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The Distribution of Vitamin D Receptor Genotypes and Haplotypes in Southern Moroccan Population

Driss Afilal[1], Mohamed Amine Basselam[1], Smail Chadli[1,2], Zahra Brakez[1] , Said Chouham[1] , António Brehm[3] , El Hassan Izaabel*[1]

[1]Cellullar Biology and Molecular Genetics Laboratory, Faculty of Sciences, University Ibn-Zohr. Agadir. Morocco
[2]Higher Institute of Nursing Professions and Health Techniques, Agadir. Morocco
[3]Human Genetics Laboratory, Life Sciences Faculty University of Madeira, Campus of Penteada, Funchal, Portugal

ABSTRACT

It is well known that biological functions of the vitamin D receptor (VDR) may be affected by genetic variations in the VDR gene. The distribution of these variants either alone or combined in haplotypes, also show remarkable differences depending on the ethnicity of the population under study. Nevertheless, still little information about these variants are available from North African and Saharan populations. Here, we investigate the distribution of four common VDR gene polymorphisms (SNPs rs2228570, rs1544410, rs7975232 and rs731236) and their haplotypes in a southern Moroccan heterogeneous population constituted by individuals of Amazigh and Arab ancestry. In this study, were genotyped 260 individuals for four common SNPs in the VDR gene using restriction fragment length polymorphism analysis. Allelic and genotype frequencies were calculated, and relationships among the four VDR gene polymorphisms were assessed by measuring pair-wise linkage disequilibrium (LD). We then compared our results to those reported in several Mediterranean and International Hap Map Project populations. In the population of southern Moroccan area that we studied, the VDR rs2228570C-allele (71,5 %), rs1544410 G-allele (60,5 %), rs7975232 T-allele (60%) and rs731236 T-allele (66 %) all occur at unexpected high frequencies. Linkage disequilibrium (LD) analysis did not show any linkage between rs2228570 and the other investigated VDR SNPs. However, strong LD was found between SNPs (rs1544410- rs7975232- rs731236) located in the 3' Untranslated Region (UTR), and that may explain the high frequencies of haplotypes GGT 36.5 % and ATC 27.2%. In spite of these results the distribution of VDR variants in the southern Morocco population was relatively not significant when compared to Mediterranean populations.

Keywords : Vitamin D receptor (VDR), Genotype-Allele frequencies, Linkage disequilibrium (LD), Amazigh, Southern Moroccan population.

I. INTRODUCTION

The vitamin D endocrine system regulates multiple routes of calcium metabolism, cellular proliferation and differentiation in various organs, including those directly involved in calcium and phosphorus homeostasis (i.e. bones, intestinal tract, kidneys and parathyroid gland) [1]. It has also been reported to play an important role in other metabolic pathways, such as those involved in the immune response, glucose homeostasis, as well as mechanisms of insulin release and physiopathology of diverse forms of cancer [2].

There is a growing interest on the role of vitamin D in a variety of pathologic conditions such as cardiovascular disorders, obesity, pulmonary, liver and kidney diseases, several forms of cancer, among many others. Moreover, epidemiological studies have shown a low vitamin D status in the human populations [1, 3-6]. Initially, vitamin D was thought to play a restricted role in the calcium homeostasis but its pleiotropic actions in biology and their clinical significances are becoming more apparent, resulting in an increasing awareness of its biological functions.

Vitamin D functions are mediated through the vitamin D receptor (VDR), a nuclear transcription factor with a DNA-binding domain belonging to the nuclear hormone receptor super-family. The VDR forms a heterodimer with the retinoid X receptor and this heterodimer regulates gene expression by interacting with vitamin D response elements VDRE in the promoter regions of target genes [7-9]. Recent evidence showed that VDR may bind to over 2000 binding sites, resulting in an up-regulation of genes controlling the immune response, intracellular signaling cascades and genes of the reproductive tissues [6, 10].

The Vitamin D receptor is widely distributed in more than 38 tissues where it controls the expression of crucial genes: (i.e. skeletal tissue, intestine, kidney, thyroid, parathyroid, hypophysis, testis, ovary, pancreas, immune cells, muscles, cardiac cells, prostate, skin, mammary gland, digestive tracts, brain). The ubiquitous distribution and expression of VDR support the vitamin D diverse effects in skeletal and extraskeletal tissues [11, 12].

The biological functions of vitamin D was shown to be affected and modulated by several genetic variations that were identified within the VDR gene, both in the regulatory and coding regions. These variations may influence the stability, quantity and activity of the VDR protein and the rate of VDR gene transcription [8, 13]. Four single nucleotide polymorphisms (SNPs) are already identified; rs2228570 is a start codon mutation that creates an alternative ATG initiation codon in exon 2, which in turn results in the production of a 424 amino acid protein, missing the three N-terminal amino acids. This short form of the polypeptide gives a 1.7 fold greater transcriptional activation compared to the other variant [14]. SNPs rs1544410, rs7975232 and rs731236 polymorphisms are located near the 3' end of the VDR gene; the first two are located in Intron 8 and do not appear to induce any changes either in the transcribed mRNA or in the translated protein. However, rs731236 induces a synonymous substitution at codon 352 in exon 9 [15].

The possible functional consequences of these common VDR allelic variants remain unclear [16]. Their high frequencies in human populations have made them targets to explain the variation and association of VDR with several common pathological conditions. A number of studies performed in different populations and ethnic groups reported a correlation between these polymorphisms and haplotypes with mineral density of bone and the risk of fracture [2, 17, 18]. Other reports suggest a possible role in the development of cardiovascular diseases [1, 5, 19], colon, breast, prostate and other cancers [15, 20-22], insulin resistance, diabetes [1, 23], metabolic syndrome, obesity [24], immune-related disorders [4, 25] and depression [26].

The contribution of the nuclear vitamin D receptor to diverse physiopathological disorders remains poorly understood and with inconsistent results among populations. This inconsistency might be in part due to the genetic structure and background of the human populations studied, especially in what concerns allele frequencies of common variants on the VDR gene. Polymorphisms of this gene have been reported in several populations and ethnic groups with important variations in frequency ([2]; Hap Map Data). It is thus important to define the haplotype structure and relative allele frequencies in different populations prior to any association study with a given pathologic condition.

Up to now there is no data regarding the frequencies of VDR polymorphisms and their haplotypes in the southern Moroccan population which is itself an assortment of peoples from diverse ethnic origins: Arabic, Amazigh and sub-Saharan. In this study we examine the distribution, frequency, and linkage disequilibrium of four common VDR SNPs in different groups from the Souss-Massa-Drâa region of south of Morocco.

II. METHODS AND MATERIAL

Study population

Our target population is from the Souss-Massa-Drâa region of southern Morocco and reflects a heterogeneous group of individuals from Arabic, Amazigh and sub-Saharan origin. Two hundred sixty healthy individuals (men and women) representing different subpopulations living in the area of southern Moroccan, all autochthones and born in the south of Morocco whose parents are from this same region for at least four generations were included in the present study. All subjects provided written informed consent and the study was conducted according to the legal guidelines from the Ibn Zohr University.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using the salting out procedure as described elsewhere [27]. VDR SNPs rs2228570, rs7975232 and rs731236 were assessed in 260 individuals and rs1544410 for 254. Genotyping was performed using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) approach. Primer sequences used for amplification of the SNPs were as previously described [28].

PCR was performed in a 15 µl reaction volume containing 2.4 µM of each primer, 10 mM of dNTP, 1x PCR buffer, 1.5 mM MgCl2, 1 U of Taq DNA polymerase (Promega), and 100 ng of genomic DNA, diluted to the final volume with H_2O. The running conditions were: pre-denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C, annealing temperature at 60°C for rs2228570, 67°C for rs1544410 and 64ºC to rs7975232 and rs731236 with an elongation period of 1 min at 72°C. A final cycle of elongation was performed at 72°C for 5 min. Amplified fragments were digested with the appropriate restriction enzyme (Biolab) according to the manufacturer's instructions and visualized on a 2% Agarose gel.

Statistical Analysis

The distribution of the four VDR SNPs was assessed for Hardy-Weinberg equilibrium and any deviation between the observed and expected frequencies was tested for significance using a Chi-squared-test (χ^2). A $P<0.05$ was considered to be statistically significant. Allele and genotype frequencies were calculated using Arlequin ver-3.5-software (http://cmpg.unibe.ch/software/arlequin3).
Reconstructing haplotypes from population genotypes was done using program PHASE version 2.1 which implements a Bayesian statistical method (http://stephenslab.uchicago.edu/software.html#phase) [29, 30]. For each pair-wise combination of variants, linkage disequilibrium was calculated using Genepop 4.0 [31].

The comparison of the distribution of VDR allele and haplotype frequencies between our population and other worldwide populations were assessed with χ^2 test. All computations were performed with BIOSYS-2 software (http://evolution.genetics.washington.edu/phylip/software.old.html) [32].

III. RESULTS AND DISCUSSION

Allele and genotype distributions of the SNPs surveilled are presented in (Table 1). The observed and expected frequencies in polymorphic sites showed no deviation from Hardy-Weinberg equilibrium, except rs1544410 SNP that shows significant differences (chi2=7.167, p-value=0.0074). Allele and haplotype frequencies of these polymorphisms in the population of South Morocco were compared to those reported in different worldwide populations, including Europeans, Asians, Arabics, North Africans and Sub-Saharans (Table 2, 4).

Table 1. Genotypes and allele frequency distribution of VDR gene (rs2228570- rs1544410- rs7975232- rs731236) polymorphism in Moroccan population.

	Genotype frequency (%)			Allele frequency (%)	
rs2228570	CC	CT	TT	C	T
	50	**43**	**7**	**71.5**	**28.5**
rs1544410	AA	AG	GG	A	G
	22	**35**	**43**	**39.5**	**60.5**
rs7975232	TT	TG	GG	T	G
	37	**46**	**17**	**60**	**40**
rs731236	TT	TC	CC	T	C
	43	**46**	**11**	**66**	**34**

The population of Southern Morocco showed all minor alleles for the four VDR genetic variants: rs2228570 C-allele (71,5 %), rs1544410 G-allele (60,5 %), rs7975232 T-allele (60 %) and rs731236 T-allele (66 %) were more frequent than rs2228570 T-allele (28,5 %), rs1544410 A-allele (39,5 %), rs7975232 G-allele (40 %) and rs731236 C-allele (34 %).

The rs2228570 C-allele corresponding to the short VDR protein, commonly found in our population, was associated with lower winter and summer serum 25(OH)D levels [13]. Its distribution in the southern Moroccan population did not show a significant difference compared to Europeans, Africans and Asians populations. Relatively ''ancient" polymorphisms show little variation between different ethnic groups whereas relatively new mutants might display large differences [2]. However, frequencies of intron 8 and exon 9 polymorphisms vary widely across different ethnic populations; the T and G alleles occur with higher frequencies in Asians compared to other populations. In

contrast, Mediterranean populations (except for Tunisians and Egyptians) showed little or no significant differences when compared to our southern Moroccan population (Table 2). In addition, the distribution of rs7975232 alleles was similar to that reported for Mediterranean populations (P> 0.05; Table 2), except for Tunisian and Portuguese populations who showed significant differences with our population (P< 0.001; Table 2). High significant differences were found when the southern Moroccan population was compared with Asian populations (Iran, China and Japan, P< 0.001; Table 2).

Table 2. Distribution of VDR allele's frequencies (%) compared to other worldwide populations.

		rs2228570			rs1544410			rs7975232			rs731236			
Country	N	C	T	P-value	A	G	P-value	T	G	P-value	T	C	P-value	References
Morocco	260	71.5	28.5		39.5	60.5	-	60	40	-	66	34	-	Present study
Tunisia	225	65	35	0.029	38	62	0.650	79	21	0.000	58	42	0.010	[42]
Egypt	120	80.8	19.2	0.006	61.8	38.2	0.000	58.3	41.7	0.663	56.3	43.7	0.001	[43]
Kenya (Maasai)	143	80	20	0.008	37	63	0.510	68	32	0.028	57	43	0.012	Hap Map Project [a]
Nigeria	113	81	19	0.006	28	72	0.002	63	37	0.466	71	29	0.195	Hap Map Project [a]
South Africa	88	82.1	17.9	0.007	24.1	75.9	0.000	62.5	37.5	0.557	79.5	20.5	0.001	[44]
Jordan	126	-	-	-	47.2	52.8	0.039	63.5	36.5	0.350	56.4	43.6	0.009	[45]
Syria	78	-	-	-	-	-	-	66	34	0.175	65	35	0.778	[46]
Turkey	60	65.8	34.2	0.217	32,5	67.5	0.159	51.7	48.3	0.095	70	30	0.397	[47]
Iran	100	-	-	-	-	-	-	45	55	0.000	35.5	64.5	0.000	[48]
Japon	86	67	33	0.244	12	88	0.000	34	66	0.000	88	12	0.000	Hap Map Project [a]
China	84	60	40	0.005	4	96	0.000	31	69	0.000	96	4	0.000	Hap Map Project [a]
Greece	96	82.8	17.2	0.002	62	38	0.000	54.2	45.8	0.161	43.7	56.3	0.000	[49]
French	100/189	54	46	0.000	37	63	0.550	54	46	0.071	57.5	42.5	0.034	[50, 51]
Sweden	100	-	-	-	40	60	0.887	53	47	0.088	61	39	0.212	[52]
Germany	100	60.1	39.9	0.003	57.4	42.6	0.000	55.4	44.6	0.272	58.1	41.9	0.046	[25]
Spain	310	66.5	33.5	0.359	-	-		-	-		65.5	34.5	0.887	[53]
Portugal	249	61.8	38.2	0.001	44.2	55.8	0.124	48.9	51.1	0.000	59.7	40.3	0.037	[54]
Netherland	563	66	34	0.025	57	43	0.000	54	46	0.057	58	42	0.002	[26]
Italy (Tuscan)	88	62	38	0.017	41	59	0.727	60	40	0.957	59	41	0.100	Hap Map Project [a]

[a] Hap Map Project Data.

Table 3. Estimated Haplotypes frequencies of VDR gene (rs2228570, rs1544410, rs7975232 and rs731236) in Moroccan population

Haplotype	rs2228570 (C27823T)	rs1544410 (A60890G)	rs7975232 (G61888T)	rs731236 (T61838C)	Frequency % (±S.E)
H1	C	A	T	T	7,76 (±0.0060)
H2	C	A	T	C	19,996 (±0.0074)
H3	C	A	G	T	1,238 (±0.0029)
H4	C	A	G	C	0,013 (±0.0005)
H5	C	G	T	T	11,208 (±0.0068)
H6	C	G	T	C	3,582 (±0.0059)
H7	C	G	G	T	26,973 (±0.0080)
H8	C	G	G	C	0,575 (±0.0036)
H9	T	A	T	T	2,745 (± 0.0051)
H10	T	A	T	C	7,158 (± 0.0068)
H11	T	A	G	T	0,67 (± 0.0019)
H12	T	G	T	T	5,795 (±0.0060)
H13	T	G	T	C	2,141 (± 0.0049)
H14	T	G	G	T	9,572 (± 0.0077)
H15	T	G	G	C	0,572 (±0.0034)

Table 4. Distribution of VDR Haplotype frequencies (rs1544410- rs7975232- rs731236) in Moroccan population compared to worldwide populations

	N	rs1544410- rs7975232- rs731236 Haplotypes (%)								χ^2	P-value	References
		GGT	ATC	GTT	ATT	GTC	AGT	GGC	AGC			
Morocco	260	36.542	27.171	17.010	10.451	5.753	1.959	1.112	0.002	-	-	Present study
Tunisia	225	2.6	12	36.6	16	13.3	1	9.5	7	292.91	0.000	[42]
Nigeria[a]	60	36.7	15.8	25.8	12.5	8.3	0	0	0	14.25	0.027	Hap Map project [c]
Jordan	126	31.42	40.59	20.32	3.44	1.44	1.97	0.82	0	28.58	0.000	[45]
East-Asian[b]	89	65.7	6.2	25.8	1.7	0	0	0	0	85.91	0.000	Hap Map project [c]
Greece	46	4.3	21.7	8.7	17.4	10.9	17.4	4.3	15.2	164.29	0.000	[49]
Germany	265	44.6	41	12.6	1.6	0	0	0	0	102.01	0.000	[25]
Portugal	223	7.1	0	56.9	1.7	0	1.8	0	32.3	546.06	0.000	[54]
Netherland	563	45.5	42.5	11.5	0.4	0	0.1	0	0	231.11	0.000	[26]
New Zealand	239	44.8	30.9	10.9	13.2	0.2	0	0	0	53.08	0.000	[8]
Brazilian	200	41.8	33.2	17.5	2.2	1.3	1.7	0	2.3	54.82	0.000	[36]

[a] 60 Yoruba individuals from Ibadan, Nigeria.
[b] 89 East-Asian individuals, comprising 45 Han Chinese from Beijing and 44 Japanese from Tokyo.
[c] Data from Hap Map phase I and phase II online database.

The estimated haplotype frequencies for southern Morocco are shown in (Table 3). Eight haplotypes were identified and our results indicate that the most common haplotypes for rs1544410-rs7975232-rs731236 VDR gene variants were GGT (36.5 %) and ATC (27%) followed by GTT (17%) and ATT (10%). By contrast, GTC (5%), AGT (2%) and GGC (1%) were the less frequent haplotypes present in our population. Finally, haplotype AGC has not been found. Regarding ethnicity, data from Hap Map Project showed significant interethnic differences in the distribution of rs1544410-

rs7975232-rs731236 estimated haplotype frequencies; three haplotypes were identified in Europeans, including GGT (41.7%) and ATC (47.5%) that were the most frequent. On the other hand, the haplotypes GGT and GTT were the most frequent in the Asian (65.7%, 25.8%) and African populations (36.7%, 25.8%) respectively.

Significant differences were found when southern Morocco haplotype frequencies were compared to those found in worldwide populations ($P< 0.001$; Table 4), except for Yoruba from Nigeria ($P< 0.05$; Table 4). Interestingly, and despite their close geographic localization in North Africa, the haplotype structure of Moroccans and Tunisians is clearly different ($P< 0.001$; Table 4). The reason for this may be due to the different ethnic composition in both regions: Morocco has a significant higher percentage of Amazigh and sub-Saharans than Tunisia [33-35]. Regardless of ethnicity, a significant difference in the VDR haplotype structure exists among worldwide populations and interestingly, there is a difference when considering the allelic frequencies distribution rather than haplotype frequencies.

Several studies have analyzed the extent of linkage disequilibrium among known SNPs of the VDR gene [17, 36, 37]. These SNPs are commonly found in linkage disequilibrium generating haplotype blocks. The anonymous markers rs1544410, rs7975232 and rs731236 are in strong linkage disequilibrium extending into the 3' regulatory region containing the UTR [36, 38]. Consistently, strong LD was also observed between rs1544410 and the polyA variable number of tandem repeats (VNTR) in the 3'UTR [39]. The 3'UTR of the gene is known to be involved in the gene's regulation of expression, especially through the control of mRNA stability. This might explain the observed associations of anonymous markers rs1544410, rs7975232 and rs731236 with several disease conditions.

In our southern Morocco population the linkage disequilibrium analysis of the 3 VDR SNPs located in the 3' UTR showed a high significant LD especially between rs1544410-rs731236 and rs1544410-rs7975232 ($P< 0.001$), which might explain the high frequencies of haplotypes GGT (36.5 %) and ATC (27%). Our findings indicate that the functional rs2228570 alleles did not show any linkage to any of the other three polymorphisms ($P>0.45$) a result that is consistent with other studies [17, 36]. rs2228570 SNP can be considered an independent marker in the VDR gene since there is no LD with any of the other VDR SNPs [2]. In addition, a region with no haplotype blocks and with low LD was found between SNPs rs2228570 and rs886441 in the 5' region, for which no block structure was found in any of the ethnic groups [36]. This observation corroborates previous findings and suggests a major site of haplotype breakage and recombination in the VDR gene 5' region that is apparently independent of ethnicity [17, 36, 37].

For these most common VDR variants significant haplotype differences were detected between our southern Morocco population and other populations. These differences support the idea that haplotype structure may be affected by geographic repartition and ethnicity. Haplotypes may reflect the origin of human populations, population genetic behavior and also gene-environmental interactions [2]. Haplotypes are much more informative in association studies because they avoid many problems associated with multi-SNP analysis. Several other studies have demonstrated a gain in power when considering haplotypes rather than individual polymorphisms separately [40, 41]. Defining the haplotype blocks in different populations, may explain in part the ethnic disparities in the risk on health condition. In fact, VDR polymorphisms have been studied in different populations, but often with contrasting results and inconsistent findings. These conflicting results might be partly due to the presence of variable degrees of linkage disequilibrium between the variants and the functional mutations in different populations.

IV. CONCLUSION

Our work allowed us to establish the genetic structure of the VDR gene polymorphisms in a southern Morocco heterogeneous population allowing us to better understand future association studies with different disease conditions.

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