Clinical Immunology

Title:

Lack of association of vitamin D receptor gene polymorphisms/haplotypes in Sjögren's syndrome

Running title: Vitamin D receptor polymorphisms in Sjögren's syndrome

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Abstract

The vitamin D is involved in a wide variety of biological processes including bone metabolism, modulation of the immune response, and regulation of cell proliferation and differentiation. Vitamin D has several immunomodulatory effects through vitamine D receptor (VDR). A series of common single nucleotide polymorphisms (SNPs) in the vitamin D receptor gene have been linked to numerous of diseases, including osteoarthritis, diabetes, cancer, cardiovascular diseases, tuberculosis, virus infections, urinary stones, periodontitis. Several studies have reported that genetic variations of VDR might be a risk factor for the development of autoimmune diseases such as systemic lupus erythematosus (SLE), multiple sclerosis (MS), psoriasis, autoimmune thyroid diseases (AITD). However no data is available on the possible relationship between primary Sjögren's syndrome and VDR gene polymorphisms. Our aim was to determine VDR gene BsmI, ApaI, TaqI and FokI polymorphism genotypes in pSS patients and healthy controls to analyze whether a relationship exists between polymorphisms in the VDR gene and susceptibility to Sjögren's syndrome. In the current study, 105 patients with pSS and 93 healthy controls were tested for VDR gene polymorphisms (BsmI, ApaI, TaqI and FokI) genotypes. There were no statistical differences in the distribution of BsmI, TaqI, ApaI and FokI genotypes and the common haplotypes between pSS patients and healthy controls. We hypothesized that the TaqI, BsmI, ApaI, and FokI polymorphisms of the VDR gene are not associated with the development of primary Sjögren's syndrome in the Hungarian population studied.

Keywords: SNP, Vitamin D receptor (VDR), Sjögren's syndrome, haplotypes

Introduction

Primary Sjögren's syndrome is a common, slowly progressive systemic autoimmune inflammatory disease that primarily affects the salivary and lachrymal glands leading to dry mouth and dry eye diseases [1]. Besides the typical glandular symptoms (GS), other systemic symptoms, denoted as extraglandular manifestations (EGMs) can also be found in a subset of patients [2]. The pathogenesis of Sjögren's syndrome reveals a complex and heterogeneous array of diverse immunological, genetic and environmental phenotypes, making identification of the precise autoimmune mechanisms difficult to define. Numerous genes might have been linked to the emergence of Sjögren's syndrome [3] including vitamin D receptor (VDR) gene that synthesizes the receptor of vitamin D. VDR is an immunomodulator known to affect both innate and adaptive immune responses.

Vitamin D is essential for bone and mineral homeostasis and exhibits immunoregulatory and anti-inflammatory properties [4, 5]. Vitamin D regulates cell-mediated immunity by modulating interleukin-12 (IL-12) and IFN-gamma, suppressing lymphocyte proliferation, antibody production, and cytokine synthesis [6]. Vitamin D considered as a regulator of the immune system [4, 7]. Vitamin D mediates its multiple actions via binding to its receptor (VDR), which is a 48 kDa soluble protein present on monocytes, and T and B lymphocytes [8].

The VDR gene encodes a ligand activated transcription factor which is located on chromosome 12 (12q12-q14), with numerous SNPs [9]. Some of the SNPs play a key role in the modification of the uptake of $1,25(OH)_2D$. It has been considered that these SNPs may modify vitamin D function. Although VDR gene has more than 100 restriction endonuclease recognition sites, 4 of them are known polymorphisms: FokI (rs2228570), BsmI (rs1544410), ApaI (rs7975232), and TaqI (rs731236) [9-12]. FokI is located in exon 2. Three

polymorphisms, BsmI and ApaI (both in intron 8), and TaqI (in exon 9) have been identified at the 3' end of the gene. Recent studies have reported that allelic variations of the VDR gene polymorphisms might be associated with a variety of diseases, including osteoarthritis, diabetes, cancer, cardiovascular diseases, tuberculosis, virus infections, urinary stones, periodontitis [13-18]. These findings suggest that allelic variations of the VDR gene may partially represent a genetic component associated with the development of autoimmune diseases. Several studies have reported that genetic variations of VDR might be a risk factor for the development of autoimmune diseases such as systemic lupus erythematosus (SLE), multiple sclerosis (MS), psoriasis, autoimmune thyroid diseases (AITD), type I diabetes (T1D) [19-25].

The VDR polymorphisms (BsmI, ApaI and TaqI) have been shown to be in strong linkage disequilibrium (LD) [9]. LD means the association (or co-occurrence) of alleles of adjacent polymorphisms with each other. LD in combination with one or more functional polymorphisms elsewhere in the VDR gene is believed to explain observed associations between the VDR gene and diseases.

In general, all polymorphisms start as mutations which occur perhaps due to a DNA damage incident, then can grow in the population and become true polymorphisms. Allele frequency differences between ethnic groups most likely result from evolutionary processes and population genetic behavior. The same holds true for the LD between the polymorphisms and haplotype structure. To illustrate this, Table 1 shows the frequencies of the BsmI-ApaI-TaqI haplotypes which Uitterlinden et al. [9] investigated in different ethnic groups. In general, most of the population studies of VDR gene were performed in Europeans and Asians and the results have been inconsistent. The results of VDR polymorphisms genotype studies show discrepancies in the different populations studied probably because of confounding factors related to ethnicity and environment.

To our knowledge, there are no information in the literature about the VDR gene polymorphisms and their connection with Sjögren's syndrome. In our study, we determined VDR gene BsmI, ApaI, TaqI and FokI polymorphisms in pSS patients and healthy controls to analyze whether a relationship exists between polymorphisms/haplotypes in the VDR gene and susceptibility to Sjögren's syndrome. We also established haplotype analysis which recently became important due to the newly developed bioinformatics background.

Materials and methods

Patients

105 Sjögren's syndrome patients [53 with glandular symptoms (GS) and 52 with EGMs, mean age 59.4 years, range from 26 to 82 years old, 100 females and 5 males] were enrolled in the present study, recruited from the Autoimmune Outpatient Clinic of the Division of Clinical Immunology, Institute of Medicine, Medical and Health Science Center, University of Debrecen. The diagnosis of pSS was established according to the European-American consensus criteria. 93 healthy individuals (mean age 41.2 years, range from 14 to 70 years, 52 females and 41 males) taking no immunosuppressive or immunomodulating medications served as controls. Informed written consent was obtained from the subjects, and the study has been approved by the ethics committee of the University of Debrecen. All experiments carried out were incompliance with the Declaration of Helsinki.

Among patients with pSS, 52 had EMGs, whereas 53 had only glandular symptoms. The distribution of EMGs patients were as follows: polyarthritis n=44, Raynaud's phenomenon n=18, vasculitis n=5, polyneuropathy n=5, myositis n=4, thyreoiditis n=5, pulmonary fibrosis n=3, primary biliary cirrhosis n=2, renal tubular acidosis n=1, pericarditis n=1, antiphospholipid syndrome n=1, neck lymphadenopathy n=1, marginal zone B cell lymphoma n=1.

Sample handling

Peripheral blood samples obtained from each study subject were collected.

Genomic DNA Extraction

High molecular weight DNA for genotyping was extracted from peripheral blood, which was collected in EDTA vacutainers. Genomic DNS was extracted according to the manufacturer's recommendation using a QiaAmp DNA Blood Mini Kit (Qiagen GmbH, Germany). DNA was quantitated by UV absorption at 260 nm and 280 nm and stored at -20ºC until analyzed.

Genotyping

Genotyping of BsmI polymorphism (rs1544410)

Genotyping of BsmI polymorphism was carried out in PCR-amplified genomic DNA by allelic discrimination using Taqman from Applied Biosystems (Foster City, CA, USA). PCR primers and TaqMan probes specific for the BsmI polymorphism were purchased from Applied Biosystems (Foster City, CA, USA). The assay enables scoring of both alleles in a single well. Real-time PCR was performed in Corbett Rotor-Gene RG-3000 equipment.

The PCR reaction was carried out in a 20 µl reaction volume containing TaqMan Universal Master Mix (2X, 4331182, Applied Biosystems), TaqMan genotyping Assay (40X) and optimized quantities of genomic DNA. The Universal Master Mix contained AmpliTaq Gold DNA Polymerase, AmpErase UNG, dNTPs with dUTP, passive reference, and optimized buffer components. Reactions were set up in duplicate. Thermal cycling was initiated by incubation at 95ºC for 10 min for optimal AmpErase UNG activity and activation of Amplitaq Gold DNS polymerase. After this initial step, 40 cycles of PCR were performed. Each PCR cycle consisted of heating to 92ºC for 15 sec for melting, and to 60ºC for 1 min for annealing and extension.

Genotyping of FokI, ApaI and TaqI polymorphisms

The genotype for FokI, ApaI, and TaqI polymorphisms of the VDR gene was determined by the digestion pattern of the amplified DNA fragment using the restriction enzymes FokI, ApaI, and TaqI.

FokI polymorphism (rs2228570)

Genotypes for the FokI polymorphisms were studied by PCR using appropriate primers as follows: 5' -AGC TGG CCC TGG CAC TGA CTC TGC TCT 3-' and 5' -ATG GAA ACA CCT TGC TTC TTC TCC CTC- 3' [26]. PCR products were amplified in a programmable thermal cycler (Eppendorf-MC-EP model). The PCR conditions were 10 min at 95ºC for initial denaturation, 30 sec at 95ºC, 30 sec at 70ºC, 30 sec at 72ºC, 40 cycles, followed by 5 min at 72 ºC for final extension. The specific PCR products were obtained 265 bp. The amplified products were digested with FokI (Fermentas Life Sciences) for 1 hour at 37ºC according to the manufacturer's instructions, and electrophoresed on 3% agarose gel and visualized by SYBR Green I staining (Figure 1). FokI genotypes were defined by capital letters in the absence of the restriction site (allele-F) and small letters where the restriction site was present (allele-f) (Figure 1).

ApaI and TaqI polymorphisms (rs7975232 and rs731236)

For the genotypes for ApaI and TaqI polymorphism the following specific primers 5' -CAG AGC ATG GAC AGG GAG CAA G- 3' and 5' -GCA ACT CCT CAT GGC TGA GGT CTC A- 3' were used by PCR as previously described [27]. The running conditions were: predenaturation at 94 ºC for 4 min, followed by 40 cycles of denaturation at 94 ºC for 30 sec, annealing at 70 ºC for 1 min, and extension at 72 ºC for 1 min. Finally, extension was carried out at 72 ºC for 4 min. Specific PCR products were obtained 740 bp. The PCR products were

digested with ApaI (Fermentas Life Sciences) for 4 hours at 37 ºC and TaqI (Fermentas Life Science) for 1 hour at 60 ºC. After digestion the fragments were separated by electrophoresis in 3 % agarose gels and visualized by SYBR Green I staining (Figure 1). For both ApaI and TaqI genotypes were defined by capital letters in the absence of the restriction site (A, T, respectively) and small letters where the restriction site was present (a, t, respectively).

Statistical analysis

Genotype frequencies were calculated by direct counting. Allele frequencies were calculated from genotype frequencies based upon Hardy-Weinberg equilibrium. For comparisons of mean values between patients and controls statistical analysis was performed by the independent samples t-test. Differences in genotypic and allelic distribution of VDR polymorphisms between patients and controls were determined by Pearson Chi-square (χ^2) test using SPSS 20.0 statistical software. The *P* value less than 0.05 was regarded as statistically significant.

Haplotype analysis was done by CHAPLIN 1.2 software. The possible haplotypes including genetic variants of three VDR polymorphisms (BsmI, ApaI and TaqI) and four polymorphisms studied (FokI, BsmI, ApaI, and TaqI) in Table 3 and Figure 2.

Pairwise linkage disequilibrium (LD) between the VDR gene polymorphisms was computed, and LD plots were constructed by Haploview software version 4.2 [28].

Results

We studied VDR-BsmI, VDR-ApaI, VDR-TaqI and VDR-FokI polymorphisms in 105 pSS patients and 93 healthy controls. Genotype analysis of the VDR gene FokI, BsmI, ApaI and TaqI polymorphisms did not show a significant deviation from Hardy-Weinberg equilibrium in the pSS patients and control groups.

Allele frequencies of VDR gene polymorphisms in pSS patients

The distribution of allelic frequencies for the four polymorphisms studied here is summarized in Table 2. The characteristics are shown separately for GS patients and EGMs patients, respectively. No significant difference was found in allele frequencies when data were compared between pSS cases and control individuals (Table 2). No significant difference was observed when the pSS cases were grouped into GS cases and EGMs cases (Table 2).

Genotype frequencies of VDR gene polymorphisms in pSS patients

No significant difference was found in the genotype frequencies when the VDR gene polymorphisms genotypes of pSS patients and healthy individuals were compared (Table 2). The same result has been confirmed between GS cases and EGMs cases (Table 2). However we recognized slightly increased prevalence of the Aa genotype in EGMs patients compared with GS patients, 57.69% and 39.62%, respectively. Likewise, we also found a mild increased prevalence of the Tt genotype in EGMs patients compared with GS group and healthy group, 53.85%, 41.51% and 39.79%, respectively. These differences have not been proven significant. Genotype frequencies of VDR gene polymorphisms did not differ between pSS cases and controls in any comparison performed (Table 2).

Linkage disequilibrium and haplotype frequencies of VDR gene polymorphisms in pSS groups

The haplotypes might provide valuable data where genotypes alone unable to do. The haplotype frequencies among the VDR-FokI, VDR-BsmI, VDR-ApaI and VDR-TaqI polymorphisms in both the patients with Sjögren's syndrome and the healthy controls were evaluated by CHAPLIN 1.2 software.

The estimated haplotype frequencies for VDR-BsmI, VDR-ApaI and VDR-TaqI polymorphisms of the Sjögren's syndrome patients and control individuals are shown in Table 3. The *baT* and *BAt* haplotypes were found the most frequent haplotypes in both the patient group (51% and 33%) and the control group (48% and 31%). These three-marker haplotype alleles were identified as the most frequent and corresponded haplotypes by Morrison et al. [10] as well. Similarly, haplotype 1 *baT* (43%); and haplotype 2 *BAt* (39%) were also found the most frequent haplotypes by Utterlinden et al. [9] in a large Caucasian population (Table 1). The *BaT* haplotype was present neither in patients nor in the controls. Three haplotypes (*BAT*, *Bat* and *bAt*) were relatively uncommon (frequency < 10%) either in the patient group or in the control group.

The distribution of the frequency of four-marker haplotype alleles (VDR-FokI, VDR-BsmI, VDR-ApaI, and VDR-TaqI) in Sjögren's syndrome cases and controls are shown in Figure 2. According to the four-marker haplotype prevalence, frequencies of the sixteen possible haplotypes do not show significant differences between the patient and the control group (Figure 2). The most common estimated haplotype was *FbaT* both in the patient and the control group (frequencies are 29.63 % and 29.78 %, respectively). The *FBAt* and *fbaT* haplotypes showed similar haplotype frequency when the patients and the controls data were compared (16.19% and 22.59%; 19.79% and 24.05%, respectively). Some of the possible haplotypes (*FBaT*, *fBAT*, *fBaT*, *fbAt*, and *fbat*) were not present neither in patients nor in the control group estimated.

Pairwise LD was computed and LD plots were constructed using the Haploview software version 4.2. LD analysis revealed a very strong LD (r^2>0.8) between ApaI and TaqI polymorphisms, a strong LD (r^2 between 0.67 and 0.7) between BsmI and ApaI or BsmI and TaqI polymorphisms, and a very weak LD (r^2< 0.3) was observed between FokI and other polymorphisms in the control group. In patients, very strong (r^2>0.8) to moderate (r^2 =0.67-

0.7) LD was found between BsmI and ApaI, TaqI polymorphisms, ApaI and TaqI polymorphisms (Figure 3). No LD was observed between FokI and other polymorphisms in patients.

Discussion

The VDR gene polymorphisms have been identified and analyzed so far in a wide variety of diseases, including osteoarthritis, diabetes, cancer, cardiovascular diseases, tuberculosis, virus infections, urinary stones, periodontitis and autoimmune diseases. Several studies have reported that genetic variations of VDR might be associated with the development of autoimmune diseases such as systemic lupus erythematosus (SLE), multiple sclerosis (MS), psoriasis, autoimmune thyroid diseases (AITD). In our best knowledge, the possible relationship between primary Sjögren's syndrome and VDR gene polymorphisms has not been investigated up to now. In the current study, our aim was to determine VDR gene FokI, BsmI, ApaI and TaqI genotypes in pSS patients and healthy controls. As the results of our investigations there were no statistical differences of the FokI (FF, Ff, ff), BsmI (BB,Bb, bb), ApaI (AA, Aa, aa), and TaqI (TT, Tt, tt) genotypes and allelic frequencies between Sjögren's syndrome patients and control individuals. Besides of these findings the haplotypes frequencies among the VDR-FokI, VDR-BsmI, VDR-ApaI and VDR-TaqI polymorphisms in both the patients with Sjögren's syndrome and healthy controls were evaluated and showed nearly the same distribution. Analysis of FokI, BsmI, ApaI, and TaqI locus haplotype frequencies failed to show any association in the study groups, suggesting that FokI, BsmI, ApaI, and TaqI polymorphisms of VDR gene are not associated with the development of Sjögren's syndrome in the Hungarian population studied. We conclude that the VDR gene polymorphisms do not seem to be a candidate genetic marker for Sjögren's syndrome according to our study which is the first study to investigate all the four polymorphisms of

VDR gene in patients with Sjögren's syndrome. The baT haplotype was found as the most frequent haplotype (Table 3) in the patient group and the control group. Table 2 shows the frequencies of the BsmI-ApaI-taqI haplotypes of VDR gene which Uitterlinden et al. [9] investigated in different ethnic groups. According to Table 2, the distribution of the most frequent haplotype (*baT*) was different among Caucasians, Asians and Africans, 43%, 75% and 26%, respectively. We calculated slightly higher frequency for the *baT* haplotype in the patient group and the control group, 51% and 48%, respectively. Despite of Caucasian origin of our study subjects, a slightly elevated distribution of the *baT* haplotype might be explained by the population genetic behavior. Haplotype frequencies of VDR gene may vary within Caucasian population as well as the strength of the LD between different VDR polymorphisms.

Alterations in VDR gene expression and VDR protein activity could lead to deregulation of vitamin D uptake, metabolism, and serum levels of the biologically active vitamin D. Furthermore, certain polymorphisms of the VDR gene may have regulatory effects on vitamin D function and metabolism [25].

Szodoray et al. [29] recently reported that the vitamin D levels were found similar in both the overall pSS patients and controls in the Hungarian population they studied. Similarly, no significant differences were found in vitamin D levels of the blood in patients with and without EGMs [29]. Allele frequencies we have found for VDR-FokI, VDR-BsmI, VDR-ApaI, and VDR-TaqI in pSS patients might help to explain the correlation between the genotypes of VDR gene of pSS patients and normal blood level of vitamin D in patients examined previously [29].

Conclusion

This is the first study to investigate all the four polymorphisms of VDR gene in patients with Sjögren's syndrome. There were no statistical differences of the FokI (FF, Ff, ff), BsmI (BB,Bb, bb), ApaI (AA, Aa, aa), and TaqI (TT, Tt, tt) genotypes and allelic frequencies between Sjögren's syndrome patients and control individuals. Analysis of FokI, BsmI, ApaI, and TaqI locus haplotype frequencies also failed to show any association in the study groups. This study suggests that FokI, BsmI, ApaI, and TaqI polymorphisms of VDR gene are not associated with the development of Sjögren's syndrome in the Hungarian population studied. The VDR gene polymorphisms do not seem to be a candidate genetic marker for Sjögren's syndrome.

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Competing interest

The authors declare that there are no conflicts of interests.

Authors' contributions

EZ designed all the experiments and performed some experiments, analyzed data, and wrote the manuscript, QJC performed research experiments, GP collected and analyzed patients data, ASZ collected data, MZ reviewed the manuscript, provided suggestions to the research. All authors read and approved the final manuscript.

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Tables

	Ethnic group (%)		
BsmI-ApaI-TaqI haplotypes	Caucasian	Asian	African
baT	43	75	26
BAt	39	7	16
bAT	11	17	59

Table 1. *Comparison of BsmI-ApaI-TaqI haplotypes across the three major ethnic groups.*

Data are from Uitterlinden et al. 1994. (9)

Enzyme analysis	Sjögren's syndrome patients	Sjögren's syndrome patients with EGMs	Sjögren's syndrome patients with GS	Controls
BsmI polymorphism	p=0.6791	p=0.3966	p=0.5868	
Genotypes				
BB	12.60 **%**	9.60 **%**	13.46 **%**	15.90 **%**
Bb	47.41 **%**	55.70 **%**	40.38 **%**	46.20 **%**
bb	40.51 **%**	34.60 **%**	46.15 **%**	37.80 **%**
Allele frequencies				
B	0.64	0.63	0.66	0.61
b	0.34	0.37	0.34	0.39
ApaI polymorphism	p=0.5331	p=0.5045	p=0.2804	
Genotypes				
AA	20.00 **%**	17.31 **%**	22.64 **%**	24.73 **%**
Aa	48.57 **%**	57.69 **%**	39.62 **%**	50.54 **%**
aa	31.43 **%**	25.00 **%**	37.74 **%**	24.73 **%**
Allele frequencies				
A	0.44	0.46	0.42	0.5
a	0.56	0.54	0.58	0.5
TaqI polymorphism	p=0.5905	p=0.2959	p=0.2804	
Genotypes				
TT	37.14 **%**	32.69 **%**	41.51 **%**	40.86 **%**
Tt	47.62 **%**	53.85 **%**	41.51 **%**	39.79 **%**
tt	15.24 **%**	13.46 **%**	16.98	19.35 **%**
Allele frequencies				
T	0.61	0.6	0.62	0.61
t	0.39	0.4	0.38	0.39
FokI polymorphism	p=0.6133	p=0.7033	p=0.6061	
Genotypes				
FF	34.28 **%**	34.62 **%**	33.96 **%**	42.25 **%**
Ff	47.62 **%**	50.00 **%**	45.28 **%**	43.66 **%**
ff	18.10 **%**	15.38 **%**	20.76 **%**	14.08 **%**
Allele frequencies				
F	0.58	0.6	0.57	0.64
f	0.42	0.4	0.43	0.36

Table 2. *Distribution of VDR-FokI, VDR-BsmI, VDR-TaqI and VDR-ApaI genotypes in our Hungarian cases and controls.*

Three-marker haplotype	Haplotype			Frequency (%)	
	BsmI	ApaI	TaqI	Sjögren's syndrome patients	Healthy individuals
baT	b	a	T	51.52	48.35
BAt	B	A	t	33.59	31.46
bAT	b	A	T	9.16	10.16
BAT	B	A	T	2.31	1.02
bat	b	a	t	1.87	4.16
bAt	b	A	t	1.55	1.69
Bat	B	a	t	0	3.16
BaT	B	a	T	0	0

Table 3. *Estimated haplotype frequencies among the VDR-BsmI, VDR-ApaI and VDR-TaqI polymorphisms in patients and controls.*

Figure legends

Figure 1. *Genotyping for the FokI, ApaI, and TaqI VDR polymorphisms.* The PCR products were digested with FokI, ApaI and TaqI digestion of the amplified region of the VDR gene producing different fragments leading to specific genotypes. The absence and/or presence of the enzyme recognition site were identified by SYBR Green I staining of fragments separated in a 3 % agarose gel. Genotypes were assigned as FF, Ff and ff for the VDR-FokI polymorphisms, AA, Aa and aa for the ApaI polymorphisms, TT, Tt and tt for the TaqI polymorphisms.

Figure 2. *Four-marker (FokI, BsmI, ApaI and TaqI polymorphisms) haplotype estimated prevalence (%) in Sjögren's syndrome patients and healthy individuals.*

Figure 3. Four *common gene polymorphisms and pattern of linkage disequilibrium (LD) of VDR gene of Sjögren's syndrome patients (A) and the healthy controls (B).* Graphical presentation of the VDR gene with the location of polymorphisms studied. Numbers in the boxes represent the correlation coefficient value of LD (r^2) value multiplied by 100. The intensity of the dark color of the boxes represents strength of linkage disequilibrium (r^2) with dark boxes having high LD and white boxes having low LD.

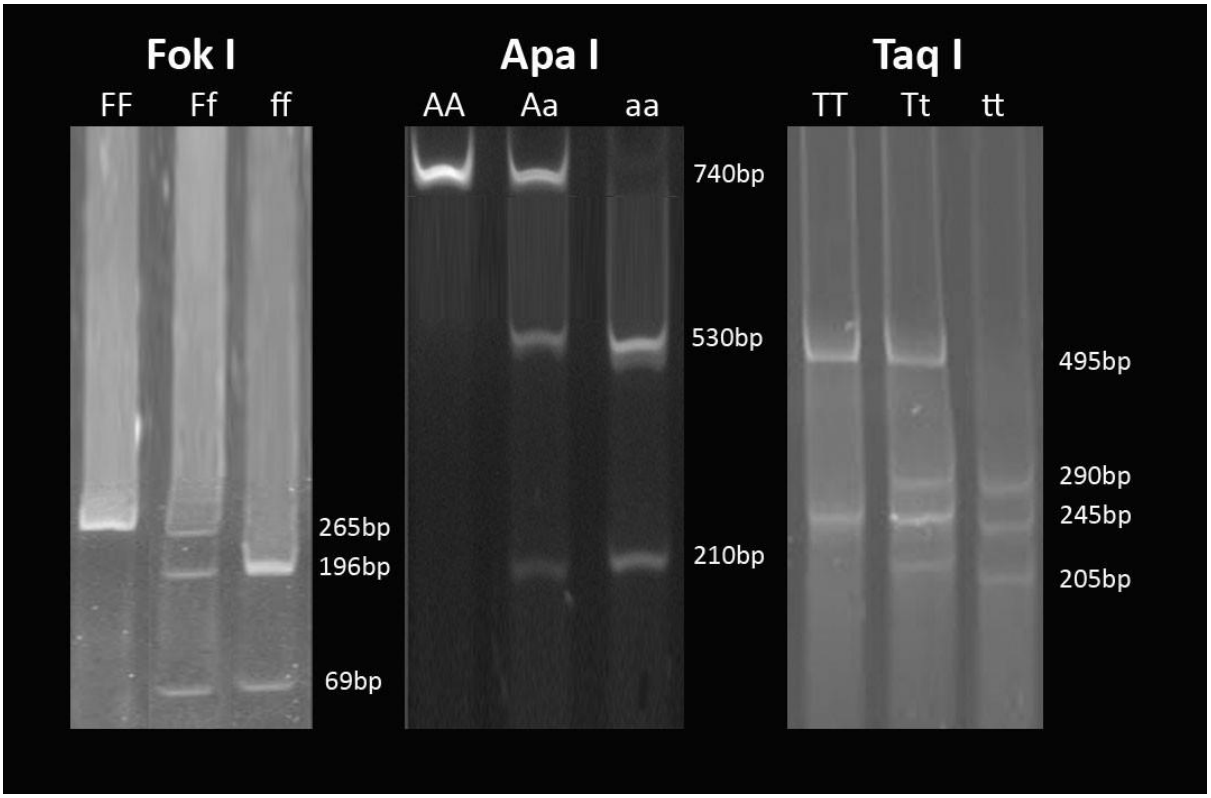
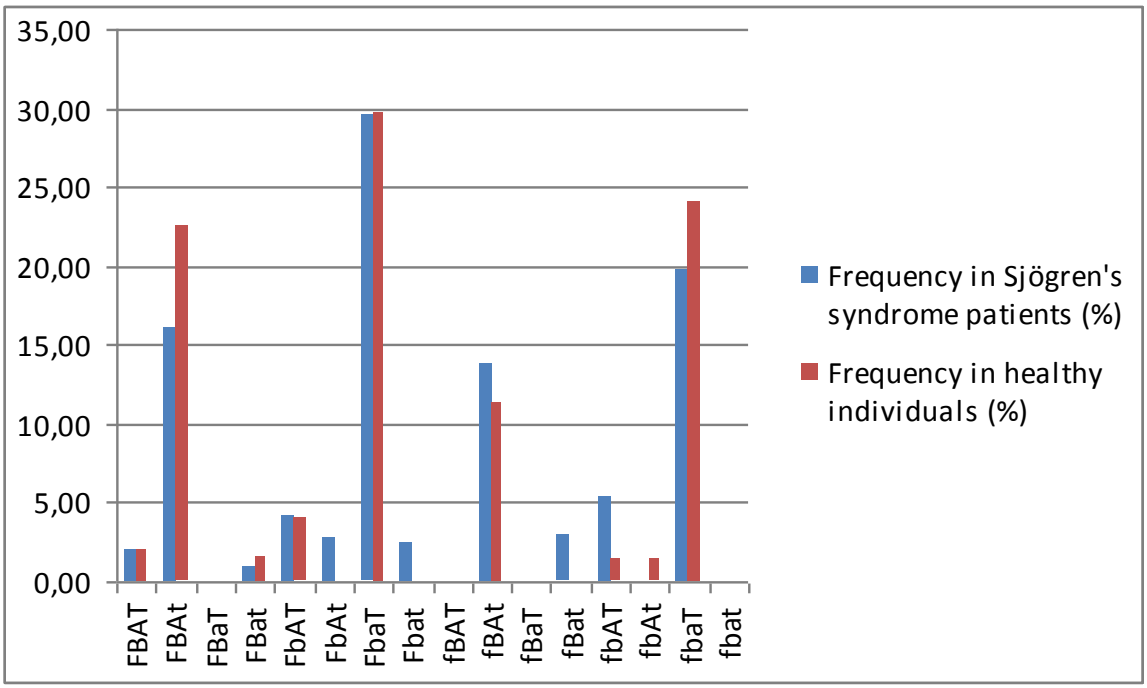


Figure 1. *Genotyping for the FokI, ApaI, and TaqI VDR polymorphisms.* The PCR products were digested with FokI, ApaI and TaqI digestion of the amplified region of the VDR gene producing different fragments leading to specific genotypes. The absence and/or presence of the enzyme recognition site were identified by SYBR Green I staining of fragments separated in a 3 % agarose gel. Genotypes were assigned as FF, Ff and ff for the VDR-FokI polymorphisms, AA, Aa and aa for the ApaI polymorphisms, TT, Tt and tt for the TaqI polymorphisms.



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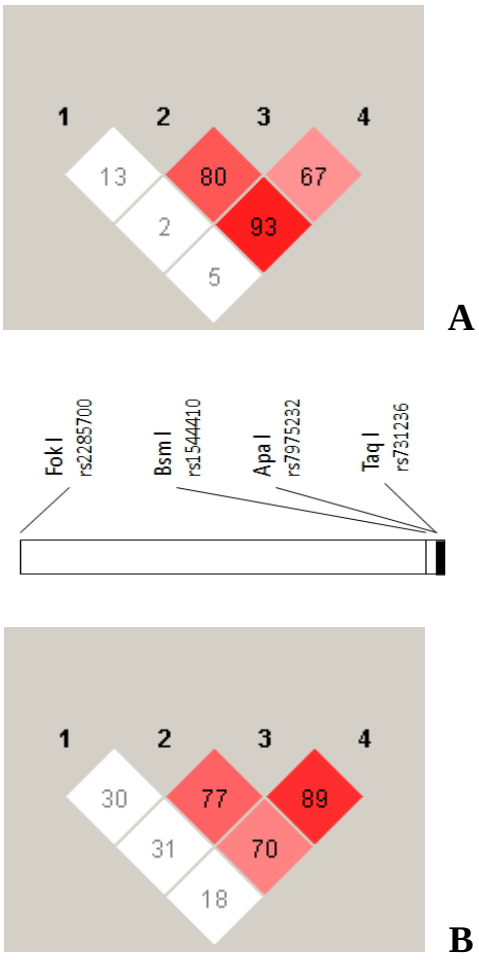

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