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Vitamin-D receptor gene polymorphisms (*TaqI* and *ApaI*) and circulating osteocalcin in patients with type 2 diabetes and healthy subjects

Polimorfizmy genu receptora witaminy D (*TaqI* oraz *ApaI*) oraz cyrkulacja osteokalcyny u pacjentów chorujących na cukrzycę typu 2 i osób zdrowych

Edgar-Alfonso Rivera-Leon[1, 6], Beatriz Palmeros-Sanchez[2, 6], Iris-Monserrat Llamas-Covarrubias[1, 6], Socorro Fernandez[2, 6], Juan Armendariz-Borunda[3], Mercedes Gonzalez-Hita[1, 6], Blanca-Estela Bastidas-Ramirez[4, 6], Abraham Zepeda-Moreno[5], Sergio Sanchez-Enriquez[1, 6]

[1]*Laboratorio de Bioquímica, Departamento de Biología Molecular y Genómica, Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara, Jalisco, México*
[2]*Laboratorio de Toxicología, Facultad de Biología, Universidad Veracruzana, Veracruz, México*
[3]*Instituto de Biología Molecular y Medicina Genómica, Departamento de Biología Molecular y Genómica, Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara, Jalisco, México*
[4]*Instituto de Enfermedades Crónico Degenerativas, Departamento de Biología Molecular y Genómica, Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara, Jalisco, México*
[5]*Instituto de Investigación en Cáncer en la Infancia y Adolescencia. Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara, Jalisco, México*
[6]*Red de Investigación Bioquímica, Molecular y Celular de la Interacción de Genes y sus Productos de Expresión en los Sistemas Biológicos (PROMEP)*

Abstract

Introduction: Vitamin D receptor (VDR) is encoded by the *VDR* gene. Several studies have supported that this gene is associated with diabetes. Heterodimer VDR/RXR functions as an enhancer of the *BGLAP* gene and increases the basal transcription rate of osteocalcin (OC) during osteoblast differentiation. OC is a regulator of glucose metabolism in mice. Moreover, OC level is decreased in patients with type 2 diabetes (T2D). Although inversely correlated with serum glucose insulin and glycated haemoglobin, it is unclear whether OC reduction is caused by diabetes or plays a role in the pathogenesis and/or progression of the disease.
In this study we analysed the association between *TaqI* and *ApaI VDR* gene polymorphisms and OC serum concentration in T2D subjects.
Material and methods: Patients underwent clinical and nutritional assessment. Genomic DNA was extracted from leucocytes using a standard salting-out procedure. The polymorphisms were genotyped by PCR-RFLP method. ELISA was used to measure OC and insulin concentrations.
Results: Association between TT genotype of *TaqI* polymorphism and low levels of OC was observed only in the population with overweight and obesity. No association between *TaqI* and *ApaI* polymorphisms and T2D was observed ($p > 0.05$). Furthermore, in T2D subjects, no correlation between *ApaI* and *TaqI* genotypes and age, sex, Body Mass Index (BMI), glucose, or OC was observed.
Conclusions: The TT genotype of *TaqI VDR* gene polymorphism was correlated with low levels of OC in overweight and obese subjects. However, *TaqI* and *ApaI VDR* gene *polymorphisms* were not associated with T2D. **(Endokrynol Pol 2015; 66 (4): 329–333)**

Key words: *VDR; osteocalcin; genotypes; polymorphisms; T2D*

Streszczenie

Wstęp: Receptor witaminy D (VDR) kodowany jest przez gen *VDR*. Kilka badań potwierdziło, że gen ten jest związany z cukrzycą. Heterodimer VDR/RXR funkcjonuje jako stymulator genu *BGLAP* i zwiększa podstawowy wskaźnik transkrypcji osteokalcyny (OC) podczas różnicowania osteoblastów. Osteokalcyna jest regulatorem metabolizmu glukozy u myszy. Ponadto, stężenie OC jest obniżone u pacjentów z cukrzycą typu 2 (T2D). Pomimo odwrotnej korelacji między stężeniem OC a stężeniem glukozy i insuliny w surowicy, a także hemoglobiną glikowaną, nie jest wiadome czy obniżenie stężenia OC jest wywołane przez cukrzycę lub odgrywa rolę w patogenezie i/lub progresji choroby.
W niniejszym badaniu autorzy przeanalizowali związek pomiędzy polimorfizmami genów *VDR TaqI* oraz *ApaI*, a także stężenie OC w surowicy u pacjentów z T2D.
Materiał i metody: Pacjentów poddano ocenie klinicznej i żywieniowej. Genomowe DNA pobrano z leukocytów dzięki standardowej procedurze wysalania. Polimorfizmy genotypowano metodą PCR-RFLP. Testem ELISA zmierzono stężenia OC oraz insuliny.
Wyniki: Związek pomiędzy genotypem TT polimorfizmu *TaqI* a niskim stężeniem OC zaobserwowano jedynie u populacji z nadwagą i otyłością. Nie wykazano związku pomiędzy polimorfizmami *TaqI* i *ApaI* oraz T2D ($p > 0,05$). Co więcej, u pacjentów z T2D nie wykazano korelacji pomiędzy genotypami *ApaI* i *TaqI* oraz wiekiem, płcią, wskaźnikiem masy ciała (BMI), glukozą lub OC.

Sergio Sánchez-Enríquez M.D., Ph.D., MSc, Departamento de Biología Molecular y Genómica, Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara, Sierra Mojada No. 950, Colonia Independencia, C.P. 44340 Guadalajara, Jalisco, México, e-mail: serlucis@hotmail.com; serlucis@yahoo.com

Wnioski: Genotyp TT polimorfizmu genu *VDR TaqI* jest skorelowany z niskim stężeniem OC u pacjentów z nadwagą i otyłością. Jednakże, polimorfizmy genu *VDR TaqI* i *ApaI* nie są związane z T2D. **(Endokrynol Pol 2015; 66 (4): 329–333)**

Słowa kluczowe: *VDR; osteokalcyna; genotypy; polimorfizmy; T2D*

Introduction

Type 2 diabetes mellitus (T2D) is a public health problem. It is estimated that there are over 347 million people living with diabetes worldwide, most of them having T2D [1]. T2D is a leading cause of premature death, mainly from cardiovascular disease [2].

Furthermore, T2D seems to be influenced by genetic factors [3]. Several genetic polymorphisms have been studied to explain this genetic susceptibility, for example genes associated with oxidative stress, growth factors, and bone mineral density, among others (Mark I et al. 2010).

Among these polymorphisms, genes associated with vitamin D metabolism have emerged as interesting candidates to be studied in the context of T2D T2D development because Vitamin D not only affects calcium metabolism, but also immunomodulation and insulin secretion [4].

The VDR gene encodes vitamin D receptor (VDR). Certain allelic variants in *VDR* have been related to diabetes. In a German population, combinations of *BsmI/ApaI/TaqI* influenced susceptibility to type 1 diabetes (T1D) [5]. In a Taiwanese population the AA genotype of *ApaI* polymorphism was associated with T1D. The aa genotype of *ApaI* has been associated with defective insulin secretion in Bangladeshi Asians, a population that have an increased risk of having T2D (Ogunkolade BW et al. 2002). These findings suggest that the *VDR* gene may contribute to susceptibility in both T1D and T2D. However, there is no data reporting its direct causality in T2D.

VDR forms a heterodimer with the retinoid X receptor (RXR) that binds to the vitamin D response element (VDRE) in the promoter region of the *BGLAP* gene to enhance its transcription [6]. It has been described that the coexistence of the *TaqI* T/C (rs731236) polymorphism and the *ApaI* G/T (rs 7975232) polymorphism result in marked linkage disequilibrium. This may affect one of the zinc fingers of the nuclear signalling heterodimer that binds to VDRE [7]. VDRE/VDR/RXR complex is associated with an increase osteocalcin (OC) basal transcription rate during osteoblast differentiation [7].

The OC peptide is encoded by the *BGLAP* gene [8]. A biological variant of OC has been described to be a regulator of glucose metabolism in mice, while its expression seems to be reduced in humans with T2D [9]. Although inversely correlated with serum glucose, insulin levels, and glycated haemoglobin, it is unclear whether the OC underexpression is caused by diabetes or if it plays a role in the pathogenesis and/or progression of the disease [10].

Since both VDR and OC are somehow related to the metabolic alterations present in T2D, it is reasonable to hypothesise that *VDR* polymorphisms could confer susceptibility to T2D and alter the OC concentration levels. This physiological axis correlating *VDR* polymorphisms and OC concentration has not been studied in T2D.

In this study we analysed the association between *TaqI* and ApaI VDR polymorphisms in relation to OC serum concentration in patients with T2D.

Material and methods

Subjects

In a case-control study, a total of 125 patients with T2D (63 women and 62 men) and 125 healthy subjects (HS) (85 women and 40 men) were recruited consecutively at the program for detection and treatment of metabolic disease in the Molecular Biology Department of the University of Guadalajara, Mexico. All Subjects included in the study were living on the western of Mexico at the time of the study. The study was approved by the Ethics, Biosecurity, and Research committee of the University Centre for Health Sciences of the University of Guadalajara, and written an informed consent was obtained from all participants. Patients that were included had the following characteristics: T2D patients and HS between 30 and 60 years old, both genders, not using drugs such as insulin, vitamin D, vitamin K, thiazolidinediones, thiazides, bisphosphonates, coumarin, steroids, oral contraceptives, and calcium. Subjects with haemolysed serum samples, loss of laboratory results, or insufficient blood sample were excluded. The medical history of all subjects included in the study was obtained and a physical examination was performed. Height was measured with a stadiometer (SECA Inc., DF, Mexico), and body composition was analysed by bioimpedance (Body Composition Analyzer, TBF-300A, TANITA Inc., IL, USA). Blood samples were collected after overnight fasting in dry tubes for biochemical analysis of glucose, total cholesterol, triglycerides, HDL-c, and VLDL-c using enzymatic colorimetric methods (Biosystems, BCN, Spain); insulin (ALPCO diagnostic, MA, USA) and total OC, (TakaraBio Inc., Shiga, Japan) were measured by ELISA method.

Genotyping

Blood samples were collected after overnight fasting in EDTA-coated tubes for genotyping. Genomic DNA was extracted from peripheral blood samples according to the Miller method [11]. *ApaI* SNP in intron 8 and *TaqI* SNP in exon 9 (ATT-ATC, Ile352Ile) of the VDR gene were examined by polymerase chain reaction/restriction fragment-length polymorphisms (PCR-RFLPs). The PCR for amplification of both polymorphisms was conducted with single pair primers designed for the proximity between polymorphic sites. These primers were, forward: 5′GGGATGGACAGAGCATGG3′, and reverse: 5′CCACCTCCCCTATCCACC3′. PCR was performed in a final volume of 50 µL, containing 500 ng of gDNA, 20 µM of each primer, 1.5 U/µL Dream Taq polymerase (Fermentas, Thermo Sicentific, MA, USA), 2.5 µL of 10X buffer, and 2.5 mM of each dNTP (Dongsheng Biotech Co., Guangdong, China). The amplification was performed on a programmable thermocycler (Techne TC-300, Staffs, UK). The fragments obtained were analysed on 2% agarose gel. Twenty microlitres of the amplified fragment and were incubated with 3U *ApaI* restriction enzyme and 20 µl with 3U *TaqI* enzyme in a thermal bath according to the supplier's specifications. PCR fragments and digestion products were analysed in 2% agarose gel stained with Gel Red™ (Biotium, Inc., CA, USA). Genotyping was done in duplicates.

Genotypes were designated conventionally by the first letter of the name of the enzyme. A capital letter indicates the absence of the cut site, whereas a lowercase letter indicates its presence.

For haplotype analysis, SNPStats software was used [12].

Statistical analyses

Genotype and allele frequencies of differences between groups were tested using Chi-square test (χ^2) and odds ratio (OR) with 95% confidence interval (MedCalc™ Statistical Software). A Student *t* test was used for two-group means comparison, and one-way ANOVA was used to compare the laboratorial and clinical assessment according to each genotype. Logistic regression analysis was performed to evaluate associations between clinical and genetic parameters. Ages were adjusted using Kruskal-Wallis regression and then compared using the Chi-square test (χ^2). Probability (*P*) values < 0.05 were considered significant. Statistical analyses were performed with SSPS 20 software (IBM©, NY, USA).

Results

The basal characteristics of the studied population are show in Table I.

Table I. *Clinical and biochemical characteristics in the studied population*

Tabela I. *Cechy kliniczne i biochemiczne badanej populacji*

		T2D (n = 125)	HS (n = 125)	p*
Gender	Female n (%)	63 (50.4)	85 (68)	
	Male n (%)	62 (49.6)	40 (32)	
Age (years)		50.8 ± 7.3	44.5 ± 8.2	0.001
BMI [kg/m^2]		29.6 ± 6.4	28.4 ± 6.2	0.07
Glucose [mg/dL]		181.1 ± 74.8	84 ± 14.5	0.001
VLDL-c [mg/dL]		44.8 ± 28.5	32 ± 19.5	0.04
Total cholesterol [mg/dL]		206.8 ± 67.4	180.8 ± 53.8	0.006
Triglycerides [mg/dL]		224.8 ± 140.8	165.2 ± 108.5	0.01
OC [ng/mL]		6.61 ± 3.5	10.8 ± 6.5	0.008

BMI — Body mass index; VLDL-c — cholesterol bound to very low density lipoproteins; OC — osteocalcin. Values are described as mean ± standard deviation. *Age-adjusted Kruskal-Wallis test

Table II. *Vitamin D receptor genotypes in T2D and HS*

Tabela II. *Genotypy receptora witaminy D w T2D i HS*

	T2D (n = 125) n (%)	HS (n = 125) n (%)	p*
TaqI			
TT	38 (30.4)	34 (27.2)	
Tt	62 (49.6)	72 (57.6)	
tt	25(20)	19 (15.2)	0.061
ApaI			
AA	47 (37.6)	31 (24.8)	
Aa	64 (51.2)	78 (62.4)	
aa	14(11.2)	16 (12.8)	0.981

Genotypes showed Hardy-Weinberg equilibrium; *Chi-squared test

The genotypic and allelic distributions are shown in Table II. No significant differences between T2D and HS in the genotypic frequencies of *TaqI* ($p = 0.061$) and *ApaI* ($p = 0.98$) SNPs were observed. Both allele and genotype proportions were in equilibrium according to Hardy-Weinberg's law.

VLDL-c, total cholesterol, and triglycerides were significantly different in T2D compared to HS (44.8 ± ± 28.5 *vs.* 40 ± 5, ($p < 0.05$), 206.8 ± 67.4 *vs.* 180.8 ± 53.8, ($p < 0.01$), 224.8 ± 140.8 *vs.* 165.2 ± 108.5, ($p < 0.01$), respectively.

In T2D patients, no significant differences in age, gender, body mass index (BMI), glucose, and OC concentration according to genotypes in *TaqI* and *ApaI* were observed (Table III).

Table III. *Vitamin D receptor genotypes and clinical profile of T2D patients*

Tabela III. *Genotypy receptora witaminy D i profil kliniczny pacjentów chorujących na T2D*

Genotype		Age (years)		BMI [kg/m²]		Glucose [mg/dL]		OC [ng/mL]	
	N	Mean ± SD	p*	Mean ± SD	p*	Mean ± SD	p*	Mean ± SD	p*
TaqI									
TT	38	51.0 ± 7.9	0.74	29.7 ± 6.9	0.80	188.4 ± 82.8	0.74	8.9 ± 3.2	0.80
Tt	62	49.9 ± 7.3		29.1 ± 6.4		183.5 ± 73.7		9.5 ± 4.7	
tt	25	50.9 ± 7.0		28.3 ± 3.6		170.5 ± 75.8		8.2 ± 5.9	
ApaI									
AA	47	51.3 ± 7.4	0.16	29.6 ± 6.9	0.36	186.2 ± 76.4	0.12	9.1 ± 5.0	0.61
Aa	64	50.5 ± 6.8		28.3 ± 5.2		169.7 ± 72.5		8.7 ± 3.7	
aa	14	47.3 ± 8.5		30.9 ± 7.0		216.1 ± 86.3		11.0 ± 4.7	

* Age-adjusted Kruskal Wallis test. After adjustment Chi-square was calculated (p)

A model in which the whole study population were stratified according to BMI as normal weight (1), overweight (2), and obese (3) and its association with the *TaqI* and *ApaI* genotypes was analysed. In this analysis we found that the homozygous TT genotype of *TaqI* was correlated with BMI and serum OC. Since a reference baseline concentration for OC has not been described, in our case we used the average concentration of OC in the control group. A significant correlation ($p = 0.025$) between TT genotype of *TaqI* polymorphism and lower levels of OC (under 10.32 ng/dL) was observed only in overweight and obese subjects.

No correlation between waist-to-hip ratio and haplotype was observed.

Discussion

Polymorphisms in the *VDR* gene may contribute to the genetic predisposition to certain diseases. As vitamin D modulates insulin secretion, it is likely that genetic variants of the *VDR* gene may contribute to the development of T2D. Since patients with T2D exhibit subtle alterations in glucose metabolism long before the onset of the disease, genetic factors contributing to its pathogenesis or development could be detected early in the disease process [13]. Our study not has shown a significant association between genotypes of *TaqI* and *ApaI* and T2D, probably due to the small sample size. However, previous studies investigating the association among *VDR* polymorphisms and diabetes risk have produced inconsistent results [14]. This biochemical evidence may partially explain our results and even those of a meta-analysis of similar works performed in Asia.

Our study suggests that the *TaqI* polymorphism is associated with susceptibility to obesity in subjects with T2D. Studies are needed over a larger sample size to clarify the role of these *VDR* gene polymorphisms in T2D.

In our study, subjects with TT genotype showed higher BMI. Previous studies have reported the association between TT genotype of *TaqI* polymorphism and obesity. This genotype accounted for a difference of about 9 kg of body weight in that group of subjects [15]. The pathophysiological mechanisms of this association remain unexplained. There is *in vitro* evidence that vitamin D directly inhibits the differentiation of preadipocytes [16, 17] and stimulates the terminal differentiation of adipocytes [18]. In addition, vitamin D stimulates the secretion of insulin [19] and lipoprotein lipase [20].

It is important to emphasise that genotypic analysis reflects an apparent association with BMI and serum OC.

The TT of *TaqI* genotype was associated with BMI and low levels of OC in overweight and obesity subjects. Our results are consistent with data reported by Dilmec F et al. [21], who suggests that VDR plays an important role in the regulation of lipids, possibly mediated by its activity in adipocyte calcium metabolism; therefore, there would be an association between bone metabolism and energy homeostasis. The clinical relevance of the association of lipid profile and the OC is not entirely clear.

OC metabolic effects may be partially mediated by an organ directly involved in insulin secretion, such as the pancreas. However, an indirect action mediated by adipose tissue and the liver has not been ruled out.

Conclusions

In conclusion, the TT genotype of *TaqI VDR* gene polymorphism was associated with low levels of OC in overweight and obese subjects. However, *TaqI* and *ApaI VDR* gene polymorphisms were not associated with T2D.

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