Association of Vitamin D Receptor Gene Polymorphisms in the Occurrence and Spectrum of Leprosy in Filipino Patients Seen at Jose R. Reyes Memorial Medical Center

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Abstract

Introduction / Background: Host genetic factors including major histocompatibility complex (MHC) polymorphisms influence both susceptibility to leprosy and also to leprosy type. Recent studies have implicated variation in the vitamin D receptor (VDR) gene in susceptibility to several diseases, including osteoporosis and pulmonary tuberculosis. Putative polymorphisms at the VDR gene, which potentially modifies VDR mRNA stability and/or activity, have been implicated in susceptibility to intracellular pathogens.

Objectives: The general objectives of the study are to determine the effects of Vitamin D receptor gene polymorphism on the occurrence and spectrum of leprosy among Filipino patients seen at Jose R. Reyes Memorial Medical Center, Department of Dermatology Hospital.

Methods: Five (5) cc of whole blood samples obtained from patients diagnosed with Hansen's disease by smear and histological confirmation as well as healthy controls seen in the outpatient department. DNA was isolated from white blood cells using the phenol/chloroform method and was used for PCR amplification. The primers 5'-CAGAGCATGGACAGGGAGCA-3' and 5'-GGTGGCGGCAGCGGATGTACGT-3' yielding a product of 352 base pairs in the 61675 and 62026 positions of the VDR gene was used (Goulart et al., 2005). The PCR cycle conditions would be 94^{o}C for 20 seconds, 60^{o}C for 30 seconds, and 72^{o}C for 30 seconds (35 cycles), using 2 m*M* MgCl2, 0.2 m*M* dNTPs, 0.009 m*M* of each primer, 100 ng of DNA, and 1 U of Taq polymerase in a 25-µL reaction (Roy et al., 1999).

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Results: Sixty (60) subjects were included in the study. A total of 43 (71.67%) patients who were diagnosed case of Leprosy with a spectrum of tuberbuloid and lepromatous type. Fifteen patients (25%) were classified as tubercuoid type and 28 (46.67%) patients were of lepromatous type. Seventeen 17 (28.33%) patients in the control group were composed of healthy volunteers unrelated to patients. The distribution of VDR genotypes at codon 352 in exon 9 was highly significant in between the control and lepromatous, tuberculoid and lepromatous type of Hansen's disease. Heterozygous type of VDR (Tt) were found to be highly significant among controls at 82.35% while 66.67% of tuberculoid has homozygous recessive type (tt) of VDR and 96.43% of lepromatous type has homozygous dominant type VDR (TT) . Significant difference were observed between the HD group (tuberculoid and lepromatous) and control. Fstat = 31.32, ($p < 0.001$). Post-hoc analysis of genotypes between the clinical groups showed that tuberculoid vs lepromatous had significantly different genotypes ($p =< 0.001$) and lepromatous type of HD vs control had significantly different genotypes ($p =< 0.001$).

Conclusion: The polymorphism of VDR genotypes at codon 352 in exon 9 was highly significant in between the control, lepromatous, and tuberculoid type of Hansen's disease. Homozygous TT was highly associated with lepromatous type (odds ratio = 16, $p =< 0.001$), and homozygous tt is highly related to the tuberculoid type (odds ratio = 5.8, $p =< 0.001$).

INTRODUCTION

Leprosy is a chronic, infectious, and slowly progressive disease caused by the bacillus *Mycobacterium leprae* characterized by granulomas and neurotropism with a predilection for the skin and peripheral nerves. The diagnosis of the disease is often delayed and aggravated by subclinical infection, presence of healthy carriers and inconsistent evidence of reduced transmission following multidrug therapy.[1]

Once worldwide in distribution, leprosy is now seen primarily in tropical and subtropical regions of Asia, Africa, and Central and South America. In endemic countries, the vast majority of new cases are in children and young adults who have close relatives with contagious forms of the disease. Elimination of leprosy as a public health problem is defined as a prevalence rate of less than one case per 10 000 persons. Efforts currently focus on eliminating leprosy at a national level in the remaining endemic countries and at a sub-national level from the others. The number of new cases detected in 2008 was 240,007 globally, and has fallen by 9126 (4% decrease) compared with 2007. However, there was a slight increase at the end of 2009 to 244,796.

The National Leprosy Control Program (NLCP) of the Philippines was established in 1986 under the supervision of the National Center for Disease Prevention and Control (NCDPC) of the Department of Health (DOH). Leprosy Control Program envisions to eliminate leprosy as a human disease by 2020. The program thrust is towards finding hidden cases of leprosy and putting them on MDT, emphasizing the completion of treatment within the WHO-prescribed duration.[28] In 1986, there were 38,570 registered patients with leprosy in the Philippines, with a prevalence rate of 7.2 per 10,000 Filipinos. There was a dramatic decrease in prevalence by the end of 1998, to 0.90 per 10,000 population, and leprosy was no longer considered to be a public health problem of the country. In 2004, the prevalence was further reduced to 0.38 per 10,000 population, translating to a total decline of 3,146 from 7,005 in 1998. In 2006, the prevalence of leprosy in the Philippines was more than 3000 cases (0.42%), while the number of new cases detected was 2,517. There was a decrease in the number of patients in 2007. However, an upward trend was observed in 2008 to 3,338 patients (0.35%).

The three requirements for the spread of leprosy are: a contagious patient, a susceptible person, and close or intimate contact. The organism is spread predominantly via nasal and oral droplets from the bacilliferous patient and much less often from eroded skin and even after 1 to 7 days, the bacillus is still viable in dried secretions. Inoculation is via the nasal mucosa or, less commonly, through breaks in the skin barrier. Transmission also depends on the infectivity of the contagious patient.

Leprosy is mainly a clinical diagnosis. Laboratory techniques may serve as adjuncts in the diagnosis. AFB microscopy is still being used in the Philippines; samples for bacilloscopy may be obtained from the earlobes, forehead, chin, extensor forearms, and dorsal fingers, as well as buttocks and trunk. The smear is usually stained by the Fite (or Ziehl-Neelsen) method and a search is made for red rods (against a blue background) at 100x with oil immersion. Routine skin punch biopsy with hematoxylin-eosin stain and Fite-Faraco are also being requested in training institutions in the Philippines but not routinely done in leprosy control programs and rural health centers. In subclinical cases seen in clinics, a clinicopathological correlation may deem necessary. A biopsy specimen of the skin lesions should be obtained, especially in patients with suspected tuberculoid leprosy. There are three basic histopathologic patterns observed in leprosy: lepromatous, tuberculoid and borderline.

Clinical manifestations depend on the relationship of the bacillus and the host immune response. Therefore, patients present a spectrum of clinical and histological characteristics, ranging from multibacillary form, which corresponds to lepromatous leprosy with a high bacillus load in tissues, to the paucibacillary form or tuberculoid leprosy, in which the bacillus is rarely found in skin smears.[2]

Host genetic factors including major histocompatibility complex (MHC) polymorphisms influence both susceptibility to leprosy and also to leprosy type.

Recent studies have implicated variation in the vitamin D receptor (VDR) gene in susceptibility to several diseases, including osteoporosis and pulmonary tuberculosis.[3] Putative polymorphisms at the VDR gene, which potentially modifies VDR mRNA stability and/or activity, have been implicated in susceptibility to intracellular pathogens. The vitamin D receptor (VDR) is traditionally described as responsible for calcium regulation and bone metabolism mediated by 1,25-dihydroxyvitamin D3.[1] Few studies have reported on the interaction between the VDR gene polymorphism and leprosy susceptibility in other countries; however, there are evidence of interpopulation heterogeneity[4] suggesting that variability may also be observed in associations with infectious diseases, perhaps related to variation in calcium intake or other gene-environment interactions,[3] and studies in other populations will be required to determine the generality of these VDR-leprosy associations.

We report herein an analysis of the Vitamin D receptor gene polymorphism on the susceptibility and spectrum of leprosy confirmed by slit-skin smear examination and histopathologically among Filipinos seen at the Department of Dermatology of the Jose R. Reyes Memorial Medical Center. The VDR gene polymorphism can be used as a predicting tool among persons without symptoms or manifestations of leprosy especially those in contact with diagnosed leprosy patients.

MATERIALS AND METHODS

Subjects

An Institutional Review Board / Institutional Ethics Committee of the Jose R. Reyes Memorial Medical Center approved the study prior to its initiation following the guidelines of good clinical practice. Sixty (60) subjects were included in the study. A total of 43 (71.67%) patients who were diagnosed case of leprosy by acid-fast bacilli in slit-skin smear examination with a spectrum of tuberculoid and lepromatous type. Tuberculoid leprosy was defined by the presence of asymmetrical well-defined lesions with a dry surface and the absence of detectable acid-fast bacilli. Lepromatous leprosy was defined by large number of of skin lesions and with numerous acid-fast bacilli in slit-skin smears. Both conditions were confirmed by histopathology and fite-faraco stain. Fifteen (15) patients (25%) were classified as tuberculoid type while 28 (46.67%) patients were of the lepromatous type. A total of 17 (28.33%) patients in the control group composed of healthy volunteers unrelated to patients. The mean age for all the subjects was 34.92 years with a standard deviation of 8.54 years. There were 31 (51.67%) males and 29 (48.33%)were females (Table 1).

Table 1. Demographic data of patients seen at the Jose R. Reyes Memorial Medical Center, 2014.

Subjects (n=60)	Patients	Age (mean years)	Gender M:F
Control	17 (28.33%)	35.58 +/- 8.03	8:9
Hansen's Disease	43 (71.67%)		
Tuberculoid	15 (25%)	36.533 +/- 7.51	7:8
Lepromatous	28 (46.67%)	33.64 +/- 9.42	16:12

Vitamin D Receptor Genotyping

DNA was isolated from white blood cells using the phenol/chloroform method (Sambrook et al., 1989) and was used for PCR amplification. The primers 5′-CAGAGCATGGACAGGGAGCA-3′ and 5′-GGTGGCGGCAGCGGATGTACGT-3′ yielding a product of 352 base pairs in the 61675 and 62026 positions of the VDR gene was used (Goulart et al., 2005). The PCR cycle conditions would be 94^{o}C for 20 seconds, 60^{o}C for 30 seconds, and 72^{o}C for 30 seconds (35 cycles), using 2 m*M* MgCl2, 0.2 m*M* dNTPs, 0.009 m*M* of each primer, 100 ng of DNA, and 1 U of Taq polymerase in a 25-µL reaction (Roy et al., 1999).

VDR gene with the restriction fragment length polymorphism (RFLP) technique was used in genotyping. In the RFLP procedure, PCR amplicons was submitted to TaqI enzyme digestion according to the manufacturer's recommendations. The results was ascertained according to the presence or absence of specific bands visualized after agarose gel electrophoresis (1.5%), ethidium bromide staining, and UV transillumination (Martinez et al., 2006).

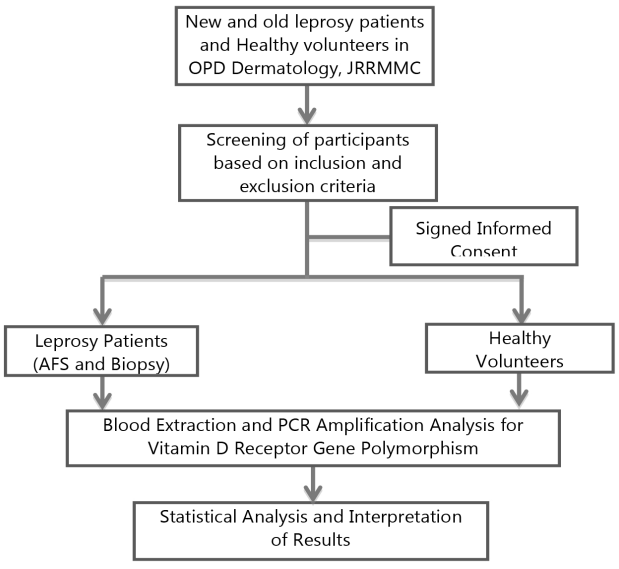
For the primers, it was ordered directly from IDT Tehcnologies which provides primers/ oligonucleotide synthesis for PCR. The PCR was done at the Philippine Genome Center DNA Sequecing Core Facility (DSCF) which is based at the New NIMBB Building, University of the Philippines, Diliman and offers both the extraction of blood and as well as the testing of the gene polymorphism.

To assure the validity of RFLP method, 75 random samples / each sample would be tested twice, and the read-out could be given (98% agreement between the tests). When results for the same sample will be different, a third PCR with that sample will be performed for confirmation.

Statistical Analysis

The raw data obtained was processed using the STATA software version 12. Descriptive analysis of the continuous data was done by getting the mean with standard deviation to describe the demographic profile of all the subjects while proportions was used for the categorical data.

Because the aim of the study was to identify the association between the Taq 1 polymorphisms and Hansen's Disease susceptibility, Chi-square test was used to determine the association of the gene polymorphism with the presence of disease. Also, the strength of association was estimated by crude odds ratios (OR), with 95% confidence interval (95% CI). The statistical significance was accepted if values are $p<0.05$.



RESULTS

The distribution of VDR genotypes at codon 352 in exon 9 is highly significant in between the control, lepromatous, and tuberculoid types of Hansen's disease. 82.35% of the control has heterozygous type (Tt) of VDR while 66.67% of tuberculoid has homozygous recessive type (tt) of VDR and 96.43% of lepromatous type has homozygous dominant type VDR (TT) . Chi-square was done and showed that there was a significant difference in between the control and cases *(X2* = 41.6564, p =<0.0001) while the the tuberculoid and lepromatous showed a significant difference in the VDR genotype (*X2* = 72.2468, p =<0.0001) with odds ratio = 16, interpreted as TT genotype has 16x increased risk among patients with lepromatous spectrum versus control and tuberculoid type, 95% confidence interval [CI = 2.48-22.76; x2 = 10.61, p=<0.001.] Odds ratio of 5.8 was noted in the tt genotype interpreted as 5.8x increased risk among patients with tuberculoid type versus control and lepromatous type, 95% confidence interval [CI = 1.26-18.55; x2 = 21.72, p=<0.001.] (Table 2)

Table 2. Hansen's Disease and Vitamin D receptor genotype

Group N=60	Genotype			Total	
	TT	**Tt**	**tt**		p-value
Control	2 (11.76%)	14 (82.35%)	1 (5.88%)	17	<0.0001
Tuberculoid	5 (33.33%)	0	10 (66.67%)	15	<0.0001
Lepromatous	27 (96.43%)	1 (3.57%)	0	28	<0.0001

***** *There was significant difference observed between the HD group and control. Fstat = 31.32, p <0.001. Post hoc analysis of genotypes between the clinical groups showed that tuberculoid vs lepromatous had significantly different genotypes (p=< 0.001) and Lepromatous type of HD vs control had significantly different genotypes (p=< 0.001).*

The significant difference in between the VDR genotype among the groups seen in this study provided an evidence that Vitamin D receptor gene plays a role in the immune response and regulates one's immune system in vulnerability to infectious disease

such as Hansen's Disease among Filipino patients. Studies have shown that deficiency in Vitamin D has an association to susceptibility to tuberculosis at low concentrations of 1, 25 hydroxyvitamin D3 as it weakens the human macrophages. Studies from different countries have shown that VDR with leprosy and its type has a controlling effect in the VDR pathway on leprosy. The results in this study showed the same outcomes as done among leprosy patients of the same studies where the genotype homozygous (TT) was highly related among lepromatous type and the heterozygous (Tt) and homozygous (tt) was highly associated with control group and tuberculoid type of Hansen's disease correspondingly.

The polymorphism was located around the ~260th base. The expected frequency of fragment length for all of the sequences were 352 bp. VDR sequence-specific oligonucleotides for T (5′-GCGCTGATTGAGGCCATC-3′) and t (5′-GCGCTGATCGAGGCCATC-3′) alleles were used (Roy, et al 1999). The control group (healthy volunteers) did not show the T (5′-GCGCTGATTGAGGCCATC-3′) and t (5′-GCGCTGATCGAGGCCATC-3′) alleles.

Homozygous (TT) was highly associated with the lepromatous spectrum of leprosy where T (5′-GCGCTGATTGAGGCCATC-3′) was seen but t (5′-GCGCTGATCGAGGCCATC-3′) alleles was not noted (Figure 1). Homozygous (tt) was highly associated with the tuberculoid type of leprosy where T (5′-GCGCTGATTGAGGCCATC-3′) was not seen but t (5′-GCGCTGATCGAGGCCATC-3′) alleles was noted (Figure 2).

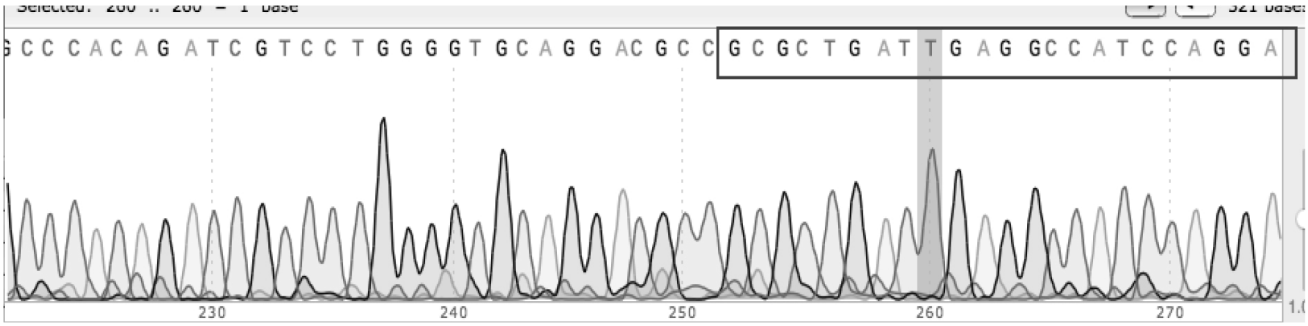


Figure 1: Homozygous TT, T (5'-GCGCTGATTGAGGCCATC-3') was seen and t (5'-GCGCTGATCGAGGCCATC-3') alleles was not seen in this sequence.

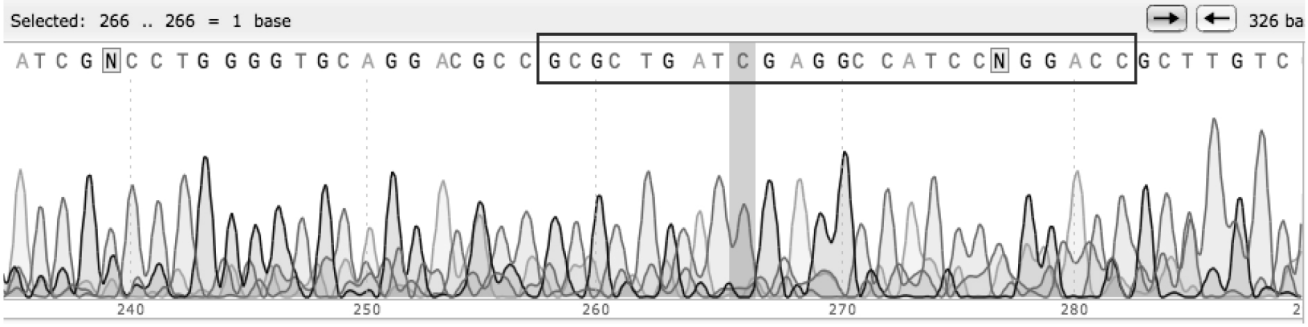


Figure 2: Homozygous tt, t (5'-GCGCTGATCGAGGCCATC-3') alleles was seen and T (5'-GCGCTGATTGAGGCCATC-3') was not seen in this sequence.

DISCUSSION

The association between VDR genotype and susceptibility to leprosy observed in this study provides further evidence for the proposal that the vitamin D receptor may be an immune response gene regulating susceptibility to infectious disease in humans. There is epidemiologic evidence linking vitamin D deficiency and susceptibility to tuberculosis and in vitro data indicating an effect of the active metabolite of vitamin D (1,25 D3), on mycobacterial growth.[3] Addition of physiologic concentrations of 1,25 D3 impairs growth of *Mycobacterium tuberculosis* in human macrophages and monocytic cell lines.[5] Such in vitro studies are not possible for *Mycobacterium leprae,* but early studies of the treatment of leprosy with medications containing vitamin D are consistent with a possible immunomodulatory effect on this bacterium.[6]

This case-control study identifying associations of VDR with leprosy and leprosy type in Filipino patients provides evidence for a modulatory effect of the VDR pathway on leprosy as well as further data supporting the functional relevance of variation in or near the VDR gene. The differential association of VDR genotype with leprosy type is of particular interest in relation to the genetic control of cellular and humoral immune responses.[7] Leprosy provides a model for understanding human immune responses to infection in that the disease presents as a spectrum in which the clinical manifestations correlate with the level of cell-mediated immunity to the bacterial pathogen.[1, 3] Tuberculoid spectrum presents with strong cellular immune response with skin lesions that contain well-organized granulomas with very few bacilli. In contrast, lepromatous leprosy patients have a stronger humoral immune response but a weak cellular response with bacilli-laden macrophages.[8]

Increasing evidence indicates that tuberculoid leprosy is associated with a predominantly Th1-type pattern of cytokine production in T cells from skin and peripheral blood and conversely, lepromatous leprosy is associated with a more Th2-shifted pattern of cytokine production.[9, 10] There is considerable interest in identifying the genetic and other factors that determine whether a Th1- or Th2-type response is made to an antigen or foreign pathogens, as this often correlates with resistance or susceptibility. These leprosy data suggest that one of the genetic factors influencing this Th1-Th2 shift in humans may be VDR genotype, with tt homozygotes tending to produce a TH1-type immune response and TT homozygotes producing a Th2-type response. This possibility is compatible with the observation that tt homozygotes have been found to be strongly correlated with the tuberculoid spectrum of leprosy and TT homozygotes were related to the lepromatous type, as seen in this study. Of interest, in leprosy, the tt genotype is associated with tuberculoid type characterized by a strong cellular response but not with resistance to leprosy per se, which was associated with heterozygosity for VDR genotype.

The role of the VDR gene in regulating the immune response may have a potential application with regard to alternative diagnostics and therapeutics that could solve the public health problem that leprosy still represents. Moreover, the genetic association study of the VDR gene polymorphism with infectious diseases like leprosy and tuberculosis can be a starting point for a better understanding of these diseases.

CONCLUSION

The polymorphism of VDR genotypes at codon 352 in exon 9 was highly significant in between the control, lepromatous, and tuberculoid type of Hansen's disease. Homozygous TT was highly associated with lepromatous type (odds ratio = 16, $p =< 0.001$), and homozygous tt is highly related to the tuberculoid type (odds ratio = 5.8, $p =< 0.001$).

These data suggesting that VDR genotype may influence the Th1-Th2 pattern of immune response in leprosy encourage assessment of the role of this genetic locus in a wide variety of human infectious and autoimmune diseases.

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