Polymorphisms within Exon 9 but not intron 8 of the Vitamin D Receptor are associated with the nephropathic complication of type-2 diabetes

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Abstract

Background: The impact of several environmental and genetic factors on diabetes and its complications is well documented but there is an urgent need to understand more about genetic risk factors associated with this disease. The present study was aimed at examining the two single nucleotide polymorphisms (SNP) in intron 8 and exon 9 of the vitamin D receptor (VDR) gene in nephropathic and non-nephropathic type-2 diabetic patients.

Material and methods: In this clinical study, peripheral blood samples were obtained from 100 type-2 diabetic patients, 100 nephropathic type-2 diabetic patients and 100 healthy controls. DNA was extracted and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was performed to examine two SNP polymorphisms within the VDR gene.

Results: Our results showed a significant difference in the *Taq-1* evaluated genotypes of exon 9 within the VDR gene of diabetic individuals with ($p=0.012$) and without ($p<0.001$) nephropathy. Analysis of the *T*aq-1 evaluated alleles of nephropathic ($p=0.917$) and none-nephropathic ($p=1.000$) did not show a significant difference. We also evaluated the intron 8 *A*pa-1 alleles in patients with ($p=0.480$) and without nephropathy ($p=0.543$) and determined there were no differences between these groups. Our results also showed that the frequency of *A*pa-1 genotypes did not differ in nephropathic ($p=0.224$) and none-nephropathic ($p=0.236$) diabetic patients.

Conclusion: Based on our results, it can be concluded that VDR and its functional polymorphism in exon 9 may play an important role in pathogenesis of type-2 diabetes and more investigations are required to clarify their role in nephropathy.

Key words: VDR, Polymorphism, Type 2 diabetes, Nephropathy.

Introduction

The frequency of diabetes mellitus is increasing globally and it is expected that this latent disorder will affect 200 million people by 2010 and 300 million by 2025 (Steyn *et al.* 2008). Type-2, sometimes referred to as non-insulin dependent diabetes mellitus (NIDDM) or adult-onset diabetes is the most prevalent type of the diabetes (Arababadi *et al.* 2009a) and is often caused by decreased insulin production by the pancreas. Current studies showed that several genetic and environmental parameters are associated with type-2 diabetes and its complications (Arababadi *et al.,* 2010; Nathanson & Nystrom, 2008). The cause of the agents that induce type-2 diabetes and its complications, such as nephropathy, are yet to be clarified. However, it has been suggested that diabetes is an immune dependent disease (Arababadi *et al.,* 2010; Cruz *et al.,* 2008). For example, in type-2 diabetes, peripheral blood monocytes produce inflammatory cytokines (Giulietti *et al.,* 2007) and serum levels of interferon-γ (IFN-γ) and interlukin-17A (IL-17A) are found to be higher in type-2 diabetes both with and without nephropathy (Arababadi *et al.,* 2009b). However, it has also been demonstrated that vitamin D and polymorphisms contained within its receptor can affect kidney function in diabetic and non-diabetic patients (Amato *et al.,* 2008, Baz-Hecht & Goldfine, Martin *et al.,* 2009). Furthermore, vitamin D has crucial effects on the function of insulin, and may act via a number of pathways which

appear to be of importance in the development of type 2 diabetes (Ozfirat & Chowdhury, 2010). Recent evidence demonstrated that the interaction of 1, 25-dihydroxy vitamin D (the active form of vitamin D) and its receptor (VDR) have a supportive and regulatory impact on the immune system (Fleet, 2008). The immunoregulatory effects of 1, 25-dihydroxy vitamin D and polymorphisms within VDR on immune system were also demonstrated by several investigators (Evans *et al.* 2006; Fleet, 2008). The association of VDR polymorphisms in immunological disorders such as hepatitis B (Arababadi *et al*., 2009c), asthma (Daley *et al.*, 2009), multiple sclerosis (Arababadi *et al*., 2009d) and type 1 diabetes (Boraska *et al.*, 2008; Garcia *et al.*, 2007) are well established. Previous studies demonstrated that intron 8 and exon 9 polymorphisms of the VDR gene have an impact on VDR expression (Larcombe *et al.*, 2008). Therefore, this aim of this study was to investigate the prevalence of two functional single nucleotide polymorphisms (SNP) (Arababadi *et al*., 2009c) within intron 8 and exon 9 of the VDR gene and correlate this with the occurrence of type-2 diabetes in patients with and without nephropathy.

Material and methods

Subjects:

Peripheral blood samples were collected from 100 type-2 diabetic patients without nephropathy, 100 type-2 diabetic patients showing nephropathic complications and 100 healthy controls. The patient and

control groups were selected from within the Rafsanjan population and had similar medical and demographic characteristics including duration of diabetes, sex, age and socio-economical status. The study protocol was approved by the ethical committee of the Rafsanjan University of Medical Sciences, and written informed consent was also obtained from all of participants. Fasting blood sugar, urine albumin level, blood pressure and clinical presentations were assessed three times during a period of 6 months for each patient and the control group. The bias factors such as infections, allergic conditions and smoking were excluded from the study.

Genomic DNA extraction:

To extract genomic DNA, peripheral blood was collected on EDTA and genomic DNA was extracted using a commercial kit (Bioneer, South Korea) according to the manufacture's guide lines. Extracted DNA was aliquoted for each sample and stored at -20°C for further analysis.

Detection of polymorphisms:

VDR gene polymorphisms within intron 8 and exon 9 were analyzed as previously described (Arababadi *et al*., 2009c). In brief, primers were designed to flank a known *Taq-1* polymorphism carried within exon 9 and a known *Apa-1* polymorphism with intron 8 of the VDR gene. Amplicons were subjected to restriction digestion with the appropriate enzyme and the products separated on an agarose gel. Alleles were

scored according to the fragment patterns. Alleles digested by *Taq-1* or *Apa-1* were scored as *T* and *A* alleles respectively whereas alleles not digested by *Taq-1* or *Apa-1* were scored as *t* and *a* alleles respectively.

Statistical analysis:

The differences in genotypes and alleles were analyzed by Pearson Chi-Square test and p values of less than 0.05 were considered significant.

Results

Polymorphisms within the VDR were scored according to PCR-RFLP of exon 8 and introns 9. An example of a typical *Taq-1* digestion of exon 8 and how it is scored is shown in figure 1. Figure 2 shows a typical example of how the *Apa-1* polymorphism within intron 9 is scored.

Evaluation of the polymorphism within exon 9 of the VDR gene by *Taq-1* restriction digestion showed that the prevalence of *T/T* genotype was 4 (4%) in type-2 diabetic patients without nephropathy, 9 (9%) in diabetic patients showing nephropathic complications and 18 (18%) in controls (Table 1). Our results also revealed that the frequency of *T/t* genotype was 63 (63%), 55 (55%) and 35 (35%) in type-2 diabetic patients without nephropathy, type-2 diabetic patients with nephropathy and controls, respectively (Table 1). The value for the *t/t* genotype in type-2 diabetic patients without nephropathy was 33 (33%), type-2 diabetic patients showing nephropathy was 36 (36%) and in the control group was 47 (47%) (Table 1). Our results demonstrated that the genotype differences

were significant in both diabetic groups when compared to the control group ($p<0.001$ and $p=0.012$) but the differences between the genotypes of the nephropathic and none-nephropathic patients was not significant ($p=0.273$) (Table 1). The differences between groups regarding the frequency of *T* and *t* alleles were not significant (Table 1).

As is shown in table 1, the differences between groups regarding the frequency of the *A/A*, *A/a* and *a/a* genotypes between the three subject groups was not significant. Significant differences were also not seen regarding *Apa-1* evaluated alleles in both diabetic patient groups when compared to controls ($P=0.236$ and $p=0.224$) as well as between the two patient groups ($p=1.000$) (Table 1).

There were no significant differences between groups regarding the mean age ($P=0.85$), gender ($P=0.9$), duration of diabetes ($P=0.1$) and socio-economical status of the participants ($P=0.90$) (Table 2). The results of the clinical parameters of the different subject groups showed that proteinuria was significantly increased ($P=0.002$) and estimated GFR was decreased ($P<0.001$) in nephropathic patients when compared to controls (Table 2).

Discussion

The impact of vitamin D and its receptor on insulin action and development of type-2 diabetes and its inflammatory complications, such as nephropathy, is in urgent need of additional research to help elucidate

the mechanisms of disease progression (Baz-Hecht & Goldfine, Martin, McKnight, Patterson, Sadlier & Maxwell, 2009; Ozfirat & Chowdhury, 2010). It seems that immune related factors play important roles in the etiology and pathogenesis of type-2 diabetes and associated renal complications (Arababadi et al., 2009b). Previous studies showed that the polymorphisms within intron 8 and exon 9 of the VDR affect expression of this protein (Arababadi et al., 2009b). For example, the T allele within exon 9 is associated with decreased expression of VDR (Arababadi et al., 2009b; Larcombe et al., 2008). In this study, the patient and control groups were matched for duration of diabetes, sex, age and socio-economical status. Our findings indicated a significant difference between *Taq-1* evaluated genotypes of exon 9 within the VDR gene between both diabetic groups and controls. To our knowledge this is the first study which was performed to evaluate the VDR polymorphisms in nephropathic type-2 diabetic patients. However in a study by Oh JY *et al*., they reported that only *Apa-1* genotypes are associated with type-2 diabetes in the American population (Oh & Barrett-Connor, 2002) and a link between *Bsm-I* polymorphisms and onset of the disease were reported by Speer G et al., (Speer G et al., 2001). Therefore, it may be concluded that *Bsm-1* and *Apa-1* polymorphisms are important in type-2 diabetes through their effects on VDR gene expression levels. On the other hand, Bid HK *et al*., using *Fok-1*, *Bsm-1* and *Taq-1* demonstrated

that there is no link between polymorphisms in the VDR gene and type-2 diabetes (Bid *et al*., 2009). Moreover, other studies also failed to show any link (Valdivielso & Fernandez, 2006). Turkish investigators used *Taq-1* and *Apa-1* to evaluate VDR polymorphisms in type-2 diabetic patients (Dilmec *et al*., 2009) and they also failed to find any relationship between these polymorphisms and type-2 diabetes (Dilmec *et al*., 2009). In addition, Malecki *et al*., also found no association between *Fok-1*, *Bsm-1*, *Apa-1* and *Taq-1* polymorphisms and the disease (Malecki *et al*., 2003). One reason for the discrepancy between our results and these studies could be explained by the genetic differences in populations studied or their exposure to environmental factors. For example, several studies showed that stress is the main reason for inducing type 2 daibetes (Simmons, 2007, Simmons, 2009), therefore it is possible that environmental factors impinge on effects of polymorphisms. The different environmental conditions of our study population may give patients carrying the exon 8 T/T genotype a protection against type 2 diabetes. Association of VDR polymorphisms with other type-2 complication also were evaluated by researchers. For example, Cyganek K *et al*., but they showed that VDR polymorphisms were not associated with type-2 diabetic retinopathy in Polish population (Cyganek *et al*., 2006).

Based on our study, and results from similar works, it can be concluded that VDR polymorphisms are not associated with nephropathic complications in this type of diabetes because non-nephropathic patients also showed the same polymorphisms; thus, it seems that the VDR polymorphisms are potentially more related to diabetes than nephropathies via an affect on insulin expression or action. Our research team proposes that the role of other VDR polymorphisms in type 2 diabetes and its nephropathy should be evaluated by other researchers, simultaneously to increase the scope and power of the study.

Finally nephropathic complications of type-2 diabetes are very complex and are associated with several environmental and genetic factors which need to be taken in consideration when evaluating different population groups. Clearly larger studies need to be completed using more parameters within the study design to evaluate the independent role of each factor in relationship to the disease.

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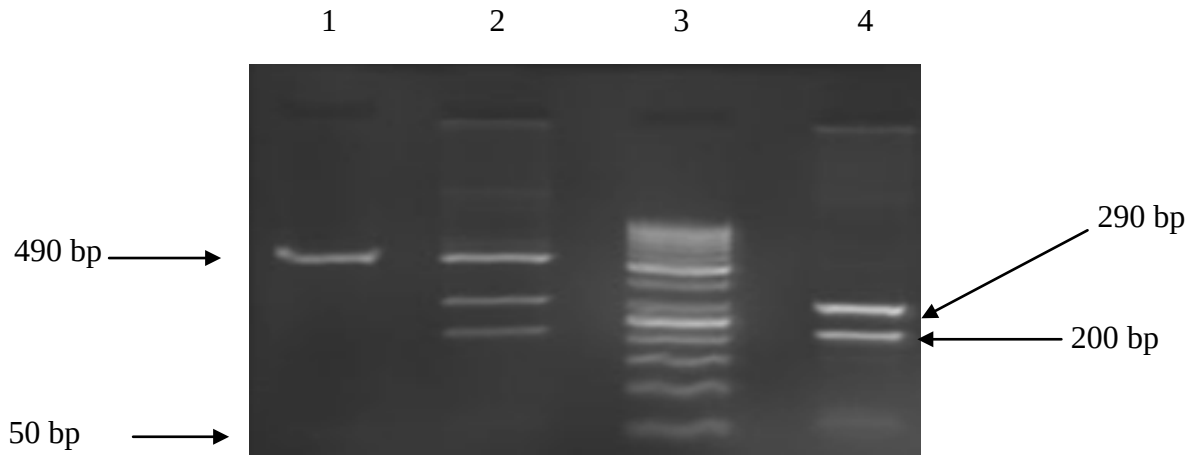

Figure 1. An ethidium bromide stained agarose gel showing PCR-RFLP analysis of exon 9 of the VDR gene. The gel illustrates *Taq-1* digestion of the VDR exon 9 amplicon which contains a known *Taq-1* RFLP. Lane 1: homozygotic PCR product, which is not susceptible to *Taq-1* digestion (*t/t*). Lane 2: heterozygotic digestion (*T/t*). Lane 3: 50 bp ladder marker and lane 4: homozygotic digestion (*T/T*).

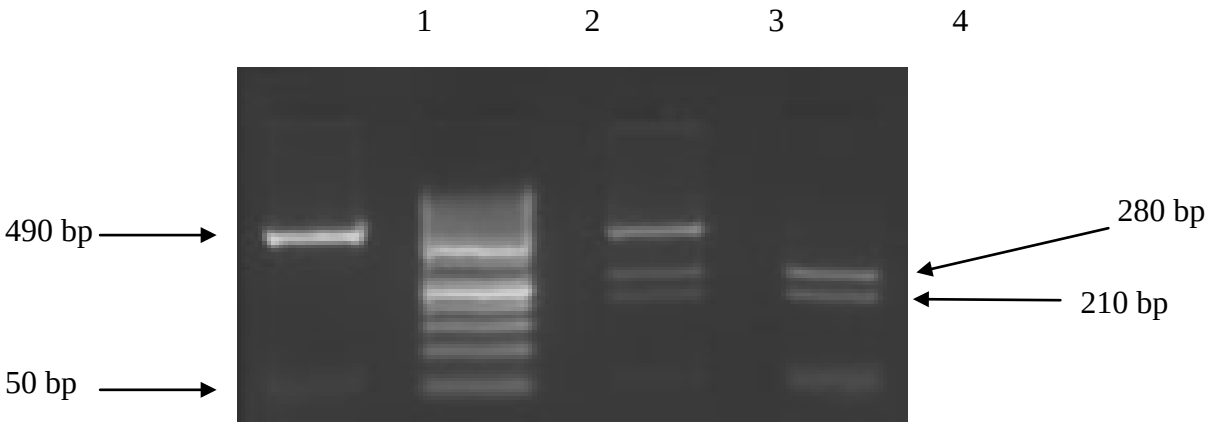

Figure 2. An ethidium bromide stained agarose gel showing PCR-RFLP analysis of intron 8 of the VDR gene. The gel illustrates *Apa-1* digestion of the VDR intron 9 amplicon which contains a known *Apa-1* RFLP. Lane 1: homozygotic PCR product, which is not susceptible to *Apa-1* digestion (*a/a*). Lane 2: heterozygotic digestion (*A/a*). Lane 3: 50 bp ladder marker and lane 4: homozygotic digestion (*A/A*).

Table 1. Frequency of polymorphisms within intron 8 and exon 9 of the VDR gene in type-2 diabetic patients with and without nephropathy and controls. *T* and *t* refer to the exon 8 polymorphism in which the alleles are cut or uncut by digestion with *Taq-1* respectively, likewise *A* and *a* refer to the intron 9 polymorphism in which the alleles are cut or uncut by digestion with *Apa-1* respectively. Data is presented as n = number of patients and % = frequency. CI: Confidence Interval. OR: Odds Ratio

P value of differences between nephropathic and none-nephropathic patients	**P value of differences between patients with nephropathy and controls**	**P value of differences between patients without nephropathy and controls**	**Control**	**Nephropathic type 2 diabetic patients**	**Type 2 diabetic patients without nephropathy**	**Condition / Genotype**
p=0.273 CI: 0.246, 0.374 OR: 1.000	p<0.012 CI: 0.000, 0.015 OR: 1.000	p<0.001 CI: 0.471, 0.609 OR: 0.521	18 (18%)	9 (9 %)	4 (4 %)	*T/T* (n (%))
			35 (35.8%)	55 (55%)	63 (63%)	*T/t* (n (%))
			47 (47%)	36 (36%)	33 (33%)	*t/t* (n (%))
p=1.000 CI: 0.246, 0.374 OR: 1.000	p=0.224 CI: 0.167, 0.283 OR: 1.000	p=0.6 CI: 0.246, 0.374 OR: 0.323	17 (17%)	9 (9%)	9 (9%)	*A/A* (n (%))
			56 (56%)	64 (64%)	63 (63%)	*A/a* (n (%))
			27 (27%)	27 (27%)	28 (28%)	*a/a* (n (%))
Alleles						
p=0.459 CI: 0.246, 0.374 OR: 1.000	p=0.917 CI: 0.246, 0.374 OR: 1.000	p=1.000 CI: 0.664, 1.506 OR: 1.000	71 (35.5%)	73 (36.5%)	71 (35.5%)	*T* (n (%))
			129 (64.5%)	127 (63.5%)	129 (64.5%)	*t* (n (%))
p=1.000 CI: 0.246, 0.374 OR: 1.000	p=0.480 CI: 0.246, 0.374 OR: 1.000	p=0.543 CI: 0.560, 1.237 OR: 0.832	90 (45%)	82 (40.5%)	81 (40.5%)	*A* (n (%))
			110 (55%)	118 (59%)	119 (59.5%)	*a* (n (%))

Table 2. Demographic, socioeconomic conditions and clinical parameters of diabetic patients and controls.

* Significant difference in Proteinuria (P< 0.002, t-test, case VS control). Data are shown as Mean ± SE.

Significant difference in estimated GFR (P< 0.001, t-test, case VS control).

Data are shown as Mean ± SE.

Nephropathic type-2 diabetic patients	**Type-2 diabetic patients without nephropathy**	**Healthy control**	**Variant**
40 ± 6	40 ± 9	40 ± 7	**Age (year)**
Sex			
62 (62%)	59 (59%)	60 (60%)	Female
38 (38%)	41 (41%)	40 (40%)	Male
10 ± 4	9 ± 3	**Duration of diabetes (years)**	
Socio-economic status			
24 (24%)	21 (21%)	22 (22%)	Weak
46 (46%)	49 (49%)	47 (47%)	Medium
30 (30%)	30 (30%)	31 (31%)	High
50 ± 9	50 ± 7	60 ± 7	**Weight (kg)**
insulin	Metformin	-	**Drug therapy**
Lipid levels			
350 ± 12	210 ± 6	100 ± 4	Triglyceride (mg/dl)
290 ± 10	170 ± 5.7	150 ± 6	Cholesterol (mg/dl)
24 ± 2	35 ± 3	40 ± 3	HDL (mg/dl)
180 ± 11	140 ± 6	100 ± 9	LDL (mg/dl)
160-205	140-190	95 ± 105	Glucose level (mg/dl)
899 ± 50*	36.6 ± 3	25 ± 1.5	**Proteinuria (mg/dl)**
72 ± 3#	101 ± 5	120 ± 5	**Estimated GFR**