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Association between Vitamin D Receptor (VDR) Gene Polymorphisms and Type-2 Diabetes Mellitus in Population of Pakistan

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Abstract

Vitamin D is known to have antiproliferative, antioxidant and immunosuppressive effect and has known association with diabetes risk. Vitamin D acts through its receptor, the Vitamin D Receptor (VDR). VDR has many Single Nucleotide Polymorphisms (SNPs) of which *Apa I* and *Taq I* have been widely studied. The present study is based on correlation of VDR gene polymorphisms with diabetes patients from Southern Punjab, Pakistan. A sum of 250 samples comprising normal controls (100) and diabetes patients (150) was collected from different areas of South Punjab, Pakistan. All of the samples were genotyped for VDR genotypes at allelic positions of rs7975232 (*Apa I)* and rs731236 (*Taq I)*. Allelic frequencies were not different between controls and patients. Statistical correlation was insignificant between control and diabetics. Enrolled subjects were assayed for biochemical parameters and the results were evaluated for statistical correlation with *TaqI*, *ApaI* and with diabetes. Random Blood Sugar (RBS) showed a very strong positive correlation with glycated hemoglobin (HBA1C). In conclusion, our study showed a non-significant correlation of *Apa I, Taq I* with type-2 diabetes.

Keywords: Type-2 Diabetes (T2DM); VDR; SNPs; Tetra ARMS PCR

Introduction

Diabetes mellitus is one of the commonly occurring diseases of the world [1]. It has two types: type 1 and type [2]. Both cause some major complications like cardiovascular diseases, retinopathy, renal failure and neuropathy [3]. It is recognized all over the world that genetic factors pose important role in common diseases such as Ischemic Heart Disease (IHD), Cancer and Diabetes Mellitus [4-7]. The role of genetics in diabetes is now indisputable [8]. Vitamin D acts through VDR, a super-family steroid hormone [9].

Insulin secretion and VDR polymorphisms are reported to have an association [10]. It has been reported that VDR can regulate and develop T2DM [8,11]. The VDR gene is polymorphic in many ethnic groups [12-15]. Genetic variation may influence susceptibility to DM and treatment response [16-17]. In Pakistan, very few studies are conducted yet to correlate VDR polymorphism with Type 2 Diabetes (T2D); therefore we planned to determine the genotypes distribution and correlation of VDR alleles with T2D patients. Different biochemical parameters like random blood sugar, Urea, Creatinine, Triglyceride, ALT, Uric Acid were also assayed and finally correlated them with diabetes and VDR SNPs.

The SNPs of VDR mostly studied "*Taq I, Apa I, Fok I, Bsm I*" are known by their corresponding Restriction Endonucleases [18]. Most of the studies done so far relied upon PCR-RFLP assays, which are difficult to perform, costly, and occasionally results are not properly interpretable. New techniques were developed for DNA amplification with advancement in technology, one of them was Amplification Refractory Mutation System (ARMS). It is allele specific type of PCR. It is specific, rapid and cost-effective [19-22].

Materials and Methods

Study design and population

This case-control study was performed on volunteers attending Chughtais Lahore Lab (Pakistan) between 2014 and 2015. Both groups were from the similar region. 250 samples were collected in total. 150 were T2D patients and 100 healthy controls. All the patients selected had classical clinical presentation and have been initially diagnosed by an endocrinologist with Type 2 Diabetes according to World Health Organization (WHO) and American Diabetes Association (ADA) criteria for T2D and attending Chughtais Lahore Lab for follow-up diagnostic testing. T2D was diagnosed in patients having glycated hemoglobin level of ≥ 6.5% and fasting glucose level ≥ 126 mg/dL along with clinical representation and associated risk factors e.g. obesity, polyuria [23-26].

Control population was healthy blood donors and or healthy individuals having their HbA1C level below the threshold value defined by WHO, international association for diabetes and ADA [23,24,26]. Volunteers with current or previous history of other pathological complications including gestational diabetes mellitus, chronic renal or liver disorder, patients with any type of malignancy and patients having some anti-inflammatory or anti-depressant drugs were excluded from the study to avoid wrong interpretations. 6 mL blood was collected from each subject, 3 mL in Ethylene-Diamine-Tetraacetic Acid (EDTA) vial for DNA extraction and 3 mL in clot activator gel vial for biochemical analysis. We used the parameters of WHO for different age groups. Teen: 10-14 years, young: 15-24 years, adult: 25-59 years.

Biochemical analysis

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Blood samples (both control and diabetes patients) were processed for biochemical parameters including HbA1c, Random Blood Sugar, Urea, Creatinine, Total Cholesterol, Triglyceride, Alanine Amino Transferase, Total Billirubin and Uric Acid using state of the art Abbott Architect ci4100 (fully automated chemistry and immunology analyzer).

DNA extraction

DNA from blood was extracted through salting out technique [27]. The DNA quality regarding purity and integrity was assessed by optical density measurement at 260 nm wavelength. The DNA was stored at -20°C until further processing.

Primer designing & genotyping

The sequence for mentioned SNP's was taken from "ucsc genome browser" and http://primer1.soton.ac.uk/primer1.html (online software) was used for primer designing [20].

Genotyping of VDR polymorphisms was done using tetra-ARMS PCR employing four primers simultaneously. Both allele specific amplification products were obtained using two primer pairs: One amplicon representing one haplotype and vice versa. Allele specificity was achieved by a mismatch between 3'terminal end of inner primer and template. For specificity enhancement, another mismatch was placed at -2 on 3' terminus of primer. The primer size was long enough to minimize non-specific attachment. Each PCR was carried out in 20 µL reaction mixture.

Annealing temperature for *Apa I* was 57°C and for *Taq I* it was 62°C while time of annealing for each cycle was 40 sec, total number of cycles was 35 with final extension of 7 mins.

Statistical analysis

Allelic frequencies of VDR were calculated in cases and controls using "SNPStat". SNPs correlations, exact test for Hardy-Weinberg equilibrium, pair-wise linkage and their effects were calculated using "SNPstat" Software. Mean, SD, SEM, r^2, p value, Histograms, 3D plots and correlations between different Biochemical Parameters were calculated using "Statistica V 13.0". Correlogram was drawn using "MedCalc V 16.2.1" by applying Spearman's rank correlation coefficient. A "p value ≤ 0.05" was considered significant.

Results

Mean age of the enrolled subjects was 39.7 ± 10.2 ranging from 10 to 59 years. We observed that *Taq I* Ancestral Allele (AA) frequency was higher in adult age group (n=181) among all three age groups i.e. teen, young and adults as compared to the *Apa I* in which mutant allele dominate (n=178). No significant statistical association was observed for allelic frequency and age groups. The gender percentage of the enrolled patients was 54.8% males and 45.2%, females. The incidence of diabetes mellitus was higher in males (62%) as compared to females (58%). No statistical association was observed between gender and diabetes (p=0.49) (Tables 1 & 2).

SNP1: rs7975232 (ApaI)

Control samples showed homozygosity (AA+aa) in 56% and 21% of the samples for *Apa I* while 23% were heterozygous (Aa) for *Apa I*. 19% and 66% of the enrolled patients were homozygous (AA+aa) for ancestral (AA) and mutant (aa) allele and 15% of the controlled patients were heterozygous (Aa). In diabetes patients, 62.7% and 12% were homozygous for *Apa I* ancestral and mutant allele while 38% had heterozygous genotype. 16% and 68% were homozygous (TT+tt) for *Taq I* ancestral (TT) and mutant (tt) allele while just 16% were heterozygous. Both of the alleles showed statistically insignificant correlation with T2D (Tables 3 & 4).

Cross-classification interaction table (n=250, adjusted by Sex+Age)						
	Controls			Diabetes Patients		
	N	Response mean (s.e.)	Difference (95% CI)	N	Response mean (s.e.)	Difference (95% CI)
AA	57	5.3 (0.05)	0.00	94	10.56 (0.14)	5.25 (4.87-5.62)
A/a	21	5.28 (0.11)	-0.02 (-0.59 - 0.55)	38	10.9 (0.25)	5.58 (5.11-6.05)
aa	22	5.41 (0.11)	0.12 (-0.45 - 0.68)	18	10.34 (0.28)	5.04 (4.44-5.64)
Interaction p-value: 0.35						

Note: Correlation of all three genotypes with diabetes and control groups was found non-significant with p value of 0.35.

Table 1: Interaction analysis with covariate Status rs7975232 (*ApaI*)

rs7975232 (*ApaI*) association with response HbA1C (n=250, adjusted by Sex+Age)					
Model	**Genotype**	**n**	**Response mean (s.e.)**	**Difference (95% CI)**	**p-value**
Codominant	AA	151	8.58 (0.23)	0.00	0.091
	A/a	59	8.9 (0.39)	0.32 (-0.53-1.16)	
	Aa	40	7.63 (0.42)	-0.92 (-1.91-0.06)	
Dominant	AA	151	8.58 (0.23)	0.00	0.62
	A/a-aa	99	8.39 (0.29)	-0.18 (-0.90-0.53)	
Recessive	AA-A/a	210	8.67 (0.2)	0.00	0.039
	Aa	40	7.63 (0.42)	[-1.01 [-1.96-(-0.06)].	
Over-dominant	AA-aa	191	8.38 (0.2)	0.00	0.23
	A/a	59	8.9 (0.39)	0.51 (-0.32-1.33)	

Note: While analyzing the phenomenon of different models for dominance, the combination of AA with A/a in recessive model showed a highly significant correlation with glycated haemoglobin. It means if a person has this allelic combination, he will be more susceptible to diabetes.

Table 2: Single-SNP analysis: SNP: rs7975232 *Apa1*

SNP2: rs731236 (TaqI)

Samples collected for current setup were analyzed for HbA1C. It was found that males (n=84) had higher percentage of T2D as compared to females (n=66). Moreover, adults (n=133) had a higher frequency of disease as compared to teen and young subjects. The frequency of ancestral *Taq 1* (n=133) was greater in diabetes patients as compared to other genotype.

Correlation of diabetes with cholesterol and triglyceride suggests the possibility of cardiac diseases and other lipid-related diseases can have higher incidence in diabetic patients, which is according to published scientific studies [28,29] (Table 5).

Table 6 show the correlations between different biochemical parameters. Out of 250 samples, 33% had elevated level of Triglyceride. Elevated Triglyceride concentration was higher in females. Sum of 32 samples had elevated level of Creatinine suggesting impaired kidney function. Elevated Creatinine concentration was higher in females as compared to males which signify that females are at higher risk of having impaired kidney function as compared to males. Among 250 samples, 14% samples had elevated level of Cholesterol. Elevated Cholesterol concentration was higher in males.

Cross-classification interaction table (n=250, adjusted by Sex+Age)						
	Controls			Diabetes Patients		
	N	Response mean (s.e.)	Difference (95% CI)	N	Response mean (s.e.)	Difference (95% CI)
tt	68	5.34 (0.05)	0.00	101	10.61 (0.14)	5.26 (4.90-5.61)
T/t	14	5.16 (0.16)	-0.15 (-0.81-0.50)	25	10.93 (0.34)	5.58 (5.05-6.11)
TT	18	5.38 (0.1)	0.03 (-0.56-0.63)	24	10.34 (0.25)	5.02 (4.48-5.55)
Interaction p-value: 0.34						

Note: Correlation of all three genotypes with diabetes and control groups was found non-significant with p value of 0.34.'

Table 3: Interaction analysis with covariate Status (*TaqI)* rs731236

Taq1 rs731236 association with response HbA1C (n=250, adjusted by Sex+Age)					
Model	Genotype	N	Response mean (s.e.)	Difference (95% CI)	p-value
Codominant	Tt	169	8.49 (0.22)	0.00	0.62
	T/t	39	8.86 (0.5)	0.38 (-0.61-1.38)	
	TT	42	8.22 (0.41)	-0.23 (-1.19-0.73)	
Dominant	Tt	169	8.49 (0.22)	0.00	0.87
	T/t-TT	81	8.53 (0.32)	0.06 (-0.69-0.82)	
Recessive	tt-T/t	208	8.56 (0.2)	0.00	0.53
	TT	42	8.22 (0.41)	-0.30 (-1.24-0.64)	
Over-dominant	tt-TT	211	8.44 (0.19)	0.00	0.39
	T/t	39	8.86 (0.5)	0.43 (-0.54-1.40)	

Note: While analyzing the phenomenon of different models for dominance, no combination showed a significant correlation with glycated haemoglobin.

Table 4: SNP2: rs731236 *Taq1*

	Controls (N=100)			Diabetics (N=150)		
	Mean	SD	SEM	Mean	SD	SEM
Alanine Amino-Transferase	36.8	14.17	1.42	39.1	23.98	1.96
Total Bilirubin	0.4	0.17	0.02	0.5	0.21	0.02
Cholesterol	180.9	29.01	2.90	207.4	55.24	4.52
Creatinine	0.97	0.31	0.03	0.9	0.38	0.03
Glycated Haemoglobin	5.3	0.45	0.04	10.6	1.42	0.12
Random Blood Sugar	117.8	17.68	1.76	295.3	99.19	8.09
Triglyceride	153.3	53.91	5.39	281.9	247.46	20.27
Uric Acid	4.3	1.06	0.10	4.6	1.32	0.10
Blood Urea	32.1	7.09	0.70	34.3	10.51	0.86

Table 5: Correlation of Diabetes with biochemical parameters

Discussion

The allelic frequency of 'A' *vs.* 'a' was 72% and 28% respectively. Allelic frequency of 'T' *vs.* 't' was 25% and 75% respectively. Genotype distribution was in Hardy-Weinberg equilibrium agreement. We compared the genotype frequency and allele SNP's of VDR from different population genetics studies with ours (Tables 7 & 8).

In the case of *VDR ApaI* distribution, our results for homozygous wild AA, homozygous mutant aa and heterozygous Aa genotypes were in accordance with Spanish population [30], Italian population [31] and Japanese population [32]. They have a reasonable sample size and allelic variation was found homogenous in all these ethnic populations. Our study was in discordance with Finnish population [33].

In the case of VDR *TaqI* distribution our results for homozygous wild TT, homozygous mutant tt and heterozygous Tt genotypes were in discordance with Spanish population [30] and Italian population [31]. Ethnic variations in allele distribution and sample size of the mentioned studies can also be a reason.

So far, many studies have been conducted to know expected correlation between VDR and T2DM, however, results were inconsistent [34-37]. But in Pakistan, very few studies are done to correlate VDR with T2DM. A non-significant association was observed in studies on several populations between T2DM risk and polymorphisms of VDR (*Fok I, ApaI, Taq I, Bsm I*) gene [20,34,36,38-40]. All of these studies and our studies have compatible results and support findings with few exceptions of the studies involving some special conditions [20,36,41]. Both Indian and Pakistani population have diverse ethnic groups, somewhat similar in lifestyle and share same Asian region. This study strongly supports the studies done so far on Indian population [35].

Polymorphism of VDR gene is reported to be associated with obesity in early onset T2DM patients [20] and glucose intolerance in the Caucasian population [36]. Keeping in view the results of these studies, it may be concluded that, although there is no significant correlation between VDR and T2DM in general but there may be a significant correlation between different risk factors of T2DM.

Different biochemistry parameters were analyzed for statistical correlation between T2DM patients and control samples (Correlogram 1). Blood glucose level and HbA1C showed a strong association with each other suggesting reliability of HbA1C for diabetes diagnosis. Blood cholesterol and triglyceride level showed a mutual positive association. Having increased levels of triglyceride and cholesterol in the blood increases the chances of a heart disease. Blood urea and creatinine were also associated with each other suggesting a good mutual diagnosis in renal diseases. In most of the patients with renal disorder, both urea and creatinine will rise.

Most of the previous studies used PCR-RFLP methods with some post-PCR manipulations which are time-consuming and not very easy to carry out. We adopted a cost effective, rapid and reliable (Tetra-ARMS PCR) method for genotyping of VDR SNPs. Using two reactions at the same time with internal controls ensures its reliability to avoid false results. Although this study showed non-significant results, it is worth mentioning the technique used can be adopted for further studies involving large demographic population producing cost effective and reliable results excluding post-PCR manipulations and restriction enzymes.

The studies on genetic variations will have many future implications regarding early intervention, prevention and treatment of diseases. We can make a database integrating information obtained from different population genetic studies for public health strategies and personalized treatment. As we know, there are huge differences between VDR alleles and its genotype frequencies between different ethnic populations; it will be useful to find an anthropological correlation between different ethnic populations. Last but not least is worth to mention that, our study was one of the earliest studies performed on Pakistani population for correlation of VDR genotypes and T2DM.

Conclusion

The Aim of our study was to correlate VDR SNP's with T2DM. Non-significant association of VDR SNP's with T2DM was observed which strongly supports the studies done so far on related ethnic populations. Polymorphism of VDR gene has been reported to be associated with obesity in early onset T2DM patients and glucose intolerance.

Random Blood Sugar		0.826	0.358	0.068	-0.035	0.162	0.064	0.163	0.002
HbA1C	0.826		0.319	0.054	-0.051	0.146	0.031	0.108	-0.055
Triglyceride	0.358	0.319		-0.032	-0.104	0.482	0.074	0.092	0.093
UREA	0.068	0.054	-0.032		0.512	-0.013	0.143	0.011	0.146
CREATININE	-0.035	-0.051	-0.104	0.512		-0.176	0.23	0.048	0.018
CHOLESTOL	0.162	0.146	0.482	-0.013	-0.176		0.01	0.016	0.074
Uric Acid	0.064	0.031	0.074	0.143	0.23	0.01		0.059	0.095
BILIRUBIN	0.163	0.108	0.092	0.011	0.048	0.016	0.059		0.183
ALT	0.002	-0.055	0.093	0.146	0.018	0.074	0.095	0.183	
	RBS	**HbA1C**	**Trigl.**	**UREA**	**CREAT**	**CHOL**	**U A**	**BILI**	**ALT**

Note: Strong Positive correlation represented by shades of red and strong negative correlation by shades of blue.

Table 6: Correlations of Biochemical Parameters 1.0 (Strong Positive) -1.0 (Strong Negative)

Study	Study Population	Genotype Distribution							
		Diabetes Patients				Controls			
		AA	Aa	Aa	p- value	AA	Aa	Aa	p- value
Our Study	Pakistan	94	38	18	Ref*	57	21	22	Ref*
Pedro et al. [30]	Spain	15	37	19	0.83	28	43	17	0.91
Bianco et al. [31]	Italy	18	11	02	0.80	11	20	05	0.55
Turpeinen et al. [33]	Finland	57	106	35	0.28	204	441	153	<0.01
Yokota et al. [32]	Japan	16	46	46	0.52	14	44	62	0.22

Note: Ref* In the case of *VDR ApaI* distribution, our results for homozygous wild AA, homozygous mutant aa and heterozygous Aa genotypes were in accordance with Spanish population, Italian population and Japanese population.

Table 7: Genotype distribution

Study	Study Population	Genotype Distribution							
		Diabetes Patients				Controls			
		TT	Tt	Tt	p- value	TT	Tt	Tt	p- value
Our Study	Pakistan	24	25	101	Ref*	18	14	68	Ref*
Pedro et al. [30]	Spain	24	36	11	0.81	31	43	14	0.95
Bianco et al. [31]	Italy	10	15	6	0.84	10	21	5	0.38
Gyorffy et al. [34]	Hungary	44	34	27	<0.01	42	27	32	<0.01
Yokota et al. [32]	Japan	90	13	5	<0.01	101	18	1	0.71

Note: Ref* In the case of VDR *TaqI* distribution our results for homozygous wild TT, homozygous mutant tt and heterozygous Tt genotypes were in discordance with Spanish population and Italian population.

Table 8: Genotype distribution

Ethics Statement

This study was approved by Ethical committee of Institute of Molecular biology and Biotechnology, Bahauddin Zakariya University Multan (Pakistan) and informed consent was taken from all volunteers.

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