[11]**.

The aim of current study to investigate the association of VDR with HCV related HCC and to find a possible relation to insulin resistance state in sample of Egyptian patients attending Mansoura University Hospitals.

Materials and Methods:

This hospital-based case-control study included 147 HCC patients recruited prospectively from out and inpatient clinics of Tropical Medicine Department, Mansoura University during the period from March 2011 to March 2014. The study was approved by the ethical committee of the faculty and informed consents were obtained from all subjects.Subjects in this study were classified into patients 103 patients with HCC on top of chronic HCV infection, 44 non-viral HCC. 100 Healthy volunteers as a control group. They are sero-negative for hepatitis C virus& HBs-Ag antibodies, also, abdominal ultrasound examination revealed nothing abnormal.

HCC was diagnosed according to the diagnostic guidelines of the European Association for the Study of the Liver **[12].**Exclusion criteria included are: Patients with liver diseases other than HCC such as (Primary Billary Cirrhosis, Primary Sclerosing Cholongitis, Toxic hepatitis, Wilson disease, Tyrosinemia), also Patients with HCC but with no cirrhosis or with AFP level less than 200 ng/dl, patients with liver metastasis as well as HIV infected patients. also usage of Nexavar was excluded.

Samples collection: 2 ml venous blood samples were delivered to sterile collection tubes containing K2EDTA (Stored as EDTA anticoagulated blood Samples at -70^{o}C for DNA extraction and genotyping of Vitamin D using the specific restriction enzyme (RFLP). Another 5 ml blood samples were delivered to Vacuum blood collection tubes and allowed to clot for 15 minutes and centrifuged at 7000 r.p.m for 10 minutes for serum separation then serum collected in other sterile tubes and stored at - 70^{o}C until used to determine serum α fetoprotein Serum vitamin D level and serum liver function tests ,as well as fasting blood glucose and insulin levels for estimation of HOMA-IR.

DNA Extraction: Genomic DNA was extracted from EDTA-anticoagulated peripheral blood leucocytes using G-spin™ Total DNA Extraction Kit supplied by intron biotechnology, IBT-QMS-T1704 (R01-2012-01).The average DNA concentration was 0.127±0.005µg/µl determined by measuring the absorbance at 260 nm and 280 nm (Jenway, Genova Model, UK). All samples had a 260/280 nm absorbance ratio between 1.6 and 1.79. The integrity of the DNA was checked by electrophoresis on 0.8 % agarose gel stained with ethidium bromide using Gel electrophoresis apparatus.

Genotyping of VDR single nucleotide polymorphism FOKI rs10735810 (#K1081)-Polymerase Chain Reaction (SNP-PCR) [13]: The primers sequences used for DNA amplification are Forward:5'-AGCTGGCCCTGGCACTGACTCTGCTCT-3', Reverse: 5'-ATGGAAACACCTTGCTTCTTCTCCCTC-3'**.** For FOKI-SNPs, PCR was carried out in 50 µL final reaction volume using 2X PCR Master mix Solution Dream Taq thermo scientific(#K 1081 - 200rxns US patent NO 6,127,155. Applied biosystems,850 lincoln Centre Drive Foster city California).The following mixture was prepared for each sample: 25 µl PCR Master mix solution (2x), 1µl (20 pmole) of forward primer, 1µl (20 pmole) of reverse primer, 2µl (200ng) of genomic DNA and 21 µl of double distilled deionizer water. Amplification was performed in a Thermal Cycler (TECHEN TC-312, Model FTC3102D, Barloworld Scientific Ltd. Stone, Stafford Shire St., 150 SA, UK) using the following program Initial Denaturation at 94°C for 5 minutes35 cycles of: Denaturation at 94°C for 30 sec then, annealing at 61°C for 30 seconds then, extension at72°C for 1 minute. Amplified samples were digested with the specific restriction enzyme;(Fast Digest FOKI for 100rxns #FD2144, Lot :00185288 ,thermoscientific 20,000 units /ml) according to the manufacturing instructions1µL (2 units) of the Fast Digest enzyme(Thermo scientific)10 µL of amplified PCR product (1 µg)5 µL (10X) of Fast Digest Green Buffer (20 mM Tris-acetate, 10 mM Magnesiumacetate50 mM Potassium acetate,1 mM DTT (pH 7.9@25°C) 34.0 µL of sterile distilled waterto obtain 50 µl total Reaction Volume incubated at 37C° in heat block for 5 min.This enzyme the digestion products were electrophoresed on a 3.0% agarose gel for 60 min, stained with ethidium bromide using Gel electrophoresis apparatus, visualized via Light UV Transilluminator (Model TUV-20, OWI Scientific, Inc. 800 242-5560, France) and photographed .FOKI restriction enzyme digestion productsare : homogenous F/F shows one band at 265bp, F/f heterogenous genotype show the three bands (265, 196 & 69),and f/f shows two bands at (196, 69) bp.

Estimation of serum Human (1,25 (OH)2D3) : The authors used the commercially available Kit (Catalog Number: MBS733782 ,96 tests , San Diego, CA 92195-330 8 USA): This essay employs the quantitative sandwich ELISA technique. It was performed according to the manufacturer's instructions. The absorbance of each sample was read on plate ELISA Reader (Tecan, Sunrise, Austria) at 450 nm wave length.

Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) : In each sample, the degree of insulin resistance was estimated by the homeostasis model assessment (HOMA-IR) as described by **[14].** HOMA-IR was calculated by taking into account fasting insulin and blood glucose levels according to the equation (HOMA-IR) = fasting insulin (µU/ml) × fasting blood glucose mg/dl) ×0.0551/ 22.5. Serum insulin level was estimated using ELISA Kit **[15].** The kit was provided from Diagnostic Systems laboratories. Inc. Corporate Headquarters, 445 Medical Center, Blvd. Webster, Texas 77598-4217 USA. Fasting blood glucose level was done according to method of **[16]** and the kits were provided from Elitech diagnostics, Zone Industrielle- 61500, Sees France.

Estimation of serum α-faetoprotein (AFP) : Serum level of α-faetoprotein (AFP) was estimated using alpha Fetoprotein Human ELISA Kit (catalog number ab108838, Abcam, alpha Fetoprotein Human). HCV antibody and HbsAg were estimated by An enzyme immunoassay (ELISA) for the qualitative detection of IgG antibodies to Hepatitis C Virus (HCV) **[17]** and HBsAg **[18]** in human serum using RUO kits (catalog number; 6307125& 6307105 respectively- LINEAR CHEMICALS S.L. Joaquim Costa 18 2ª planta. 08390 Montgat, Barcelona, SPAIN).Diagnosis of HCV infection was confirmed by the Cobas TaqMan HCV test real-time RT quantifiable PCR for HCV.

Estimation of serum liver function : Liver functions estimation was performed colorimetrically using the commercially available kits. Alanine and aspartate amino transferase serum alkaline phosphate (ALP) were estimated according to the method [19],Total-Billirubin, Direct Billirubin[20] and Albumin(Alb)[21].The kits were provided from Elitech diagnostics, Zone Industrielle 61500, Sees France.

Statistical Analysis : The data were expressed as mean, standard deviation (±SD) for the studied groups. Statistical analysis was performed using statistical package for social science (SPSS) program version 17 (SPSS

Inc., Chicago, IL, USA). The studied groups were compared by Chi square (X2) test to evaluate Hardy-Weinberg equilibrium in allele and genotype frequencies in the study group.

The association of VDR gene receptor polymorphisms with hepatocellular carcinoma with steatosis risk was estimated using odds ratios (OR) and 95% confidence intervals (95% CI) for the comparison of genotype and allele contrast. The difference was considered statistically significant when *P* value was less than 0.05.

Results

Table 1: Age and Biochemical parameters in the studied groups:

	Groups				P
	Control (n=100)		Cases(n=147)		
	Mean	±SD	Mean	±SD	
SGOT(U/L)	26.53	7.48	77.70	47.81	<0.0001
SGPT(U/L)	22.66	6.72	62.60	40.89	<0.0001
T.Bilirubin(mg/dl)	0.93	0.30	3.10	4.73	<0.0001
D.Bilirubin(mg/dl)	0.30	0.10	1.19	0.95	<0.0001
Albumin(g/dl)	3.95	0.80	3.08	0.80	<0.0001
ALP(U/L)	107.04	31.25	310.20	41.22	<0.0001
A.F.P(ng/ml)	9.01	2.02	356.10	612.78	<0.0001
Serum vit.D level (ng/ml)	49.535	13.246	37.461	18.475	<0.0001
BMI(Kg/m^2)	25.80	4.62	37.01	5.40	<.0001
HOMA-IR	2.19	0.73	6.28	1.10	<0.0001
Age(year)	56.55	11.01	57.69	8.68	0.3

BMI: *Body Mass* ***AFP*** :α-faetoprotein **P** < 0.05
HOMA-IR *:Index Homeostasis Model Assessment of Insulin Resistance*

Table 2: The genotyping and allelic frequency of VDR-FOK1 gene polymorphism in all studied groups:

		HCC cases(147)		Normal Control(100)		P value	OR (CI95%)
		No	%	No	%		
Genotypes	**FF**	37	25.17%	54	54.00%	-	1(Ref)
	Ff	62	42.18%	39	39.00%	0.004	2.3 (1.3-4.1)
	ff	48	32.65%	7	7.00%	<0.0001	10.0 (4.08-24.5)
	Ff+ff	110	74.8%	46	46.0%	<0.0001	3.49 (2.03-5.99)
Allels	**F**	136	46.3%	147	73.5 %	<0.0001	1(Ref)
	f	158	53.7%	53	26.5 %		3.2 (2.18-4.75)

Table 3: Genotyping and allelic frequency in VDR-FOK1 gene polymorphism virus positive versus virus negative HCC patients:

		HCC cases(147)				P value	OR (CI 95%)
		Virus positive(103)		Virus negative(44)			
		No	%	No	%		
Genotypes	FF	18	17.48%	19	43.18%	-	1(Ref)
	Ff	44	42.72%	18	40.91%	0.026	2.58(1.1-6.02)
	ff	41	39.81%	7	15.91%	<0.0001	6.18(2.2-17.3)
	Ff+ff	85	82.5%	25	56.8%	0.001	3.58(1.6-7.85)
Allels	F	80	38.8%	56	63.6%	<0.0001	1(Ref)
	f	126	61.2%	32	36.4%		2.75(1.6-4.60)

Table 4: Association of Vitamin D serum level and FOKI genotyping in HCC cases versus controls :

ELISA of Vit D		Genotyping			P1
		FF	Ff	ff	
Control (n=100)	Median	50.814	45.999	49.904	0.7
	Minimum	28.860	12.402	41.845	
	Maximum	78.859	80.441	58.467	
Cases (n=147)	Median	54.000	32.758[a]	24.319[bc]	<0.0001
	Minimum	16.783	12.001	12.001	
	Maximum	82.248	82.248	59.000	
	P2	0.9	0.001	<0.0001	

Table 5: Association of Vitamin D serum level with FOKI allele in HCC patients versus the controls :

ELISA of VitD		Alleles						P1
		F			F			
		Median	Minimum	Maximum	Median	Minimum	Maximum	
Groups	Control (n=100)	49.98	12.40	80.44	47.96	12.40	80.44	0.46
	Cases (n=147)	42.58	12.00	82.25	26.31	12.00	82.25	<0.0001
	P2	0.002			<0.0001			

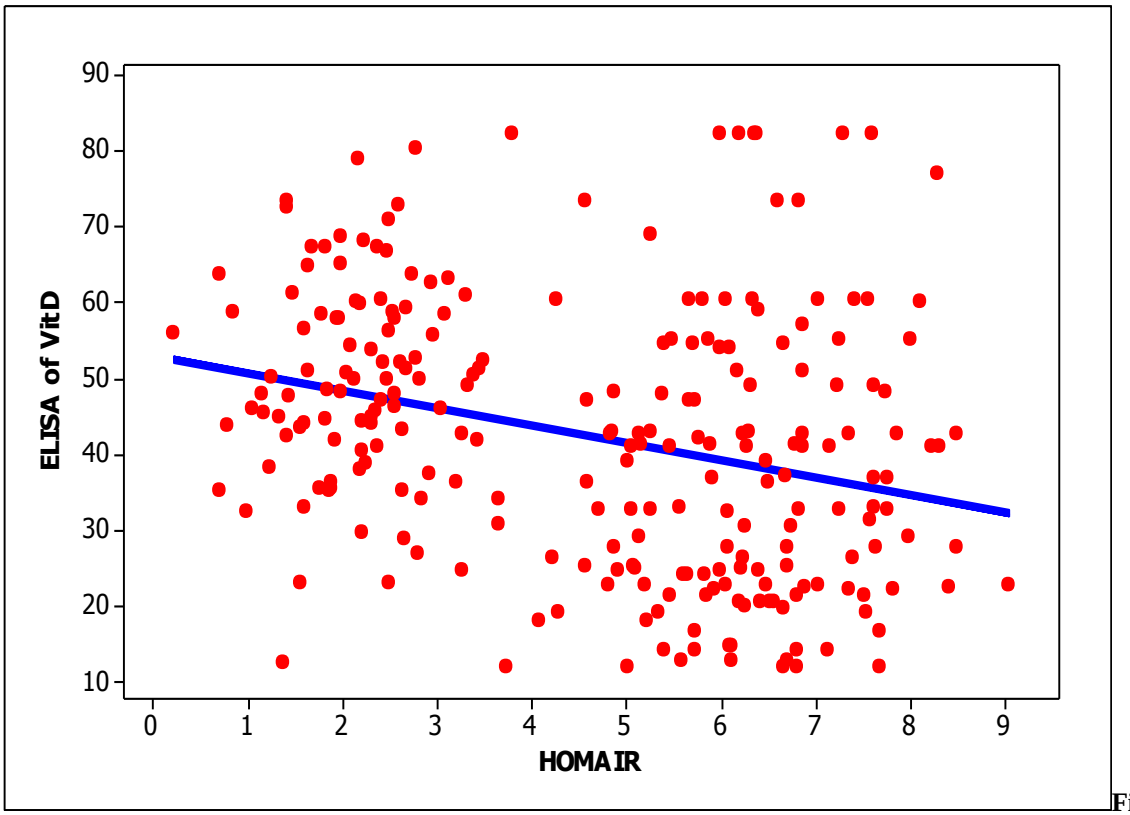

Fig 1: Correlation of serum vitamin D level with HOMA-IR

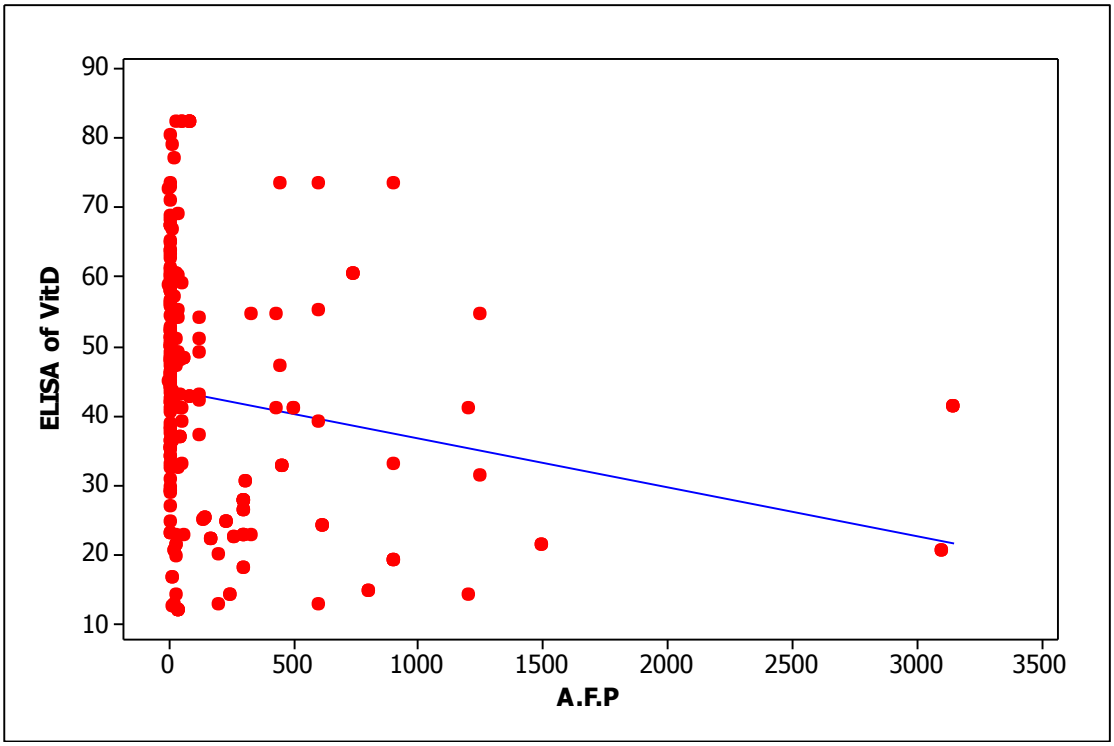

Fig 2: Correlation of serum vitamin D level with A.F.P

In Table 1 There is asignificant difference for the measured parameters Body Mass Index (BMI), homeostasis model assessment (HOMA-IR), Total Billirubin ,Direct Billirubin ,albumin(Alb),alkaline phosphatase(ALP),alpha featoprotein(AFP), Aspartate aminotransferase (AST), Serum glutamic pyruvic transaminase(SGPT)and VitaminD with pvalue <0.0001and age show no significant(with p=0.3) when compared HCC groups to control .In table 2 ,the genotyping and allelic frequency of vitamin D receptor gene distribution shows asignificant difference when compared HCC to control in (FF,Ff,ff,Ff+ff)and alleles frequencies with p<0.0001).

Table 3 show the frequencies of VDR genotypes in patients with and without HCV infection .We find a significant difference between HCC with HCV and HCC without HCV with (p<0.001). In Table 4,study of the serum vitamin D level in the different genotype grouping of FOKI vitamin receptor gene in HCC cases and controls .It shows no significant in controls .whereas in HCC cases show significant decrease with p<0.0001 FF/ff, FF/Ff show statically signicant decrease (p<0.0001) regarding FF shows no statically significant(p=0.9),Ff shows statically significant decrease with (p=0.001) ,ff shows statically significant decrease (p<0.0001) in cases to controls. In **table 5** ,study of the serum vitamin D level in the different genotype grouping with FOKI alleles. It's clear that control normal groups shows no statically significant (p=0.46) ,while in cases show statically significant decrease with p<0.0001) However,in comparison of vit D in F allele with regard to cases show statically significant decrease in cases to control with p =0.002 ,also in f allele show statically significant decrease in cases to control with p <0.0001 . When we make correlation serum vitaminD level shows negative significant negative correlation in all biochemicals as shown in Figure 1, 2: (HOMA-IR, A.F.P) except albumin shows positive significant positive correlation.

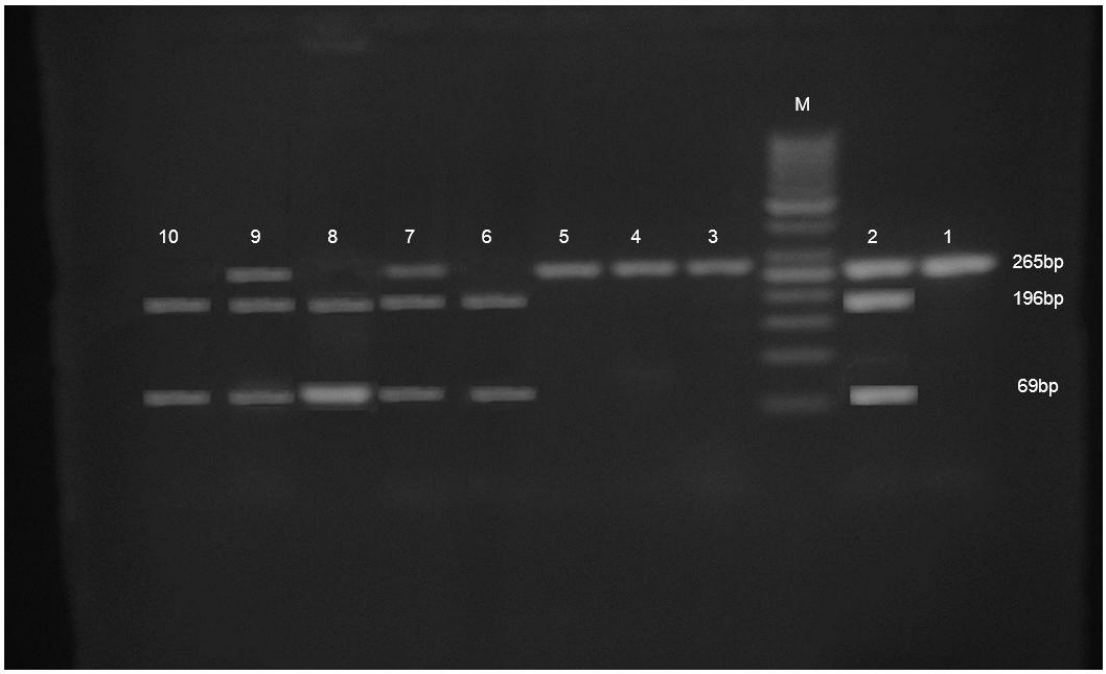

Fig 3: Agarose gel electrophoretic analysis of FOKI F/f polymorphism after Fast Digestion analysis Lane M (100bp) represents the molecular marker Lanes ,(3, 4, 5)have F/F showing one band at 265bp, lane (2, 7, 9) have F/f heterogenous genotype showing the three bands (265, 196 & 69). Lane (6, 8, 10) have f/f showing two bands at (196, 69) bp.

Discussion

Hepatocellular carcinoma (HCC) is the fifth most common cause of cancer-related death worldwide. Primary hepatocellular carcinoma can be found most frequently (80-90%) in patients with liver cirrhosis. The most frequent causes of liver cirrhosis are chronic hepatitis B and C viral infections and chronic alcohol consumption. The occurrence of hepatocellular carcinoma is about 3-5% in patients with alcoholic liver disease. Other predisposing causes can be: non-alcoholic steatohepatitis (NASH), obesity, diabetes mellitus, autoimmune hepatitis, intrahepatic biliary inflammations, copper and iron metabolic diseases, congenital alpha-1-antitripsin deficiency, and other

pathogenic factors as smoking and different chemical agents . The burden of HCC has been increasing in Egypt with doubling in the incidence rate in the past 10 years **[22] .**In Egypt, it is the second most common malignancy in males and the sixth in females **[1] .** Over a decade, there were nearly two-fold increases in proportion of HCC among chronic liver disease patients in Egypt with a significant decline in HBV infection and a slight increase of HCV infection as a risk factor **[2].**

Nonalcoholic fatty liver disease is strongly associated with diseases of affluence such as obesity, DM2 or insulin, hypertension, and dyslipidemia which are regarded as the liver manifestation of the metabolic syndrome **[23].** Indeed, 75% of obese individuals have hepatic steatosis **[24].** The prevalence of the metabolic syndrome is rapidly increasing, due to hyper alimentation and a sedentary life style **[25]** , and is reflected by the increasing prevalence of NAFLD and associated complications **[26]** . NAFLD affects a third of adults, while NASH occurs in about 3% of the adult population in the western world **[27].** Numerous population studies have revealed strong links between obesity and the development of liver cancer. Obesity can alter hepatic pathology, metabolism and promote inflammation, leading to nonalcoholic fatty liver disease (NAFLD) and the progression to the more severe form, non-alcoholic steatohepatitis (NASH).NASH is characterized by prominent steatosis and inflammation, and can lead to HCC **[6] .**Currently, NAFLD is considered a benign condition that can progress to NASH in approximately 20% of cases. In turn, 20% of NASH cases are thought to progress to cirrhosis. HCC can develop in individuals with NASH combined with cirrhosis and also albeit less often in cases with NASH occurring in the absence of cirrhosis. The actual rate of HCC development in NASH-associated cirrhosis is estimated to be similar to that occurring in alcoholics with cirrhosis (3–4%/year). There are no current estimates of the rate of HCC development in cases of NASH without cirrhosis but increasingly, such cases are being recognized **[28] .**

This case control study aimed to investigate the association of VDR polymorphism with the occurrence of HCC and their relation to insulin resistance which has some incrimination to HCC development.

Vitamin D is a fat soluble secosteroid which is involved in a wide variety of biological processes like bone metabolism, modulation of immune response, cell proliferation and cell differentiation **[29].**

The genomic actions of vitamin D are mediated through its binding to the Vitamin D Receptor (VDR), which allows it to modulate the expression of genes in a cell-and tissue-specific manner **[30]** . VDR directly or indirectly regulates the expression of more than 200 genes that influence cell proliferation, differentiation and apoptosis, as well as immunomodulation and angiogenesis **[31] .**The gene coding for vitamin D is located on chromosome 12q12–q14 and is highly polymorphic **[32]** and consisting of 4 SNPs .We study one of them is FOKI and its susceptibility of HCC in this study **[33] .**Many investigators suggest a role of vitamin D in pathogenesis of many cancers including breast cancer **[34]** , prostate **[35]** , pancreatic cells **[36]** colorectal **[37]** And its role in angiogenesis, tumors growth and metastasis.VDR polymorphism is used as a molecular marker to predict the risk and to evaluate the disease severity of HCC in patients with chronic hepatitis HCV in Chinese **[38]**

In the current study, we found a significant association between HCC with FOKI-VDR gene polymorphism and FF as a reference. Our data showed that (ff) carriage had a significantly higher risk for development of HCC after adjustment with age, HCV infection, BMI and HOMA-IR with F allele as a reference. The OR of F allele carriage was 3.2($P<0.0001$).

This finding was previously confirmed by Falleti **[39]** and Vogel **[40]** . Falleti **[39]** demonstrated an association between the VDR genetic polymorphism and HCC development **[39]** while, Vogel **[40]** found an association between VDR genetic polymorphism chronic autoimmune hepatitis **[40]**.Moreover, Xing **[38]** reported in their study that only FokI C>T(F>f) was associated with HCC susceptibility in Chinese patients with chronic HBV infection; other SNPs, including BsmI, were associated with the development of HCC. HCC patients had a significantly higher cirrhosis rate than non-HCC patients suggesting that the VDR polymorphisms at FokI locus may relate to HCC risk by enhancing the occurrence of liver cirrhosis while, Fan had the opposite result observed by them **[41]**.

HCV has been identified as a cause of metabolic syndrome that includes dyslipidemia, diabetes and insulin resistance (IR) **[42].** In the present work, we demonstrate High HOMA-IR in HCC cases than normal control with $p<0.0001$. Chen et al., **[43]** suggested a strong synergistic effect of metabolic factors and viral hepatitis in HCC development in HCV-infected patients, our result support this synergistic metabolic effect of HCV in HCC susceptibility as the group of chronic HCV patients complicated by IR has more association with hepatocellular carcinoma**[43].**

Kawaguchi and Sata **[44]** reported that the development of intrahepatic complications, including hepatocellular carcinoma (HCC) is linked to insulin resistance state and metabolic syndrome **[44]** .It is known that insulin resistance, hepatic lipid accumulation and oxidative stress, could be considered to be pathophysiologic mechanisms responsible for the development of HCC in patients with IR **[45].** Besides these possibilities, insulin has a mitogenic effect, suggesting that insulin may be directly linked to hepatocarcinogenesis **[46].**

In the present study, there in a negative correlation between HOMA-IR with vitaminD level with ($p<0.0001$).This could be another possible mechanism that links IR to HCC.

The VDR is expressed by macrophages; 1,25(OH)2D3 upregulates the inhibitor of nuclear factor(NF)-κB (IκB-alpha) by increasing mRNA stability and decreasing IκB-alpha phosphorylation, suggesting that 1,25(OH)2D3 has an anti-inflammatory effect on macrophages **[47].** The association of vitamin D status and cardio-metabolic disorders (cardiovascular disease, diabetes, and metabolic syndrome) was reviewed in a meta-analysis of 28 independently published studies **[48].** An important mediator of vitamin D action is the VDR which functions as a transcription factor when bound to1,25(OH)2D. There is evidence that VDR genotype may affect insulin resistance, both in regards to insulin secretion (the Apa1 VDR polymorphism) and insulin resistance (theBsmI VDR polymorphism) **[49].**The association of the FokI,ApaI, BsmI and TaqI polymorphisms of the VDR gene withtype 2 diabetes was explored in a case–control study in Korean population . It was reported that individuals with lower 25(OH)D(3) levels are presented with a significantly increased risk for NAFLD compared with those of higher levels, even after adjusting for the body mass index and metabolic syndrome. Accordingly, individuals with higher serum 25(OH) D3presented with a significantly reduced risk for NAFLD independent of obesity and metabolic syndrome **[50].** This is confirmed by the results of the current study as vitamin D level was significant lower in HCC with IR in HCC cases than normal control**.** This indicated that serum vitamin D level is inversely associated with the presence of dys-metabolic conditions **[51]** and may play a role in both NAFLD and cirrhosis outcomes though its anti-inflammatory and insulin-sensitizing activities.

In the current study, we presented the frequencies of VDR genotypes in patients with and without HCV infection .We found a significant difference between HCC with HCV and HCC without HCV with ($p<0.001$).This is supported by **[38].**

Association studies of several polymorphisms in the VDR gene have been performed to investigate their implication with severity of chronic liver disease **[38].** They found a significant association of VDR ApaI polymorphism with the development of HCC in chronic HCV infection. The characterization of VDR genetic polymorphisms in HCV carriers may help to identify those who are at high risk of developing HCC **[11].** A significant association between the polymorphisms in VDR which serves as the physiological target to mediate vitamin D effects and HCV-induced HCC suggests that an impaired vitamin D metabolism contributes to hepatocarcinogenesis in chronic HCV infection **[11].** VDR polymorphisms have been associated with liver diseases such as primary biliary cirrhosis, autoimmune hepatitis**,** alcohol-related hepatocarcinoma, and HBV **[41]**. VDR expression in normal liver cells has been demonstrated in several studies **[52].**

This observation in accordance with previous studies suggests a strong synergistic effect of metabolic factors and viral hepatitis in HCC development among HCV-infected patients **[43]**.There are several possible mechanisms by which obesity, insulin resistance, and T2D increase risk of HCC **[53]**liver vitamin D receptor (VDR) expression has a strong association with the NASH diagnosis independent of BMI, insulin resistance or adiponectin, suggesting a loss in the hydroxylation of hepatocytes and the hepato-protective role of vitamin D **[54]**. In the same study, liver VDR expression was strongly associated with a diagnosis of NASH independently from other metabolic determinants such as BMI, insulin resistance or adiponectin; additionally, CYP27A1 and CYP2R1 enzymes that catalyse the 25-hydroxylation of vitamin D was preserved in NASH patients the diagnosis of NASH as independent variables confirmed the presence of a strong association between low liver VDR expression and the diagnosis of NASH independently from all other metabolic parameters**[54],**

Finally, Hammad et al., **[55]** indicated that vitamin D, as well as IL-6 and IL-17, was a potential biomarker for the development of HCC in patients with hepatitis C [55]. Lange et al.,**[56]** also, demonstrated that vitamin D deficiency was associated with the development of HCC in patients with hepatitis C**[56].**

Insulin resistance measured by HOMA-IR, regardless of the presence of diabetes, is significantly associated with HCC development in patients with chronic HCV infection. HCV-associated insulin resistance is involved in the development of various complications associated with Hepatic steatosis that is commonly observed **[57]** and is an independent risk factor for disease progression in patients with HCV infection **[58].** Insulin resistance is associated

with a poor response to anti-viral treatment in patients with HCV genotype 1, 2, and 3 infections **[59].** Insulin resistance is closely associated with progression of hepatic fibrosis in patients with HCV infection **[60].**

Conclusion:

The current study demonstrates that VDR genetic polymorphisms are significantly associated with the occurrence of HCV related HCC especially f allele carriers which could be considered as a risk factor of hepatocellular carcinoma in Egyptian patients. Moreover, IR is associated with the course of chronic HCV infection and considered to be an additional predisposing factor for progression of the metabolic liver disease in the form of NAFLD and NASH to HCC in such patients**.**

Limitation of the study :

Some limitation should be addressed, the limited case numbers included in the current study. Secondly, no information was available regarding vitamin D intake (dietary or supplemental) that influences the circulating vitamin D levels of patients. Thirdly, this work dealt only with one site of VDR- SNP gene polymorphism.

Recommendation :

Further studies with larger sample sizes are needed to validate the genetic effects of the VDR polymorphisms on HCC in Egyptian patients. Also, other single nucleotide polymorphism sites are needed to be subjected for investigation in Egypt and to correlate them with the insulin resistance and hepatic steatosis state.

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