ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2021; 26(1): 2249-2254
doi: 10.25083/rbl/26.1/2249.2254

Received for publication, December, 26, 2018
Accepted, May, 2, 2019

Original paper

Study on VDR Polymorphism Influence in Associating with Diabetes Mellitus

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Abstract The aim of this study was to evaluate the association between vitamin D receptor, IL6 polymorphisms and DM. DNA was used to genotype the VDR FokI (rs2228570), TaqI (rs731236) and ApaI (rs7975232) polymorphisms by PCR RFLP and IL6 G-174C (rs1800795) by tetra-primer ARMS PCR. The presence of torque teno viruses DNA was assessed with heminested-PCR. For this study T1DM (n = 107) and T2DM (n = 124) patients and matched clinically healthy subjects (n = 200) were recruited. T1DM patients have a tendency to be more frequent carriers of the C allele and TTV infection than controls (OR = 1.9, p = 0.03). VDR tt genotype and VDR "FAt" haplotype are risk factors for T1DM. VDR "fAt" haplotype may increases the risk for T2DM. These associations were not changed after exclusion from statistical analysis of patients with hypertension, myocardial infarction, stroke or breast cancer.

Keywords PCR – RFLP, diabetes, IL6, vitamin D receptor, haplotypes.

To cite this article: BERCA LM, MOSOIU CE, NICA S, APOSTOL PP, NICA R, CIMPONERIU D. Study on VDR Polymorphism Influence in Associating with Diabetes Mellitus. *Rom Biotechnol Lett*. 2021; 26(1): 2249-2254. DOI: 10.25083/rbl/26.1/2249.2254

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Introduction

Interleukin-6 (IL-6) can protect β-cell from cytokine-induced death and also may contribute to insulitis. The presence of -174 C variant or of CC genotype of IL-6 -174 G>C (rs1800795) polymorphism were considered risk factors for type 1 diabetes mellitus (T1DM) in some populations. Also, the -174C variant was considered protective (HUTH & al [1], STEPHENS & al [2]), while the G variant or the GG genotype may represent risk factors for type 2 DM (T2DM) among Indians (MUKHOPADHYAYA & al [3]) and Europeans (WILLER & al [4]). However, these positive associations with DM has not been reconfirmed by meta-analyses (YIN & al [5], QI & al [6]).

Vitamin D and the nuclear receptor for vitamin D (VDR) seem to be involved in insulin secretion and sensitivity, glucose tolerance and metabolism, modulation of the immune system, secretion of several cytokines (e.g. IL1, IL2, IL6, IL12) and immune responses to viral infection, inflammation and adiposity. Thus VDR may contribute to predisposition for insulin resistance or DM, especially in some population (QIN & al [7], ZHU & al [8]).

Torque teno virus (TTV), *Torque teno midi virus* (TTMDV) and *Torque teno mini virus* (TTMV) are human anelloviruses which can influence IL6 production and secretion (ROCCHI & al [9], ZHENG & al [10]). In addition, viral genome may encode for miRNAs (KINCAID & al [11]) and for some short proteins with incompletely understood functions. Chronic viral infections may represent a stress factor for the host and thus an aggravating factor for disease onset or evolution.

The aim of the present case-control study was to test the association between four genetic polymorphisms and DM in the Romanian population.

Materials and Methods

Subjects

Data and biological samples from unrelated T1DM (n = 107) and T2DM (n = 124) patients were selected from National Institute of Diabetes, Nutrition and Metabolic Diseases "Prof. N. Paulescu", Bucharest. The patients were selected based on the age (>18 years old), the duration of diabetes (>5 years) and the negative history for other auto-immune disorders, chronic pancreatitis, overt nephropathy or declared blood transfusions.

Clinically healthy subjects matched for age and gender with the T1DM and the T2DM patients were distributed into two control groups: HC1 (n = 100) and HC2 (n = 100).

Genotyping methods

DNA (AxyPrep™ Blood Genomic DNA Miniprep Kit, Axygen Biosciences, California) was used to genotype the VDR *Fok*I (rs2228570), *TaqI* (rs731236) and *ApaI* (rs7975232) polymorphisms by PCR RFLP and IL6 G-174C (rs1800795) by tetra-primer ARMS PCR (YE & al [12]). The presence of torque teno viruses DNA was assessed with heminested-PCR (NINOMIYA & al [13]).

Statistical analysis

The differences between the clinical parameters and the alleles and genotypes frequencies between lots were compared using the StatsDirect software. The SHEsis online platform and Plink software (version 1.07) have been use for haplotype analysis (SHI & al [14]). A Bonferroni corrected p value threshold was employed for correction of type I error.

Results and Discussion

The distributions of the four polymorphisms were similar in diabetic patients and in the control lot. Haplotype analysis revealed a significant association between T1DM and VDR Ft or Fat haplotypes (OR = 2.1, p <0.003). For T2DM, a trend of association with the VDR fAt haplotype was observed (OR = 2, p < 0.05). Seven patients with T1DM (which had hypertension) and twenty-four patients from T2DM lot (which had, in different association, hypertension – 15, myocardial infarction – 4, stroke – 12, or breast cancer – 1) were excluded from statistical analysis to avoid potential confounding effects; the characteristics of the subjects which remained for further analysis are listed in Table 1.

Table 1. Clinical and biochemical data and genotype distributions of subjects selected for this study

Investigated characteristics	T1DM		HC1		T2DM		HC2	
	Male	Female	Male	Female	Male	Female	Male	Female
Number	51	49	51	49	47	53	47	53
Age (years)	25.2 ± 3.5	25.67 ± 3.7	25.2 ± 3.4	25.7 ± 3.7	55.7 ± 5.0	54.4 ± 4.6	55.6 ± 4.9	54.5 ± 4.6
Duration of diabetes	14.5 ± 4.2	14.5 ± 4.0	N.A.	N.A.	7.5 ± 1.8	7.1 ± 1.9	N.A.	N.A.
Body Mass Index	23.1 ± 1.2	21.4 ± 1.8	23.3 ± 0.8	21.4 ± 1.1	27.4 ± 1.4[a]	26.5 ± 1.4[a]	24.4 ± 0.4	22.9 ± 1.1
Hemoglobin A1c (%)	8.5 ± 0.6	8.5 ± 0.7	N.A.	N.A.	7.9 ± 0.6	7.8 ± 0.5	N.A.	N.A.
"A jeune" blood glucose	N.A.	N.A.	87.4 ± 6.2	88.0 ± 5.7	119.0 ± 16.6[a]	106.4 ± 15.6[a]	91.0 ± 7.7	92.0 ± 7.3
Cholesterol (mg/dl)	188.1 ± 32.21[a]	176.9 ± 29.6[a]	149.3 ± 23.6	146.6 ± 21.3	211.6 ± 35.2[a]	201.3 ± 27.7[a]	171.8 ± 16.5	169.9 ± 17.1
Triglycerides (mg/dl)	139.8 ± 38.9[a]	121.2 ± 31.3[a]	96.2 ± 17.4	96.5 ± 16.9	183.6 ± 65.6[a]	160 ± 56.4[a]	111 ± 24.3	111.6 ± 23.6
Smokers[#]	6	5	6	8	8	5	5	3
Drinkers[##]	0	0	0	0	4	0	3	0
TTV infections	38	32	29	30	37	36	35	29
TTV	38	32	29	30	37	36	35	29
TTMDV	23	23	18	25	25	22	27	22
TTMV	33	28	26	28	31	30	31	27

a: the two-tailed P value for T test < 0.0001; b: the two-tailed P value for T test < 0.05;
N.A.: not available; #: more than five cigarettes per day, for at least one year; ##: more than 5 units of alcohol per day, for at least one year

The distribution of the positive samples for torque teno viruses infection presented no significant differences between subjects. Some differences were found when data regarding other characteristics were tested in addition to the presence of viral DNA. First, healthy males without TTV (34.32 ± 14.15 *vs*. 42.58 ± 16.19, p = 0.014), T1DM females without TTMDV (24.35 ± 3.47 *vs.* 27.17 ± 3.55, p = 0.007) and T2DM females without TTMDV (53.39 ± 4.87 *vs.* 56.05 ± 3.92, p =0.039) were younger compared with infected subjects from the same group. Second, healthy males infected with TTMDV had higher BMI (24.13 *vs.* 23.68, p = 0.008) and triglycerides levels (108.09 *vs*. 99.26, p < 0.05) than uninfected subjects from the same sub-group. Males with T2DM infected with TTV (217.54 *vs.* 189.80, p = 0.025), TTMDV (225.76 *vs.* 195.59, p = 0.002) or both of them (225.76 *vs.* 189.80, p = 0.002) had higher total cholesterol levels compared to uninfected subject from the same group. The age of these sub-groups did not differ significantly.

The distribution of IL-6 genotypes has not deviated from Hardy-Weinberg equilibrium. The -174C allele was more common in patients diagnosed with T1DM in the first 10 years of life than in those with later onset (41/13 *vs.* 26/20, OR = 2.43, 95% CI: 1.03 - 5.69, p = 0.04). T1DM patients were also more frequent carriers of the C allele and TTV infection than controls (46 *vs.* 31, p = 0.03, OR = 1.9). The IL-6 G-174C was not associated with T2DM, even if the gender, BMI, the age of diabetes onset or the presence of TTV infection were included in the analysis.

Single locus analysis revealed that VDR *Taq*I tt genotype (OR tt = 2,45; 95% CI = 1,29 - 4,62; p = 0,005) and VDR t variant (OR t = 1,99; 95% CI = 1,06 - 3,72; p = 0,02) increased the risk for T1DM whereas the TT genotype (OR TT = 0,50; 95% CI = 0,26 - 0,93; p = 0,03) and the T variant (OR T = 0,40, 95% CI = 0,21 - 0,76, p = 0,005) appeared as protective factors. A similar result was detected in the sub-group of women (OR tt = 2,72; 95% CI = 1,15 - 6,44; p = 0,02; OR T = 0,36, 95% CI= 0,15 - 0,86, p = 0,02), yet not in the case of male. Single locus analysis revealed no other significant associations with T1DM or T2DM (Table 2).

Table 2. The distribution of VDR and IL6 polymorphisms in investigated lots

Polymorphisms	T1DM			HC1			T2DM			HC2		
	Male	Female	total	Male	Female	total	Male	Female	total	Male	Female	total
IL16 GG	17	16	33	24	19	43	28	33	61	29	27	56
IL16 GC	26	24	50	20	25	45	16	17	33	13	23	36
IL16 CC	8	9	17	7	5	12	3	3	6	5	3	8
VDR FF	26	23	49	18	19	37	16	21	37	14	24	38
VDR Ff	19	20	39	23	25	48	25	23	48	28	22	50
VDR ff	6	6	12	10	5	15	6	9	15	5	7	12
VDR AA	21	17	38	14	13	27	17	18	35	14	16	30
VDR Aa	21	21	42	22	24	46	24	26	50	23	25	48
VDR aa	9	11	20	15	12	27	6	9	15	10	12	22
VDR tt	15	**23** [b]	**38** [d,e]	8	12	20	14	19	33	10	15	25
VDR Tt	**23** [a]	17	40	23	21	44	28	27	55	28	26	54
VDR TT	13	**9** [c]	**22** [f,g]	20	16	36	5	7	12	9	12	21

a: OR t *vs.* T = 1,75, 95% CI = 1,001 - 3,04; p = 0,048; b: OR tt = 2,72; 95% CI = 1,15 - 6,44; p = 0,02; c: OR T = 0,36; 95% CI = 0,15 - 0,86, p = 0,02; d: OR tt = 2,45; 95% CI = 1,29 - 4,62; p = 0,005; e: OR C = 1,99; 95% CI = 1,06 - 3,72; p = 0,02; f: OR TT = 0,50; 95% CI = 0,26 - 0,93; p = 0,03; g: OR T = 0,40; 95% CI = 0,21 - 0,76, p = 0,005.

No significant results for T1DM and T2DM were obtained when allelic by allelic epistasis between the four SNPs were performed for case – control samples (p > 0.08). However, VDR haplotype-based association analyses revealed some significant results for T1DM. The most significant results were estimated for Ft haplotype (OR = 2.36, p = 0.0003). Overall, these results indicated that VDR Taq polymorphism considered independent or in association with VDR *Fok*I polymorphisms increased the risk for T1DM in Romanian population (Table 3).

Table 3. The logistic regression for VDR haplotye-based association analysis (plink software, haplotype frequencies >= 0.01)

Phenotype	SNPs	Haplotype	Freq	OR	T from Wald test	p
T1DM	*Apa*I-*Taq*I	aT	0.342	0.59	6.13	0.01
	*Apa*I-*Taq*I	At	0.387	1.65	6.13	0.01
	*Fok*I-*Taq*I	FT	0.328	0.64	4.19	0.04
	*Fok*I-*Apa*I	FA	0.345	1.65	4.83	0.03
	*Fok*I-*Taq*I	Ft	0.319	2.36	13.1	0.0003
	*Fok*I-*Apa*I-*Taq*I	Fat	0.235	2.21	8.69	0.003
T2DM	*Fok*I-*Apa*I-*Taq*I	fAt	0.161	2.05	4.57	0.03

The *Taq*I and *Apa*I VDR polymorphisms displayed linkage disequilibrium (D' = 51) (Figure 1). Genomic coordinates of polymorphisms were established according to human genome assembly GRCh38.p2. A significant LD was identified only between rs731236 (TaqI) and rs7975232 (ApaI) (D' = 51). The estimation of linkage disequilibrium (LD) was computed for pairs of SNPs ussing Haploview 4.2 software.

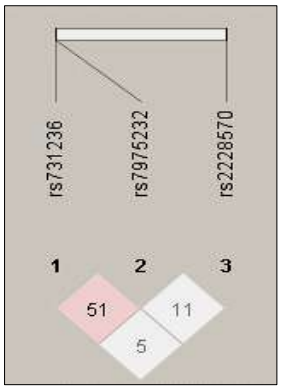

Figure 1. The Linkage Disequilibrium (r2) between the VDR Taq1 (rs731236; chr12:47844974), VDR ApaI (rs7975232 chr12: 47845054) and VDR FokI (rs2228570, chr12: 47879112).

Although carriers of risk genotypes in both VDR *Taq*I and *Apa*I or *Fok*I polymorphisms seemed to be associated with T1DM, the results were not significant, if correction for multiple tests have been applied. In addition the conclusions regarding these associations may be interpreted with caution because only a low number of carriers of these combinations have been detected in this study.

The investigation of the relation between IL6 -174 polymorphism and DM provided contradictory results. The -174 C allele and the -174 CC genotype were considered risk factors for T1DM in some studies. These markers seemed to increase the disease risk in both gender (COOPER & al [15]) and, may predispose to the onset of disease at younger age in women (GILLESPIE & al [16]). In this study the IL-6 -174C allele was found to be more common in patients diagnosed with T1DM in the first 10 years of life than in those with later onset (41/13 vs 26/20, OR = 2.43, 95% CI: 1.033 - 5.698, p = 0.04). This observation was not confirmed when each gender was tested independently.

The prevalence of torque teno viruses depends on the methods used for the detections and on the investigated population. TTV is considered to be a ubiquitous virus that is present in up to 95% of the tested samples (HSIAO & al [17]). The percent of healthy subjects which have TTV (61.5%), TTMDV (46%) and TTMV (56%) was in the range of values described previously for other populations (SPANDOLE & al [18]). We observed that carriers of IL6 -174C allele and TTV infection are more common in T1DM patients than in controls (46 *vs.* 31, p = 0.03, OR = 1.9). In a previous study, TTV was more common in T2DM patients than in controls, however the difference was not statistically significant (p > 0.05) (GUNEY & al [19]). In the present study we found a similar result; however this retrospective study do not allow as to test if TTV is an opportunistic virus or it may contribute to disease onset.

There are controversial (TSIAVOU & al [20]) evidences that the IL-6 *G-174C* polymorphism can be considered a risk factor for T2DM (MUKHOPADHYAYA & al [3], WILLER & al [4]). The association with T2DM may be restricted to thin males carriers of the IL6 -174G allele (ILLIG & al [21]), or to overweight or obese subjects carriers of the -174C allele. The associations between IL6 -174GC and T2DM or BMI were not confirmed [1]. In the present study, IL-6 G-174C was not associated with T2DM, not even when the gender, BMI or the age of onset were included in the analysis. Nevertheless, in the investigated groups 91% of the T2DM subjects and 5% of the T1DM subjects were overweight. These results suggest a strong interaction between T2DM and obesity in the Romanian population.

VDR *Fok*I polymorphism (C>T, rs2228570), mapped at the first ATG start codon of VDR gene, was considered a functional polymorphism (JURUTKA & al [22]). VDR *Apa*I (T>G, rs7975232) and VDR *Taq*I (T>C, rs731236) polymorphisms, identified in the 3′ end of the gene are frequently found in linkage disequilibrium and have no functional postulated effect (UITTERLINDEN & al [23]).

A 2002 study done in Romania showed a trend of preferential transmission of VDR alleles in T1DM patients: variant F seemed to be predisposing, variant A has not modify the risk, and variant T seemed to be protective; however these results did not reach the statistical significance (GUJA & al [24]). Our findings supported the hypotheses that variant T of VDR *Taq*I may be protective for T1DM, yet did not found any association between the other two polymorphisms and T1DM or T2DM in Romanians. Also, T alleles were found to be protective against T1DM in Korean (CHEON & al [25]) and Iranian (MOHAMMADNEJAD & al [26]) subjects. Dalmatian T1DM patients had a higher occurrence of ff genotype (ZEMUNIK & al [27]), while genotype FF was associated with a higher risk of T1DM in the West, yet not in the East Asian population (WANG & al [28]). In other studies none of the VDR FokI, *Apa*I and *Taq*I taken individually were associated with T1DM risk (ABD-ALLAH & al [29], HAMED & al [30], TIZAOUI & al [31]). The risk for T1DM conferred by VDR polymorphisms may be gender specific, with an increased risk in girls (GYORFFY & al [32]). This sex-specific risk was not previously evaluated in Romanian population.

McDermott et al noted that in Asian subjects, yet not in North American subjects, the "bAT" haplotype ("b" variant of VDR BsmI) conferred risk for T1DM, while haplotype "BAT" provided protection (MCDERMOTT & al [33]); nevertheless these associations were not confirmed. In the Basque population the "fBAt" haplotype was significantly more frequent in T1DM patients (SAN-PEDRO & al [34]). Our result showed an association between several haplotypes and T1DM, of which haplotype Ft (OR = 2.36, p = 0.0003) with highest statistical significance, underlining the significance of other factors in modifying the disease risk.

VDR was associated with T2DM in Chinese (XU & al [35]), Saudi Arabian (AL-DAGHRI & al [36]) and Indian population (BID & al [37], MUKHOPADHYAYA & al [3]), but not in Polish (MALECKI & al [38]), Turkish (DILMEC & al [39]), North Indian populations (BID & al [37]) and Tunisians (MAHJOUBI & al [40]). Our results are in agreement with studies which detected non-significant association between VDR polymorphisms and T2DM.

The size and stratification of the population, exposure to risk factors, differences in alleles frequency within the population and age range may partially explain differences between results of this study compared to those previously published.

Conclusion

Subjects carriers of -174C allele and infected with TTV were more common in T1DM patients than in controls. This allele has a modest association with the early onset of T1DM. The polymorphism was not associated with T2DM in the Romanian population.

Our data suggest that VDR Taq tt genotype and VDR haplotype "FAt" represents a risk factor for T1DM. The presence of VDR f-A-t haplotype presented a trend of association with T2DM.

Acknowledgements

This paper was written in the frame of Core Programme PN 16 46 05 01.

ANNE MARIE CRACIUN – thanks for helping to collect the samples.

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