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The Status of Vitamin D and Its Receptor *Fok I* Gene Polymorphism Among Adult TB Patients of Mulago Hospital Kampala Uganda

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Abstract

Tuberculosis (TB) is an infectious disease causing severe problems throughout the world. However this disease is prevalent in Africa. The antimicrobial activity of vitamin D may plays a significant role in prevention of TB. The vitamin D receptor gene is associated with susceptibility. The functional Single Nucleotide Polymorphism rs2228570, *Fok I* gene has been found to be inconsistent among the TB patients. Additionally, patients with active TB have significantly lower vitamin D serum concentration than their contacts from the same ethnicity. This study investigated the relationship between vitamin D status and Vitamin D receptor *Fok I* gene polymorphism, in TB patients. A pilot case-control study was conducted in 41 newly diagnosed Tuberculosis patients and 41 non-TB healthy workers enrolled between April and June, 2013 at Mulago National Referral Hospital. Levels of Vitamin D and PTH were analyzed by Electrochemiluminescence using Cobas 6000. Genotyping of *Fok I* gene was done by Polymerase Chain Reaction and direct sequencing method using the ABI Sequencer. The Prevalence of hypovitaminosis in the TB patients was 24.4% and 26.8% in the healthy subjects. Vitamin D deficiency in TB patients was 9.7% and 4.9 in healthy subjects respectively. Severe vitamin D deficiency was only found in the TB patients. Subjects with vitamin D deficiency were only found in the FF genotype. There was no significant difference between vitamin D levels among the *Fok I* gene variants ($p = 0.78$). Although there was no significant difference between vitamin D status in the different genotypes of Vitamin D receptor *Fok I* gene polymorphism among TB patients and controls in this Ugandan cohort, the hypovitaminosis D noted dominantly in the FF genotype should not be under estimated. On the other hand optimal levels of vitamin D were predominantly found in both groups.

Keywords: *VDR; Polymorphism Fok I; vitamin D levels; Tuberculosis; Uganda*

Abbreviations: BLAST: Basic local alignment search tool; CI: Confidence Interval; DNA: Deoxy Ribonucleic Acid; EDTA: Ethylenediaminetetraacetic acid; HIV: Human immune deficiency Virus; HS: Healthy subjects; NCBI : National Centre for Biotechnology Information; ng/ml: Nanograms per milliliter; PCR: Polymerase Chain Reaction; PTH: parathyroid hormone; SNP: Single nucleotide polymorphism; TB: Tuberculosis TEA: Tris base, acetic acid; VDR: Vitamin D Receptor;

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Introduction

Tuberculosis (TB) is a severe infectious disease throughout the world but more prevalent in Africa. [1] Among the African countries Uganda remains among the 41 high TB/Human Immunodeficiency virus (HIV) burden countries. This is an indication that TB still remains a major public health problem in Uganda. The cause of reactivation from latent to active disease is still unclear [2]. Genetic predisposition to TB has been suggested in several studies [3-4] but the immunologic association of the genetic polymorphisms still requires further elucidation [5]. Vitamin D hypovitaminosis is common globally and studies have associated it with higher risk of active TB disease [6]. The VDR has been linked to TB susceptibility although findings have not been comparable across populations[7-8]. A diversity of polymorphisms have been found in the VDR gene and its polymorphic haplotypes have been associated with TB susceptibility or resistance [2,6,8,9]. Among these the functional SNP rs2228570, *Fok I* gene has been inconsistently represented [6-8] In the Ugandan population the frequency distribution of *Fok I* FF homozygous genotype was 87.6% in the pulmonary TB patients and this was not different from the control group [6]. Additionally, patients with active TB have significantly lower vitamin D serum concentration than their contacts from the same ethnicity [6,10]. However a recent study documented controversial findings in which the TB patients had optimal vitamin D levels [11]. An earlier vitamin D study that was done in Ugandan TB patients reported a high vitamin D deficiency of 44.2% [12]. According to another study in Africa, the relationship between *Fok I* gene and low vitamin D serum levels may increase the risk of active TB [13]. However no study had reported on the vitamin D receptor *Fok I* gene polymorphism and its association with vitamin D status in TB patients in the Ugandan population. This study investigated the association of vitamin D status and the VDR *Fok I* gene polymorphism in susceptibility to pulmonary TB in the Ugandan population.

Materials and Methods

Study Setting, Design, Population and Sampling Procedure

A case-control study in newly diagnosed smear positive TB patients and healthy controls was conducted between the months of April and June 2013, at the Assessment Centre of Old Mulago National Referral and Teaching Hospital with a 1500 bed capacity located north of Kampala, Uganda. Adult Patients between the age 15-60 who presented with symptoms of TB were tested for TB and were enrolled by consecutive sampling procedure.

Laboratory Analysis

HIV screening was done using Determine Rapid HIV-1/2 assay (Alere Company) on all samples and, HIV-1/2 STAT-PAK Dipstick assay (Chembio Diagnostic Systems Inc, New York, USA) and Uni-Gold assay on the positive samples.

***Fok I* gene polymorphism analysis:** Samples were collected from whole blood in an EDTA vacutainer and stored at 2-8°C until the sample size was obtained for vitamin D receptor gene sequencing. *Fok I* gene polymorphism was determined by PCR-direct sequencing as described previously [6]. Extraction of human genomic DNA was done by the column extraction using Genotype DNA isolation kit version 2.0 (HAIN life science, Germany).Genomic DNA was run on a 1.0% agarose gel in a 1X Tris Acetate EDTA (TAE) buffer containing

ethidium bromide for 1 hour at 120 volts and visualized under UV transilluminator. The primer sequences used in our study were forward 5'-AGC TGG CCC TGG CAC TGA CTC TG TCT –3' and reverse 5'- ATG GAA ACA CCT TGC TTC TTC TCC CTC-3' as described by Harris., *et al*, [1997]. PCR was performed in 15.1 µl reaction volumes which contained 7 µl of PCR water, 1 µl of Mgcl2, 1 µl of master mix 1 µl each of the forward and reverse primers. Pre heated Taq polymerase of 0.1 µl and finally 5 µl of pure DNA were added and the reaction was thoroughly mixed. The PCR GTQ Thermocycler program involved an initial denaturation at 95ºC for 5 minutes, followed by 30 cycles 70ºC annealing at for 45 seconds, elongation at 72ºC for 1 minute , for and a final elongation at 72ºC, for 10 minutes. Amplified DNA was purified using an extraction kit (JET quick company, USA) according to manufacturer instructions. Cycle sequencing PCR was done using Big Dye Terminator KIT version 3, in the Thermocycler Gene Amp PCR system 9700. The cycling program involved denaturation at 96ºC for 1 minute for 1cycle ,annealing at 96ºC for 10 minutes 30 cycles, elongation at 70ºC for 30 seconds and a final elongation at 60ºC for 5 minutes. The Dye EX 2.0 spin kit [250] (QIAGEN, Germany), was used to remove dye and other PCR products following the manufacturer's instructions. Sequencing of the PCR products was done using the ABI Big Dye Termination Kit (Applied Biosystems, USA) and the ABI Prism 310 Genetic Analyzer (Applied Biosystems). Sequences obtained were compared to those in the Gene Bank database by applying BLAST; NCBI tool. Identification of Fok I gene polymorphism was done by DNA sequence assembler software version 4.7.0.

Vitamin D measurement: Samples were collected in a plain vacutainer and serum was obtained by centrifugation and stored at -20°C and analysis was done in one batch. Serum 25 (OH) D was determined by electrochemiluminescence using Elecsys vitamin D3 assay according to the manufacturer's instructions. Briefly, serum samples were left to stand at room temperature for six hours before the analysis was done. Vitamin D was determined by the electrochemiluminescence binding assay with the COBAS 6000 immunoassay analyzer. Materials and reagents for the assay included pretreatment reagent 1 which had 1 g/L of dithiothreitol at a pH 5.5. Pretreatment reagent 2 had sodium hydroxide, streptavidin coated micro particles, vitamin D binding protein and 25-hydroxyvitamin D biotin. Other materials were vitamin D total CalSet, Precicontrol varia 1 and 2, sample diluent, a procell and clean cell, CC cups and other accessories for the COBAS analyzer. Before sample analysis, the equipment was calibrated and quality control varia was run. Three hundred microliters of sera were pipetted into the CC cups which were arranged in Calsets according to the study number. The study numbers were carefully registered in the analyzer system. The samples were then loaded and analysis began for the duration of 30 minutes. Tests out of range were automatically repeated or analyzed by auto dilution. Results were determined via a 2 point calibration curve and a master curve provided via the reagent barcode. Reagents barcodes were loaded and the machine automatically calculated the analyte in each sample. Results were displayed in the test review window and were printed out.

Parathyroid Hormone Estimation: After analysis of vitamin D, a purposive sampling was done to select ten samples, of which five had low levels and the other five had high levels of vitamin D. Among the ten samples, one sample did not have enough serum therefore 9 of the selected samples were further analyzed for PTH. Analysis of parathyroid hormone was done in the Clinical Chemistry laboratory using automated Cobas 6000 (Roche Diagnostics).

Statistical Analysis

Vitamin D was categorized into three groups; 1- the deficient which was defined as serum vitamin D concentration ≤ 20 ng/ml. 2 - the insufficient group was defined as vitamin D concentration ≤ 30 ng/ml and 3- the sufficient group with ≥ 30 ng/ml vitamin D concentrations. This has been the acceptable level according to the international standards [14]. Data was entered in Excel and statistical analyses were performed using STATA (Stata Corp. STATA 12.0, College Station, Texas, USA). Categorical variables were summarized into frequencies and percentages. Continuous variables were summarized using means and standard deviations. Mantel-Henzel odds ratios were used to measure the level of association between vitamin D concentration levels in the three categories of deficient (≤ 20 ng/ml), the insufficient (21-29 ng/ml) and the optimal (≥ 30 ng/ml) with the independent variables in the two categories of TB-patients and healthy subjects. This data was summarized into odds ratios, 95% confidence interval (95% CI), and alpha of $p < 0.05$ was considered significant. A t test was used to compare means and the Spearman's test was used to determine correlation of *Fok I* gene and vitamin D-

status, parathyroid hormone levels and vitamin D levels. A logistic linear regression analysis was done to measure the association between the vitamin D and *Fok I* gene.

Ethical Consideration

Permission to carry out the study was obtained from the Research and Ethics Committee of Mulago Hospital, ref MREC: 329, the Institutional Review Board of School of Biomedical Sciences Higher Degrees Research and Ethics Committee, ref SBS 108, and from the Uganda National Council of Science and Technology Ref No.1431. Before investigations the study participants were interviewed by the Principal Investigator and the research assistants, informed consent was obtained from the study subjects. Patients' personal information was kept confidential by using serial codes instead of names on the questionnaire.

Results

Vitamin D Status Among TB Patients and Healthy Subjects (Hs) Across Social Demographic Characteristics

A total of 82 serum and whole blood samples analyzed. Forty one of these were from pulmonary TB patients while the others were from healthy subjects. There were less of HIV/TB patients in this study (24.4%) compared to the non HIV and the control group had fewer HIV participants. However the vitamin D levels of the HIV positive was slightly higher than the HIV negative in the 82 participants although the difference was not significant [Table 1]. Vitamin D levels were compared between genders and the mean serum concentration in the males was lower than that of the females with a borderline significance observed, inter quartile range of 17.4 ng/ml and p value of 0.06, Table 1. Vitamin D levels did not decline with age p value 0.95. Other lifestyle factors did not show significant variation [Table 1]. However there was a significant variation in vitamin D mean serum levels between the employed with 38.6 ± 11.6 and unemployed 32.4 ± 11.0, p value of 0.039

Variables	N (%)	25 (OH)D mean SD ng/ml	P value
Age 18-25 26-35 > 60	 18 (21.9 %) 31 (37.8 %) 33 (40.2 %)	 38.9 ± 8.0 34.8 ±13.3 38.3 ± 11.8	 0.95
Male Female	54 (66%) 28 (34%)	35.4 ± 12.3 40.5 ± 9.6	0.06
Baganda Non Baganda	54 (66%) 28 (35%)	38.1 ± 11. 5 35.4 ± 12.1	0.32
Single Married	37 (45%) 45 (55%)	37.1 ±10.2 37.2 ± 12.7	0.99
Home shelter Small house Large house	 38 (46%) 44 (54%)	 36.3 ± 10.7 37.8 ± 12.5	 0.61
Alcohol use Non alcohol	6 (7.3. %) 76 (92.7%)	37.0 ± 16.6 37.1 ± 11.3	0.98
Smokers Non smokers	4 (5%) 78 (95%)	36.02 ± 19.3 37.2 ± 11.3	0.84
HIV positive	13 (16%)	38.5 ± 15.1	0.66
Non HIV	69 (84%)	36.9 ± 11.0	
Employed Unemployment	62 (75.6%) 20 (24.4%)	38.6 ± 11.6 32.5 ± 11.6	0.039

Table 1: *Vitamin D levels according to social demographic status among all subjects. Values are means, standard deviations, percentages and p values.*

Vitamin D Status in TB Patients and Hs

The mean serum concentration of 25 (OH) D was 37.1 ± 11.7 ng/ml for all subjects (Data not shown). The overall hypovitaminosis D of both TB patients and healthy subjects n = 21 was 25.6%. The Prevalence of hypovitaminosis D in the TB patients n = 10 was 24.4% while that of normal healthy subjects n = 11 was 26.8%. The overall prevalence of optimal levels of vitamin D in the study subjects n = 61 was 74.4%, the overall prevalence of insufficiency levels, n = 15 was 18.3% and vitamin D deficiency in the overall population n = 6 was 7.3%. Among the TB patients, 75% had vitamin D optimal levels (≥ 30 ng/ml). Severe vitamin D deficiency (levels < 10 ng/ml) was only found among the TB patients n = 2, 4.9%. And the prevalence of vitamin D deficiency in TB patients, 9.76% was higher than that in the healthy Subjects as shown in table 2. Prevalence of insufficient levels in the HS was 21.9% and this was higher than that of the TB patients.

Category	TB n = 41 (%)	HS n = 41 (%)	Total n = 82 (%)
Deficiency	4 (9.76%)	2 (4.9%)	6 (7.3%)
Insufficiency	6 (14.6%)	9 (21.9%)	15 (18.3%)
Optimal	31 (75.6%)	30 (73.2%)	61 (74.4%)
Other categories			
Severe vitamin D deficiency	2 (4.9%)	0 (0.0%)	2 (2.4%)
Hypovitaminosis	10 (24.4%)	11 (26.8%)	21 (25.6%)

Table 2: *Vitamin D status in TB patients and healthy subjects.*
Values are frequencies and percentages.

Vitamin D Levels and Parathyroid Hormone In TB Patients And Hs

When a comparison of Vitamin D and parathyroid hormone was done using spearmans correlation. Nine samples were analyzed and there was a strong positive correlation rho 0.76 between Vitamin D and parathyroid hormone.

Association Between *Fok I* Gene Polymorphism and Vitamin D Levels In TB Patients And Hs

The identification of polymorphism of *Fok I* gene sequences was done by the bioinformatics DNA sequence assembler software which aligned recognition sites of 5'-GGATG/3'-3'CATCC-5'. Among the healthy subjects the heterozygous and recessive homozygous genotypes no vitamin D hypovitaminosis subjects were noted. However, majority n = 19 (90.5%) hypovitaminosis D subjects were among the FF genotype. [Table 3a and 3b] . when a logistic regression analysis was done to determine the association of *Fok I* gene polymorphism and vitamin D status no statistically significant association was found (p value 0.78) as shown in Table 4. A spearman correlation test was used to examine the correlation between *Fok I* gene polymorphism and vitamin D levels and in the insufficiency group there was a weak negative association of -0.4330 and in the optimal levels group the association was weaker with -0.1470 correlations.

Genotype	Deficiency > 20 ng/ml	Insufficiency 21-29 ng/ml	Sufficiency ≤ 30 ng/ml	Total N = 41
FF	3 (7.3%)	6 (14.6%)	27 (65.9%)	36
Ff	0 (0%)	0 (0%)	3 (7.3%)	3
ff	0 (0%)	0 (0%)	2 (4.9%)	2

Table 3a: *Correlation of Fok I genotypes and vitamin D status among healthy subjects.*
Vitamin D levels across the three categories.

Genotype	Deficiency > 20 ng/ml	Insufficiency 21-29 ng/ml	Sufficiency ≤ 30 ng/ml	Total N = 41
FF	2 (4.9%)	8 (19.5%)	28 (68.3%)	38
Ff	0 (0%)	1 (2.4%)	0 (7.3%)	1
ff	0 (0%)	1 (2.4%)	1 (2.4%)	2

Table 3b: *Correlation of Fok I genotypes and vitamin D status among TB patients. Vitamin D levels across the three categories.*

Genotype	25 (OH) D ng/ml mean	P value
FF	37.20 ± 1.9	0.78
Ff	38.85 ±8.4	
ff	34.46 ±12.7	

Table 4: *Logistic regression analysis of vitamin D levels and Fok I genotypes in TB patients and NHS. Means and standard deviations and p value .*

Discussion

In this study, the level of Vitamin D deficiency in pulmonary TB patients was 9.7%. It is comparable to a study among HIV without TB 10% and those with TB and HIV co infection was 12% deficient and none of the healthy controls in western Uganda [15]. However this study used a 12 ng/ml definition of vitamin D deficiency. It is also similar to a study in newly diagnosed TB patients in Tanzania who had 9.8% deficiency [16]. Our results, however differ from those of a more recent Ugandan study that reported a higher prevalence of 44.2% vitamin D deficiency in TB patients admitted to ward at Mulago hospital [12]. The variations in these studies may be due to different study settings and participants. The hospitalized patients could have been more malnourished and had a lack of sun shine exposure for a longer period in the latter study than the out patients in the present study.

Our obtention of no significant difference in vitamin D hypovitaminosis in TB patients and healthy subjects is quite interesting and needs further exploration. The underlying cause of this hypovitaminosis needs to be found. None the less both TB patients and healthy subjects had optimal vitamin D levels and the mean serum concentration of the study was above 30 ng/ml. similarly a more recent study in Zimbabwe among active pulmonary TB patients has been published with such findings. Both our study and this study found vitamin D mean serum levels to be above 30 ng/ml. This situation may be caused by genetic metabolic handling of vitamin D in individuals and raises interest in the bioavailability of vitamin D.A similar situation was noted in a West African study [17]. Like the Zimbabwe study again more optimal levels were found among TB patents than the control according to Table 3a and 3b [11]. A study in Indonesia also found no significant difference among vitamin D levels of the TB patients and cases [18]. The other aspect to consider is the cutoff recommendations which have not been consistent over the years. This study used the recommended cut off of the US Endocrine Society Guidelines which considers vitamin D deficiency as values less than 20 ng/ml and insufficiency as vitamin D between 21 to 29 ng/ml but recommends the safety margin to be 100 ng/ml and in this study none of the participants had level up to 100 ng/ml.

Noteworthy though no study has reported the optimal levels of vitamin D in the Ugandan healthy population, levels of which are said vary from population to population [19]. A study in 5 East African ethic groups like Masai reported a serum level of 46 ng/ml because of their lifestyle. Currently the vitamin D council has come up with a new recommendation of a lower acceptable limit of 40 ng/ml. Blood

levels above 60 ng/ml are now considered the optimal limit and 80 ng/ml is considered the safe upper limit. If such a cutoff is considered then there is a need for a study to identify the serum levels of the normal Ugandan population.

Our study did not find a significant association between *Fok I* gene polymorphism and Vitamin D levels in both TB patients and controls, (p 0.78). This finding is in agreement with a recent Egyptian study [20]. This observation is also comparable to that of a study from an African population of the Venda in South Africa [21] who reported no difference between the TB patients and controls. In a West African Gambian study [7] there was no association observed between vitamin D polymorphism and TB susceptibility however suggested associations based on haplotypes rather than individual genotypes of VDR. Studies of VDR *Fok I* polymorphism in relation to tuberculosis and other diseases have been inconsistent across populations of the world these include a predisposing role for *Fok I* FF while others report a protective factor against TB disease. The recent Ugandan study on *Fok I* gene polymorphism among TB patients did not clearly find an association of TB and VDR, however the heterozygous Ff was only found among TB patients and not the controls [6]. Based on these studies, our findings confirm the inconsistence of findings of VDR across populations. Furthermore, we confirm the dominance of the homozygous FF genotype in the African population. However, this genotype has a high transcriptional activity and high utilization of vitamin D3 which may give a clue on how *Fok I* gene influences vitamin D status and TB disease in blacks. On the contrary majority of our participants both TB and non TB had optimal levels of vitamin D. The free hormone hypothesis where the biologically active hormone is thought to be the free and bioavailable portion may therefore, be necessary to identify genetic associations.

Conclusion

Although there was no significant difference between vitamin D status in different genotypes of *VDR Fok I* gene polymorphism among TB patients and controls in this Ugandan cohort, the hypovitaminosis D noted dominantly in the FF genotype should not be under estimated. On the other hand optimal levels of vitamin D were predominantly found in both groups. Therefore there is need to elucidate the relationship between vitamin D status and *Fok I* haplotypes in a wider population. Furthermore, the odds of having high levels of vitamin D and TB disease may be another subject for debate.

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