ORIGINAL ARTICLE

(VDR) Gene Polymorphisms - Rapid and non-invasive oral detection Method

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ABSTRACT

Background: In recent years, the importance of vitamin D receptor (VDR) gene restriction fragment length polymorphisms for various types of disease and cancers has been investigated by a great number of studies. A non invasive method could be employed for extracting DNA. Oral rinse has been found to be one of the sources for collecting genomic DNA.

Objective: To develop a rapid and non-invasive method for the detection of Vitamin D Receptor (VDR-Fok1) Gene from oral rinse.

Methods: Oral rinse samples were collected normal individuals with informed consent. Individuals included were healthy adults between 20-40 years of age. Oral rinse (40ml) was taken after gentle brushing over the lesions. DNA extraction was done according to Lucky MH et al and PCR was performed using beta globin primers GH02/PC04 and VDR-Fok1 primers. A 256bp amplified products was visualized by Gel Doc Hero Lab software (Germany). The PCR-RFLP results showed the 20 or 40% FF genotype (homozygote of common allele) with one band of 265bp.

Results: The mean concentration of 60 DNA samples was 14.484±10.63ug/ml. The results of VDR-Fok1 gene polymorphism shows that out of 60 subjects 48 were normal (FF 80%), 12 were Heterozygous (Ff 20%) and 0 were mutated (ff 0%).

Conclusion: Oral rinse is a perfect medium for rapid and non invasive diagnostic applications of VDR gene may be optimized for other salivary biomarkers.

KEY WORDS: *Oral Rinse, DNA Extraction, PCR, RFLP, VDR, Fok1 Polymorphism.*

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INTRODUCTION

In recent years, the relevance of vitamin D receptor (VDR) gene restriction fragment length polymorphisms for various types of cancer has been investigated by a great number of studies.[1] It has been hypothesized that VDR polymorphisms may influence both the risk of cancer occurrence and prognosis.[1,2] However, studies investigating the associations between specific VDR polymorphisms and cancer often show controversial results. The human VDR protein contains either 427 or 424 amino acids depending upon the presence of a T to C polymorphism (ATG to ACG) in a translational start site.[3,4] The gene encoding the *VDR* is located on chromosome 12cen-q12,[5] that contains 11 exons[6] and spans approximately 75 kb of genomic DNA[7] Several polymorphisms have been identified in the gene including the *Fok I* polymorphism located in exon 2 (rs2228670) at the 5′ coding region of the gene.[8] Fok I polymorphism results in different translation initiation sites due to thymine (T) to cytosine (C) substitution in the first translation initiation codon ATG (methionine) which generates long and short variants of *VDR*. Oral rinse is one of the most accessible sources of sample collection easy and non-invasive.[9] Oral rinse also contains significant amount of DNA which can be easily assessed for a wide spectrum of genetic data and be successfully utilized for genetic research and clinical diagnostic applications.[10] The study was undertaken to find out a rapid and non-invasive method for the detection of Vitamin D Receptor (VDR-Fok1) Gene from oral rinse which can be beneficial for future studies of various polymorphisms associated with this gene.

METHODOLOGY

The study was conducted at the Ziauddin University Research Laboratories, Karachi. Oral rinse samples were collected from 60 healthy individuals. Informed consent was taken prior to sampling. Individuals included were healthy adults between 20-40 years of age. The procedure devised to get more of DNA was a use of toothpick with a small bristle on the other end used for dental floss. The subjects, after collection of oral rinse, were asked to swipe the bristle on the oral mucosa of cheeks to gather a good number of mucosal cells. The genomic DNA was isolated from oral rinse according to Lucky MH *etal*[9]. The DNA was quantified by using Qubit® dsDNA BR Assay (Qubit® 2.0 invitrogen life technologies USA) and the quality was checked by running an aliquot on 0.6% agarose gel stained with ethidium bromide. 50 µl were used in PCR. The DNA samples were amplified in BioFlux Thermal Cycler (BioFlux, Korea). The primers used were GH02/PC04[11] for beta globin gene and Fok1[12] for VDR Polymolymorphism.

Table 1. Sequences of beta globin and Fok1 primers

Gene	Primer sequence
β globin	GH20 5'-CAACTTCATCCACGTTCACC-3' PC04 5'-GAAGAGCCAAGGACAGGTAC-3'
Exon2 (T/C) FokI rs2228570	Forward 5'-AGCTGGCCCTGGCACTGACTCTGCTCT-3' Reverse 5'-ATGGAAACACCTTGCTTCTTCTCCCTC-3'

β globin PCR Cycling Conditions are as follows: DNA samples were amplified with cycling parameters as follows: Initial denaturation at 95°C for 5 minutes followed by 40 cycle of 95°C for 1 min, 51°C for 1 min, followed by 72°C for 2 min, and a final extension at 72°C for 7 minutes. The PCR reaction was carried out in 50 µl volume, containing 25 µl of GoTaq® Green master mix (GoTaq® DNA Polymerase is supplied in 2X Green GoTaq® Reaction Buffer pH 8.5, 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP and 3 mM MgCl2,Promega, USA) 4 µl of 1 µM of each primer (Genelink, USA) , 5 µl (100-200 ng) of DNA template and 12 µl of PCR graded water (Promega, USA). A 268bp Bands were

visualized on HeroLab (Germany) Gel Doc Imaging system. PCR Cycling Conditions for VDR Fok1 are as follows: Initial denaturation at 94°C for 5 minutes followed by 32 cycle of 94°C for 45 seconds, 58°C for 45 seconds, followed by 72°C for 45 seconds, and a final extension at 72°C for 7 minutes. The PCR reaction was carried out in 50 µl volume, containing 25 µl of GoTaq® Green master mix (GoTaq® DNA Polymerase is supplied in 2X Green GoTaq® Reaction Buffer pH 8.5, 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP and 3 mM MgCl2,Promega, USA) 4 µl of 1 µM of each primer (Genelink, USA) , 5 µl (100-200 ng) of DNA template and 12 µl of PCR graded water (Promega, USA). A 265bp Bands were visualized on Hero Lab (Germany) Gel Doc Imaging system. PCR-RFLP Conditions for FokI polymorphism were: 0.2ul of PCR product was digested with 1 µL of Fast Digest FokI restriction enzyme (Fast Digest FokI Fermantas, USA) and the 1X FastDigest buffer and incubated at 37^{o}C for 5 minutes. 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.

RESULTS

Out of 60 samples the PCR-RFLP results showed (Table 2) that FF genotype (homozygote of common allele) with one band of 265bp. The ff genotype (homozygote of infrequent allele) generated two fragments of 196bp and 69bp (Figure 1). The heterozygote displayed three fragments of 265, 196 and 69bp, designated as Ff. The mean concentration of 60 DNA samples was 14.484±10.63ug/ml.

Table 2. Frequency of Fok-1 polymorphism in normal subjects

Subjects	Fok-1 polymorphism			Total
	FF 365bp No. (%)	Ff 265, 196,69bp No. (%)	Ff 196,69bp No. (%)	
Normal (n=60)	48 (80%)	12 (20%)	0 (0%)	60

DISCUSSION

DNA, traditionally extracted from whole blood, though an excellent source, requires technical assistance for collection, is costly to ship, store and process. Over the past few years, saliva has become recognized as a very important and reliable alternative to blood samples for genetic research, clinical diagnostics, personalized medicine and more.[13] This study showed that the oral rinse has the perfect ability to be a non-invasive way to examine VDR gene in our setting and can be the means for an inexpensive, non-invasive and easy to use diagnostic method for monitoring health status as discovered by other studies.[14] The shortcoming in the use of saliva as a diagnostic fluid is that metabolites in general are present in lower amounts and that can be a limitation in genetic studies where sequencing is a requisite.[15] In this study the method employed for obtaining more cells provided a good DNA yield. The mean concentration of 60 DNA samples was 14.484±10.63ug/ml. This method was earlier tested on HPV with success.[9] Similarly, new and very sensitive techniques have been developed which provide better level of viral DNA, such as that of HBV, HCV or HIV in saliva[16] Furthermore saliva is far more beneficial than blood in collection, handling and storage, without causing any trauma or anxiety to the patients and researchers.

In Pakistan people at all levels suffer from vitamin D deficiency in spite of plentiful sunlight. It was imperative thus, to investigate molecular and functional aspects of vitamin D receptor polymorphisms in the population to fully appreciate the significance of its genotypes and understand their potential clinical implications in various VDR polymorphism related diseases[17].

VDR has been extensively studied focusing on seven different types of polymorphisms. Amongst these the *Fok1* single nucleotide polymorphism (SNP) is undoubtedly the most frequently studied SNP within the VDR gene.[18] The clinical significance of this SNP has been highlighted by its associations with several metabolic and pathological conditions. In this regard renal functions have been found associated with VDR genotypes (including *Fok1*).[19] In a cross sectional study Vaidya, et al[20] showed that vitamin D metabolites are inversely associated with circulating rennin. The biologic role of 1,25(OH)2D as an inhibitor of

renin expression in humans, suggests that the combination of 25(OH)D status and genetic variation at the *Fok1* SNP may enhance prediction of plasma rennin activity and Renin Angiotensin System activity, which may in turn be relevant for cardiovascular and metabolic risk.

A preliminary data on Vitamin D activity and its role in the investigation of prostate, breast, colorectal, and skin cancer, suggest that VDR polymorphisms is more frequently associated with tumorigenesis.[21] Fok1 polymorphism can be regarded as an independent prognostic factor when vitamin D levels are low. In a huge US study, the Fok1 polymorphism was found associated with the vitamin D status (measured by plasma 25(OH)D) in such a way that those with the ff genotype were found more susceptible to prostate cancer in the presence of low 25(OH)D status.[22] The risk of cancer with VDR Fok1 polymorphism was found only when it combines with exposure to environmental factors such as sun or high occupational outdoor activity (Table 3).[23]

Table 3: VDR Fok1 polymorphism studied in various cancers

Studies	Authors	Type of Cancer	Year of study
A prospective study of plasma vitamin D metabolites, *vitamin D receptor* polymorphisms, and prostate cancer.	Li H, et al[24]	Prostate	2007
Association of *vitamin-D receptor* (Fok1) gene polymorphism with bladder cancer in an Indian population.	Mittal RD, et al [25]	Bladder	2007.
Vitamin D receptor polymorphisms in colorectal cancer in New Zealand: an association study.	Bentley RW, et al[26]	Colorectal	2012
Associations between vitamin D receptor polymorphisms and breast cancer risk.	Wang J, et al [27]	Breast cancer	2013
Polymorphism in the vitamin D receptor (VDR) genes and skin cancer risk in European population: a meta-analysis.	Zhao XZ.et al[28]	Skin cancer	2014

Figure 1: Lane 1-10 showing VDR fok-1 gene band of 265bp

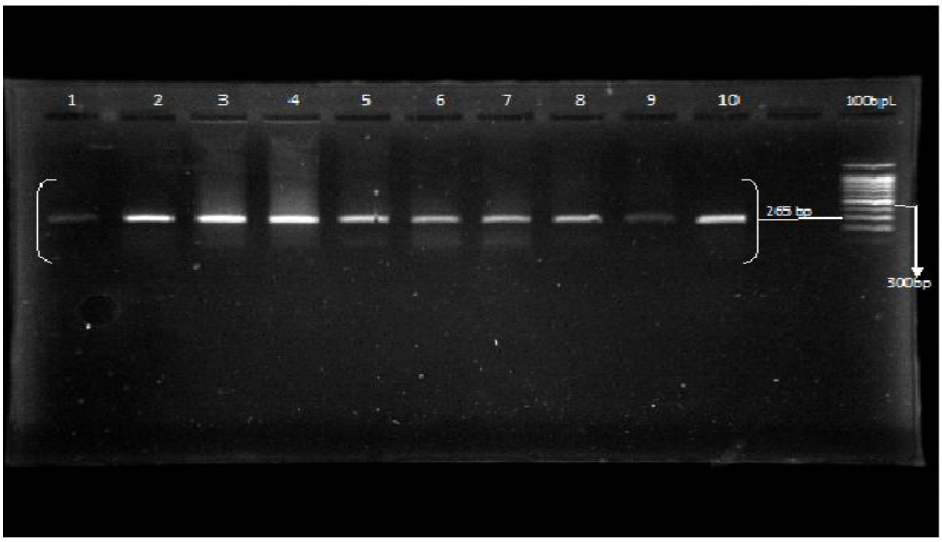
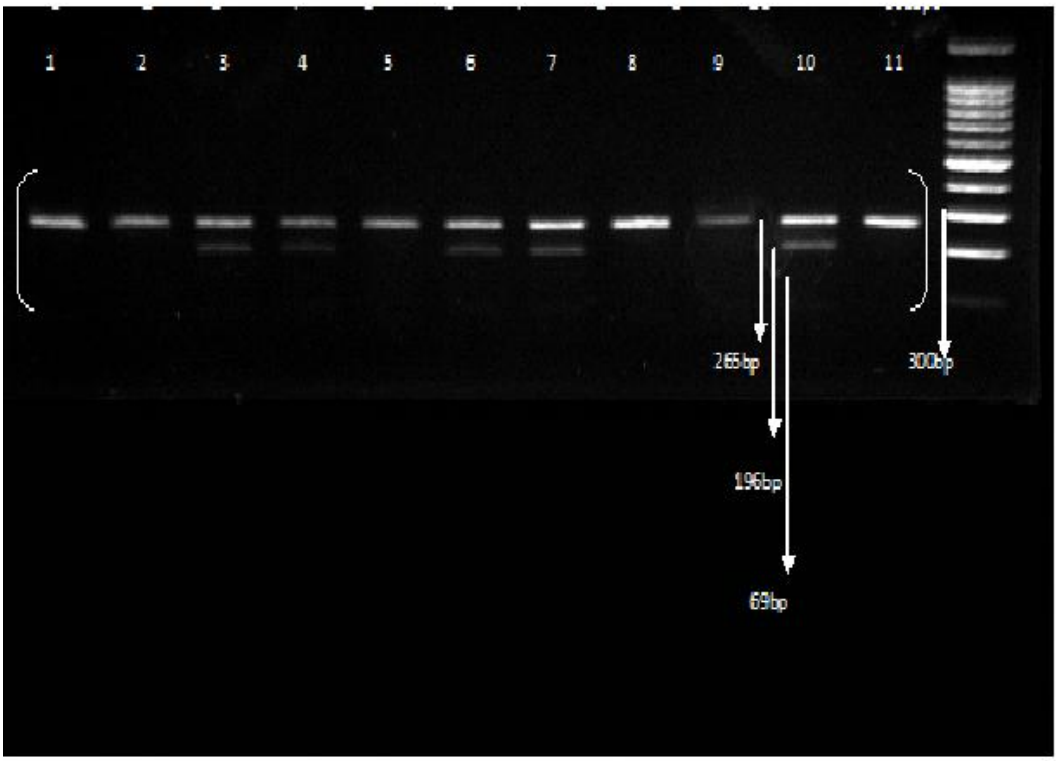


Figure: 2 Lane 1, 2,5,8,9 and 11 shows FF genotype whereas lane 3,4,6,7 and 10 shows Ff genotype of VDR fok-1 gene polymorphism.



CONCLUSION

Genetic variation in human genome is an emerging resource for studying cancer and other complex set of diseases characterized by both environmental and genetic contributions. DNA sequences of the human genome reveal that many genes are polymorphic. Saliva-based diagnostics can be employed, since they are more accessible, accurate, and less expensive and present less risk to the patient than current methodologies.

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