Introduction

The most common microvascular complication of type 2 diabetes mellitus (T2DM) is diabetic foot with the prevalence around 25% [1], [2]. The process is initiated by chronic hyperglycemia, endothelial dysfunction, and cytokines secretion which result in chronic inflammation, decreasing nitric oxide (NO) production, and triggering atherosclerosis [2], [3], [4]. Clinically, skin lesions found in diabetic foot patients can develop into gangrene and chronic inflammation, with an increased risk of secondary infections, and a 2% chance of amputation [5]. Therefore, disruption of patient movement and reduction of individual activity lead to a disabling condition.

Vitamin D, known as the sunshine vitamin, has been understood as an important key for controlling inflammation of endothelial cells, through Vitamin D receptors (VDRs). Hypo vitamin D and VDR polymorphisms are strongly associated with diabetes and cardiovascular diseases [6], [7], [8], [9]. The genetic role of Vitamin D in T2DM has been well-demonstrated, and different variants of VDR gene FokI are associated with T2DM [10], [11], [12], [13]. The human VDR genes are located in chromosome 12q12-q14, and the FokI polymorphism (ATG-ACG) are in the exon 2 of the gene, with a unique function to change the structure of the VDR protein and then produce two different protein variants. FokI gene with f allele (T amino acid) encodes 427 amino acid proteins while the F allele (C amino acid) encodes 424 amino acid proteins [11], [14]. It makes the shorter variant increases its binding capacity to 1, 25-dihydroxyvitamin D [15].

The higher level of Vitamin D might enhance pancreatic β-cell secretion function and improve insulin resistance [12]. However, the biological association between the absent of allele in FokI polymorphisms and vascular diseases is still limited.
the susceptibility to T2DM cannot be clearly determined due to the wide variety of study methods [16], [17]. This study aimed to determine the status of Vitamin D and to detect the FokI VDR polymorphisms among diabetic foot patients in sunlight-rich areas.

Materials and Methods

Research ethics
Ethics clearance was approved by the Ethics Committee of Medical Faculty of Andalas University (No: 297/KEP/FK/ 2016). Written informed consent was obtained from all subjects before the start of the study, after they got information about the procedures.

Patients
This is part of the study on the effect of Vitamin D supplementation in diabetic foot patients. Diabetic foot outpatients treated at Ibnu Sina and Dr. Rasyidin General Hospital, aged 40–65 years, had HbA1c level >6.5% (normal <6.5), and ankle-brachial index (ABI) between 0.4 and 1.2 (normal 0.9–1.3) were included in this study. Diagnosis and clinical treatment of T2DM were performed by internists or endocrinologists. T2DM patients with arterial complications: Occlusion, bleeding, and with organ diseases that affected Vitamin D metabolism such as osteoporosis, hypoparathyroidism, the complication of diabetic or hypertension, ankle lesions or ulcers, infection, and sepsis were excluded from the study.

Samples
Sample collections began with initial selection through patients medical records. Subjects fulfilled the inclusion and exclusion criteria were selected consecutively. A questionnaire for symptom-based screening and ABI examination was performed by vascular surgeons. Demographic data on age, gender, body weight, and height were recorded. Fasting blood glucose, HbA1c, Vitamin D levels, and VDR FokI genetic polymorphisms were examined. Vitamin D serum levels were measured by electroimmunoassay (ECLIA) using a COBAS analyzer at Biomedical Laboratory of Medical Faculty of Andalas University and were classified as sufficiency >30 ng/mL (75–80 nM), insufficiency 20–30 ng/mL (75–80 nM), and deficiency <20 ng/mL (50 nM) [18].

Polymerase chain reaction (PCR)
DNA isolation, amplification, and restrictions were done to get the PCR results. The isolation process was carried out using the PureLink™ Genomic DNA Mini Kit Invitrogen, through DNA incubation and homogenization at 55°C, followed by centrifuge 1000 g for 10 min to binding, washing with 500 μl wash buffer 1 twice and then DNA eluting.

Furthermore, FokI VDR gene amplification was carried out by PCR process using PCR solutions that consist of 12.5μl Go Tag Green Master Mix (Promega), 1 μl Primer Forward VDR FokI (10 μM), 1 μl Primer Reverse VDR FokI (10 μM), 3 μl DNA, and 7.5 μl Nuclease Free Water, for total volume 25 μl. The PCR cycle conditions were denaturation at 95°C for 3 min, followed by 35 cycles initial denaturation at 95°C for 30 s, annealing at 59°C for 30 s, elongation at 95°C for 30 s, annealing at 59°C for 30 s, elongation at 72°C for 5 min and the last elongation at 72°C for 5 min. Electrophoresis process was been done for 60 min on 1.5% agarose gel resulting PCR product with length 250 base pair (bp).

Restrictions process was done using Restriction Fragment Length Polymorphism-PCR methods with FokI restriction enzyme, forward primer (5’-CAGCTGACTCTGGCTCTGACCGT-3’), and reverse primer (5’-AACACCTTGCTTCTTCCCTCC-3’) [14]. The 3 μl PCR amplicon with 1 μl FokI restriction enzyme, 2 μl Buffer Green, and 24 μl Nuclease Free Water for total restriction reaction volume of 30 μl were digested overnight at 37°C. All digest products were analyzed by electrophoresis on a 1.5% agarose gel for 60 min at 120 V, resulting PCR products with length 192 and 58 bp for homozygous (TT) and 250, 192, and 58 bp for heterozygous samples (TC) and homozygous CC remained uncut. Some of the amplification results were sent for sequencing at Macrogen Laboratory, South Korea.

Data analysis
We analyzed data using SPSS Statistics version 22.0 (IBM, NY, USA). Descriptive statistics was used to describe the characteristics of the study population, including age, sex, disease duration, blood sugar levels, HbA1c, and Vitamin D levels. One-way ANOVA (for normally distributed variables) or Kruskal–Wallis (for non-parametric variables) tests were used to evaluate the difference between explanatory variables across different genotypes of VDR FokI.

Results

Characteristic of study subjects
Characteristics of diabetic foot subjects are shown in Table 1. Of 36 subjects, 52.8% were female, 61.1% <50 years, 63.9% had normal body mass index (BMI), and most of them with normal ABI (86.1%).
Vitamin D deficiency, insufficiency, and sufficiency were observed in 19.4%, 33.3%, and 47.2% of the study subjects, respectively. The majority of the subjects had FokI VDR gene polymorphisms, that is, mutant heterozygous Ff (44.4%) and mutant homozygous FF (41.7%).

Results from one-way ANOVA and Kruskal–Wallis tests showed that there were no differences (p > 0.05) of BMI, ABI, fasting blood glucose, HbA1C levels across VDR FokI genotypes (Table 2).

Table 2: Mean of variables based on VDR FokI genotypes

| Variable (mean) | f (mean ± SD) | Ff (mean ± SD) | FF (mean ± SD) | p-value |
|-----------------|---------------|----------------|---------------|---------|
| BMI (kg/m²)     | 20.8 ± 4.1    | 23.9 ± 4.4     | 23.3 ± 3.9    | 0.335*  |
| ABI             | 1.04 ± 0.09   | 1.06 ± 0.17    | 0.96 ± 0.15   | 0.004** |
| Fasting blood glucose (mg/dL) | 202.8 ± 65.7 | 174.6 ± 74.7 | 194.3 ± 75.2 | 0.345** |
| Vitamin D (ng/mL) | 35.8 ± 13.5 | 28.9 ± 9.4 | 29.0 ± 10.2 | 0.399* |
| HbA1C (%)       | 11.2 ± 2.4    | 10.2 ± 2.5     | 9.9 ± 2.0     | 0.537*  |

Table 2: Mean of variables based on VDR FokI genotypes

After stratifying subjects based on VDR FokI genotypes and Vitamin D levels, we found that most of the Vitamin D deficiency subjects had VDR FokI heterozygous mutant Ff (31.2%) and none of them had homozygous wild type ff (0%) (Figure 1), no statistical significant differences were observed (p > 0.05). When we classified as allele subgroups, all VDR FokI gene with F allele mutant had more frequent Vitamin D deficiency status than f allele (54.8% vs. 40%), on the other hand in subgroup VDR FokI with f allele wild type, Vitamin D deficiency status is not frequent as compare to F allele (45.2% vs. 60%).

Discussion

Diabetic foot is the most common micro vascular complications in T2DM. Factors associated with its development have not been fully understood, but many studies assume that it occurs secondary to micro angiopathy due to late diabetic complications. The involvement of Vitamin D and VDR FokI gene polymorphisms has been suggested in the development of worsening inflammation in T2DM [12], [13].

In the present study, 61.1% of the diabetic foot patients were <50 years old. This is relatively different with results from other studies. Two studies of the Indonesian population in other regions such as Manado and Denpasar reported that the majority of diabetic patients were ≥50 years (78.3%) and 50–59 years (46.9%), respectively [19], [20]. Other reports from India and a meta-analysis also provide data that diabetic foot was predominantly shown in older patients (aged ≥50 years) [21], [22], [23], [24]. This difference might due to the high proportion of patients (33.3%) in our study being diagnosed with T2DM when they were <40 years old. This condition might have resulted in an earlier worsening of vascular endothelium due to the long-term chronic hyperglycemia. Consistent with the other studies, a positive association was shown between foot ulcer and age [25], [26].

In our study, the proportion of females with diabetic foot was higher than males and females were more likely to develop T2DM at an earlier age than males. This is supported by another study in Lampung, Indonesia, showing that the prevalence of diabetic foot females was higher than males (65% vs. 34.7%) [27]. This is possibly due to cultural reasons, given that most of the Indonesian women are housewife, getting married at an early age, and not frequently doing outdoor activities. In addition, most of the Indonesian women are Moslems wearing full-body garments. This lack of exposure to sunlight might prevent the synthesis of Vitamin D, causing Vitamin D deficiency and diabetes. This finding is similar to another study by Malik et al. in the Kashmir valley [28]. In contrast, a meta-analysis showed that males were relatively more frequent to develop T2DM than females (11.2 vs. 9.91%) [29]. Another study also reported that males had 2.2 times higher incidence and prevalence of diabetic foot compared to females, caused by higher physical activity that contributes to diabetic ulcers [30].

In general, diabetic foot patients in our study had normal BMI. Among individuals who had VDR FokI with F allele, BMI values were higher than those with f allele, but no statistical difference was observed. Some studies provide evidence that body size (reflected by BMI) has
relationships with either Vitamin D levels or VDR [31], [32]. VDR is present in adipocytes and plays a role in modulating this active metabolic tissue in obese individuals [33]. Caron et al. reported that Vitamin D was negatively associated with adipocyte size, but this association was only shown in women [34]. This might explain our insignificant difference of BMI between these two VDR FokI alleles, as our study population was dominated by females. However, the relationship between VDR FokI polymorphisms and BMI is not consistent; some studies reported no significant association [31], [32], whereas other studies found an association between both of them [35].

Low Vitamin D levels have been associated with an increase in cytokine concentrations in diabetic foot subjects causing delayed wound healing [36]. In this study, 52.7% of diabetic foot subjects had Vitamin D levels <20 ng/mL (33.3% insufficiency and 19.4% deficiency). A study in multi-ethnic subjects at risk for T2DM also reported similar conditions where Vitamin D levels were significantly associated to insulin resistance and beta-cell dysfunction [37]. These findings highlight the importance of Vitamin D to the development of inflammation in T2DM patients [36], [38].

Our results revealed that the frequencies of both mutant VDR FokI Ff (44.4%) and FF (13.9%) genotypes were higher and tended to have lower Vitamin D levels compared to wild type alleles (ff) among diabetic foot subjects. Several reports on the effect of VDR polymorphisms to basal serum Vitamin D levels due to insulin secretion and necessary to maintain glucose tolerance [39], [40]. There has been known that the FokI single-nucleotide polymorphism is capable of changing the protein structure and produces two different protein variants with different activities [10], [11]. The T allele changing into the C allele is suspected to affect insulin sensitivity [11], [14]. It is possible that genetic variants of the VDR gene may contribute to the development of diabetes [40]. However, it remains inconsistent, since a meta-analysis showed no significant relationship between VDR polymorphisms and risk of T2DM among Asian population [13].

Our study has some limitation that should be acknowledged. As this study only aimed to evaluate the association of Vitamin D levels with VDR FokI polymorphisms, the cause of Vitamin D deficiency cannot be presented. The small number of subjects might have reduced the statistical power of the study and resulted in potential bias. Apart from those limitations above, to the best our knowledge, this was the first study evaluating the association between Vitamin D levels and VDR FokI polymorphism among diabetic foot patient in Indonesia.

Conclusion

Most individuals with diabetic foot tended to have mutant VDR FokI gene polymorphism and have low Vitamin D levels (Vitamin D insufficiency). These findings may highlight the potential role of Vitamin D in diabetic foot complications, even in the sunlight-rich areas of the tropical region like Indonesia. It would have been valuable if we had more information on Vitamin D status of our patients, and this should be considered in future studies.

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