RESEARCH COMMUNICATION

Vitamin-D Receptor (VDR) Gene (Fok-I, Taq-I & Apa-I) Polymorphisms in Healthy Individuals from North Indian Population

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Abstract

The vitamin-D endocrine system is involved in a wide variety of biological processes including bone metabolism, modulation of immune responses, and regulation of cell proliferation and differentiation. Variation in this endocrine system have, thus, been linked to several common diseases, including osteoarthritis (OA), diabetes, cancer, cardiovascular ailments, urolithiasis and tuberculosis. Activity of Vit-D is mediated by the vitamin D receptor (VDR), a ligand dependent receptor. VDR gene polymorphisms thus represent strong positional candidates for different diseases like prostate cancer, urolithiasis, inflammatory bowl disease and osteoporosis. Genetic studies provide excellent opportunities to link molecular insights with epidemiological data and can reveal modest and subtle but true biological effects. The abundance of polymorphisms in the human genome as well as high frequencies in human populations have made them targets to explain variation in risk of common diseases. The present study was carried out to determine the distribution of VDR gene (Fok-I, Taq-I and Apa-I) polymorphisms using a PCR-based restriction analysis in unrelated normal healthy individuals from a north Indian population. We obtained allelic frequencies of (68.5% vs 31.5%), (66% vs 34%) and (58% vs 42%) for (F vs f), (T vs t) and (A vs a) alleles, with 44%, 49% and 7%, respectively, for genotypes FF, Ff and ff , 49%, 40% and 11% for TT, Tt and tt and 36%, 44% and 20% for AA, Aa and aa . Our results suggest that the frequency and distribution of the polymorphisms in India are substantially different from in other populations and ethnic groups. Thus the data signify an impact of ethnicity and provide a basis for future epidemiological and clinical studies.

Key Words: VDR gene - SNPs - PCR-RFLP - north India

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Introduction

Genetic variation in human genome is an emerging resource for studying cancer, a complex set of disease characterized by both environmental and genetic contributions. DNA sequences of the human genome reveal that many genes are polymorphic. In coding or noncoding regions of a specific gene, there may be either a single base pair substitution of one nucleotide (SNPs) for another or a variable number of repeats of a short repetitive DNA sequence. (VNTR). These variations may influence the rate of gene transcription, the stability of the messenger RNA, or the quantity and activity of the resulting protein. Thus, the susceptibility or severity of a number of disorders will be influenced by possession of specific alleles of polymorphic genes. SNPs have gained popularity in recent years and are touted as the genetic markers of choice for the study of complex genetic traits (Collins et al., 1997 and Risch et al., 1996). These variants can serve as markers for fine-scale genetic mapping experiments and genome-wide association studies. Some genetic polymorphisms have functionally significant effects on the gene product and are the most useful type of polymorphism in disease association studies while others are simply useful markers.

Calcitriol, the active metabolite of Vitamin D [1, 25$(OH)_2$ D3] have several major roles in the body: hormonal regulation of calcium and phosphate-homeostasis, regulation of parathormone synthesis and modulation of the immune and endocrine systems. Due to its antiproliferative affects it also plays a role in tumour development. The calcitriol can have these differential effects as it is modulating gene expression in the cell nucleus. The effects of vitamin-D are mediated by the nuclear vitamin-D receptor, which heterodimerizes with the retinoid-X receptor and changes

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gene transcription. About five single nucleotide polymorphisms of the vitamin-D receptor gene have been identified so far.

VDR Polymorphism

Vit-D is a member of the steroid receptor family and mediates the effects of the active metabolite 1, 25$(OH)_2$ Vit D_3 by regulating transcription of a number of different cellular genes (Whitfield et al., 1995). The action of vitamin-D is mediated through binding to its nuclear receptor (VDR). The VDR gene, located on chromosome 12, is made up of 5.6 kb (Figure 1). In response to hormone binding, the VDR regulates the transcriptional activity of 1, 25$(OH)_2$ D_3-responsive genes by complexing with a vitamin-D response element located in the promoter region of target genes. The vitamin-D receptor (VDR) is a candidate locus for different disease such as Prostate cancer, Urolithiasis, Inflammatory bowl disease and Osteoporosis etc due to allelic variation which affects the activity of the receptor and subsequent downstream vitamin-D mediated effects such as calcium absorption, excretion and modulation of cellular proliferation and differentiation. Vitamin-D is a steroid hormone that maintains calcium homeostasis and modulates cellular proliferation and differentiation in a number of normal and malignant cells. It has been implicated in prostate cancer, with several epidemiological studies; linking low vitamin-D levels with increased risk of prostate cancer (Schwartz and Hylka., 1990; Corder et.al.,1993).

Several polymorphisms have been identified in the VDR gene (fig-1), and their functional significance and potential effects on disease susceptibility have been investigated (Zmuda et al., 2000). These studies have also pointed out substantial allelic variations of VDR gene in different populations. One of the known DNA sequence variants is a thymine/cytosine (T/C) polymorphism in the first of two potential start (ATG) codons separated by 3 codons. This polymorphism results in two alleles that can be distinguished by RFLP using the endonuclease Fok-I (Gross et al., 1996).

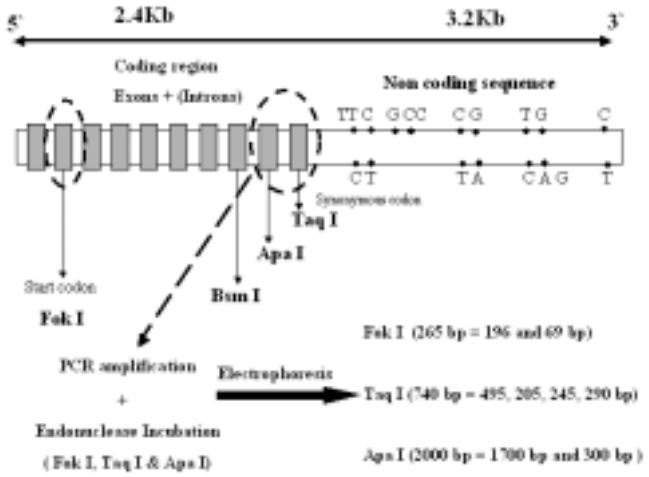

Figure 1. Schematic Map of the VDR Gene Demonstrating the Different Restriction Sites for Fok I, Taq I and Apa I Restriction Enzymes

Other polymorphisms found in the 3' region of the VDR gene, in the intron between exons 8 and 9, using endonucleases Bsm I and ApaI and at the 3' noncoding sequences in exon 9 is identified by the enzyme TaqI. The SNP sites most frequently reported have been the start codon (Fok I) and intron 8 (Bsm I) polymorphism in western and Asian populations. However, there is lack of data regarding allelic variations in VDR (Fok-I, Taq-I and Apa-I) gene from Indian subcontinent where populations/ethinicity are quite different. Therefore, the present study is an attempt to investigate normal distribution of VDR (Fok-I, Taq-I and Apa-I) gene polymorphism by using a PCR-based restriction analysis in unrelated normal healthy individuals from northern India.

Materials and Methods

Subjects

Blood samples were collected from 346 unrelated normal individuals (Male-305 & Female-41) [age range- 15-68 years] from the north Indian population with informed consent. The genomic DNA was isolated from peripheral blood by using standard phenol chloroform method (Sambrook et.al., 1989).

Polymerase Chain Reaction

Reaction mixtures of 25ml were used in PCR for the VDR gene (Fok-I, Taq-I and Apa-I) polymorphism and DNA samples were amplified in MJ Research PTC-100TM (Peltier Thermal Cycler). Gels were visualized under UV and photographed with Alpha Imager 1220 v5.5 Camera software. The primers of the VDR gene used were as reported earlier

Fok-I (Harris et al., 1997)
Forward
5'-AGCTGGCCCTGGCACTGACTCTGCTCT-3'
Reverse
5'-ATGGAAACACCTTGCTTCTTCTCCCTC-3'

Taq-I (Riggs et al., 1995)
Forward
5'-CAGAGCATGGACAGGGAGCAA -3'
Reverse
5'-CACTTCGAGCACAAGGGGCGTTAGC -3'

Apa-I (Sainz J et al., 1997)
Forward
5'- CAACCAAGACTACAAGTACCGCGTCAGTGA -3'
Reverse
5'- CACTTCGAGCACAAGGGGCGTTAGC-3'

Fok-I polymorphism

The PCR cycle conditions were Initial denaturation at 94°C for 5 min, followed by 35 cycles at 94 °C for 30 s, 61°C for 30 s and 72 °C for 1 min and one final cycle of extension at 72 °C for 7 min The reaction mixture consisted of 100-200ng of genomic DNA, 2-6 pmol of forward and

reverse primers, 1x Taq polymerase buffer (1.5mM $Mgcl_2$)(Genetix Biotech Asia Pvt. Ltd.), dNTPs (200mM each) (Bangalore genei India) and 1.5 units of Taq DNA polymerase (Bangalore genei India). The PCR product (265 bp) was verified using a 1.5% agarose gel containing ethidium bromide. The PCR product was digested with 1.0 unit of Fok-I restriction enzyme and the reaction buffer and incubated at 37°C for 4 hours; 10ml of the digested reaction mixture was then loaded into 9% PAGE (Polyacrylamide gel electrophoresis) containing ethidium bromide. The FF genotype (homozygote of common allele) lacked a Fok-I site and showed only one band of 265 bp. The ff genotype (homozygote of infrequent allele) generated two fragments of 196 and 69 bp. The heterozygote displayed three fragments of 265, 196 and 69 bp, designated as Ff.

Taq-I polymorphism

The PCR cycle conditions were: Denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 60 s, 63 °C for 60 s and 72 °C for 2 min and one final cycle of extension at 72 °C for 5 min using 100-200 ng of genomic DNA, 2-6 pmol of forward and reverse primers, 1x Taq polymerase buffer (1.5mM $Mgcl_2$) (Genetix Biotech Asia Pvt. Ltd.), dNTPs (200mM each) (Bangalore genei India) and 1.5 units of Taq DNA polymerase (Gibco BRL). PCR products (740 bp) were digested with Taq-I (Fermentas, Lithuania) restriction enzyme at 65°C for 3 hrs and 10ml of the digested reaction mixture was then loaded into 9% PAGE. Taq-I digestion revealed one obligatory restriction site, the homozygous TT (absence of the specific Taq-I restriction site) yielded bands of 245 bp and 495 bp. The homozygous tt exhibited 205, 245, 290 bp and the heterozygous Tt provided 495, 205, 245, 290 bp fragments.

Apa-I polymorphism

The PCR cycle conditions were denaturation at 94 °C for 4 min, followed by 35 cycles at 94 °C for 30 s, 65 °C for 30 s and 72 °C for 2 min and one final cycle of extension at 72 °C for 4 min using 100-200ng of genomic DNA, 2-6 pmol of forward and reverse primers, 1x Taq polymerase buffer (1.5mM $Mgcl_2$) (Genetix Biotech Asia Pvt. Ltd.), dNTPs (200mM each) (Bangalore genei India) and 1.5 units of Taq DNA polymerase (Gibco BRL). The amplified 2000-bp PCR product was subjected to Apa-I restriction enzyme (Gibco BRL) digestion. 5µl of the PCR product was digested with 5 units of Apa-I restriction enzyme in a 10µl reaction-using manufacturer's buffer at 37 °C for 3 h. The Apa-I enzyme digested product was electrophoretically (100V for 30 mins) run on a 1.5% agarose gel containing 0.5% µg/ml ethidium bromide. Absence of Apa-I restriction site (2000 bp) was assigned as a common allele A (wild-type allele) and presence of restriction site resulting in 1700 bp and 300 bp fragments was assigned as infrequent allele a (mutant allele). Genotypes were assigned accordingly as homozygotes for common allele (AA) and homozygotes for infrequent allele (aa). Presence of 2000, 1700 and 300 bp fragments was assigned as heterozygotes (Aa).

Statistical Analysis

Genotype frequencies of the VDR gene polymorphism in normal healthy controls in north Indian population were determined according to Hardy-Weinberg equilibrium. Two-tailed Fisher's exact test and chi square test was done to compare the allelic frequencies of different populations using NCSS 6.0 software program and data were analyzed by using the computer software SPSS for windows (version 10.0).

Results

The distribution of VDR genotypes (Fok-I, Taq-I & Apa-I) and allele frequencies in north Indian population is shown in (Table-1). The allelic frequency of 'F'vs 'f', 'T' vs 't' and 'A'vs 'a ' was 68.5 vs 31.5 %, 66 vs 44 %, 58 vs 42 % in our population. Genotype distribution was in agreement with Hardy-Weinberg equilibrium. We compared the frequency distribution of different genotypes and alleles of VDR gene with different populations with reference to ours (Table 2, 3 & 4) by using χ^2 tests. In case of VDR (Fok-I) genotype distribution we found significant difference between Finland, Black Pennsylvania in comparison to our population. Significant distribution of different genotypes and allele frequency was reported in VDR (Taq-I) polymorphism in Austria, Japan, China and Thailand population Genotype and allele distribution of VDR (Apa-I) polymorphism was significantly different in Japan, China, Korea and Thailand.

Discussion

SNPs are scattered throughout the genome and high degree of variability make these informative genetic markers useful for disease susceptibility. Indian population is believed to be most diverse because of different socio-cultural traditions. VDR is known to regulate cell proliferation, calcium absorption from the gut, and cell differentiation and may also influence androgen and estrogen activation. The action of VDR is not only up regulated by vitamin-D but also by protein kinase A, parathyroid hormone and growth factors. Any defect in the VDR gene could modulate the metabolism of calcium thereby increasing the risk of developing different diseases e.g. osteoporosis, calcium stones, prostate cancer etc. The VDR gene polymorphism has been widely used as a genetic marker for diseases related to calcium metabolism. In the present study, we have

Table 1. Genotypes and Allele Frequency Distribution of VDR Gene (Fok-I, Taq-I & Apa-I) Polymorphism in North India

	Genotypes (%)			Allelic	
	FF	Ff	ff	F	f
N = 346	152 (44)	170 (49)	24 (7)	68.5	31.5
	TT	Tt	tt	T	t
N = 346	170 (49)	138 (40)	38 (11)	66	34
	AA	Aa	aa	A	a
N= 150	54 (36)	66 (44)	30 (20)	58	42

Table 2. Genotypes and Allele Frequency Distribution of VDR Gene (Fok-I) Polymorphism in Various Populations and P-values of Different Allele and Genotypes in Different Populations in Comparison to North Indian Population

Country/Ethnicity	No	Age (Years)	Genotype (%)				Allele (%)			Reference
			(FF)	(Ff)	(ff)	P	(F)	(f)	P	
Europe										
North India	346	20-74	44	49	7	Ref	68.5	31.5	Ref	Present study
Finland	144	35-69	28	58	14	*	60	40	NS	Videman et al, 1998
Paris	100	64-86	43	47	10	NS	66	34	NS	Correa et al, 1999
England	108	56±20	48	41	11	NS	69	31	NS	Hutchinson et al, 2000
South Pacific										
Australia	577	59-82	37	48	15	NS	61	39	NS	Kotowicz et al, 1998
Asia										
Japan	249	13-20	37	51	12	NS	62	38	NS	Minamitani et al, 1998
Taiwan	101	40-53	36	49	15	NS	61	39	NS	Cheng et al, 1999
South India	80	NR	43	29	8	NS	72	28	NS	Selvaraj et al, 2003
Americas United States										
White, Massachusetts	82	20-40	37	45	18	NS	59	41	NS	Harris et al, 1997
Black Pennsylvania	104	≥ 65	63	31	6	*	78	22	NS	Zmuda et al, 1999
Mexican, California	100	59-82	37	48	15	NS	61	39	NS	Gross et al, 1996

* = p<0.05, ** = p<0.01, *** = p<0.001 , at 5 % level of significance NS= Not Significant (p>0.05)

reported the distribution of VDR (Fok-I, Taq-I and Apa-I) genotypes in our population and compared with the genotypes reported in different populations worldwide. The frequency of the individual alleles varies among different ethnic or geographic populations shown in (Tables. 2, 3 & 4).

The study of genetic variation can elucidate critical determinants in environmental exposure and cancer, which could have future implications for preventive and early intervention strategies. However, we are in the initial stages of characterising the tools (i.e., the single-nucleotide polymorphism, SNP) in rigorous analysis of the genetic contributions to complex diseases, such as cancer. If the promise of the genomic era is to be realised, we must integrate this information into new strategies for implementation in both public health measures and, most importantly, provision of individual cancer-related care. The number of common germ-line variants is great, on the order of 10-15 million per person, and represents a remarkable opportunity to investigate the aetiology, interindividual differences in treatment response and outcomes of specific cancers.

Although active research in this field has been started only recently, some directions (e.g. cancer pharmacogenetics) already demonstrated impressive achievements. At the same time the reliability of results

Table. 3 Genotypes and Allele Frequency Distribution of VDR Gene (Taq-I) Polymorphism in Various Population and P-values of Different Allele and Genotypes in Different Populations with Comparison to North Indian Population

Country/Ethnicity	No	Age (Years)	Genotype (%)				Allele (%)			Reference
			(TT)	(Tt)	(tt)	P	(T)	(t)	P	
Europe										
North India	346	20-74	49	40	11	Ref	66	34	Ref	Present study
France	189	31-57	33	49	18	0.05	57	43	NS	Garnero et al, 1995
Austria	163	44-78	12	49	39	***	36	64	***	Ewald et al, 1996
Sweden	100	70±1	34	54	12	NS	61	39	NS	Carling et al, 1997
Greece	53	20-70	38	41	21	NS	59	41	NS	Fountas et al, 1999
South Pacific										
Australia	518	NR	36	48	16	NS	60	40	NS	Tokita et al, 1996
Asia										
Japan	488	8-78	77	22	1	***	88	12	***	Tokita et al, 1996
China	144	30-40	90	10	0	***	95	5	***	Kung et al, 1996
Thailand	84	40-79	83	17	0	***	92	8	***	Ongphiphadhanakul et al, 1997
AmericasUnited States										
White, Minnesota	130	≥ 30	41	44	15	NS	63	37	NS	Riggs et al, 1995
Black Pennsylvania	101	≥ 65	32	53	15	0.05	58	42	NS	Zmuda et al, 1997
Mexican, California	101	59-84	51	40	9	NS	71	29	NS	McClure, 1997
White, North Carolina	162	NR	33	45	22	*	55	45	NS	Taylor, 1996

* = p< 0.05, ** = p<0.01, *** = p<0.001 , at 5% level of significance NS= Not Significant (p>0.05)

Table 4. Comparative Frequency Distribution of VDR (Apa-I) Genotypes and Alleles in Various Populations

Country/Ethnicity	No	Age (Years)	Genotype (%)				Allele (%)			Reference
			(AA)	(Aa)	(aa)	P	(A)	(a)	P	
Europe										
North India	150	20-74	36	44	20	Ref	58	42	Ref	Present study
France	189	31-57	30	50	20	NS	54	46	NS	Garnero et al, 1995
Austria	163	44-78	29	45	26	NS	52	48	NS	Ewald et al, 1996
Sweden	100	70±1	27	52	21	NS	53	47	NS	Carling et al, 1997
Greece	53	20-70	36	43	21	NS	58	42	NS	Fountas et al, 1999
South Pacific										
Australia	518	NR	26	51	23	NS	51	49	NS	Tokita et al, 1996
Asia										
Japan	488	8-78	9	48	43	***	33	67	**	Tokita et al, 1996
China	144	30-40	10	36	54	***	29	71	***	Kung et al, 1998
Korea	104	NR	3	28	69	***	17	83	***	Park et al, 1999
Thailand	84	40-79	11	50	39	***	36	64	**	Ongphiphadhanakul et al, 1997
South India	80	NR	38	46	16	NS	61	39	NS	Selvaraj et al, 2003
AmericasUnited States										
White, Minnesota	128	>30	30	46	24	NS	53	47	NS	Riggs et al, 1995
Black Pennsylvania	101	≥65	44	46	10	NS	67	33	NS	Zmuda et al, 1997
Mexican, California	100	7-12	21	55	24	NS	48	52	NS	Sainz et al, 1997

* = p< 0.05, ** = p<0.01, *** = p<0.001 , at 5% level of significance NS= Not Significant (p>0.05)

reported by many groups remain questionable mostly due to insufficient statistical power of the studies and often-random choice of polymorphisms. It is evident that large studies based upon combined analysis of groups of genes within relevant regulatory and metabolic pathways have a much higher potential value in terms of unravelling prognostically important individual polymorphism profiles.

In the present analysis, variation at VDR gene was measured solely on the basis of PCR-RFLP of the basic core sequence. Due to their highly polymorphic content, SNPs constitute useful tools in population genetic studies in understanding population and ethnic variations. As there are large differences in allele frequency and distribution of genotypes of VDR (Fok-I, Taq-I and Apa-I) SNP, it would be quite helpful to find out any linkage disequilibrium in individuals from other ethnic groups. The variation in our Indian population from the rest of the world population signifies the impact of ethnicity. Thus this kind of study may form the basis for future establishment of epidemiological and clinical databases. Allelic association studies are in progress with several chronic inflammatory and degenerative diseases in which VDR may be involved. In the long run, these studies may help in determining disease susceptibility and clinical management.

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