ASSOCIATION BETWEEN VITAMIN D RECEPTOR POLYMORPHISMS (BSMI AND FOKI) AND GLYCEMIC CONTROL FACTORS AMONG PATIENTS WITH TYPE 2 DIABETES

By

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LIST OF ABBREVIATIONS AND SYMBOLS

ABBREVIATIONS

BMI	Body mass index
CI	Confidence Interval
DM	Diabetes Mellitus
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide Triphosphate
EDTA	Ethylenediamine Tetraacetic Acid
FBG	Fasting Blood Glucose
FI	Fasting Insulin
GFR	Glomerular Filtration Rate
HbA1c	Glycosylated Haemoglobin
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance
HWE	Hardy-Weinberg equilibrium
LD	Linkage Disequilibrium
Mg^{+}	Magnesium
MgCI	Magnesium Chloride
OR	Odds Ratio
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
SD	Standard Deviation
SNP	Single Nucleotide Polymorphism
Taq	*Thermophilus aquaticus*

TBE	Tris-borate- Ethylenediamine tetraacetic acid
Tris-HCI	Tris-hydrochloride
T2DM	Type 2 Diabetes Mellitus
VDR	Vitamin D Receptor
25(OH)D	25-Hydroxy Vitamin D (Calcifediol)

SYMBOLS

bp	basepairs
mL	millilitre
mM	millimolar
mmol/L	millimoles per litre
ng/mL	nanograms per milliliter
nmol/L	nanomoles per litre
rpm	revolutions per minute
U	units
µl	microlitre
µM	micromolar
°c	degree Celsius
≥	more or equal than
<	less than

ABSTRAK

PERHUBUNGAN DI ANTARA VARIASI GEN RESEPTOR VITAMIN D (BSMI DAN FOKI) DAN FAKTOR KAWALAN GLISEMIK DALAM KALANGAN PESAKIT DIABETES JENIS 2

Pengenalan:

Beberapa kajian menyarankan bahawa gen reseptor vitamin D (VDR) berperanan dalam mempengaruhi kecenderungan individu menghidap diabetes mellitus jenis 2 (T2DM). Walaupun demikian, hubungan di antara variasi VDR dan T2DM masih belum dapat disimpulkan. Kami mengkaji genotip VDR rs1544410 (BsmI) dan rs2228570 (FokI) polimorfisme dalam kalangan pesakit T2DM di Malaysia dan hubungannya dengan faktor kawalan glisemik (paras vitamin D, kalsium, magnesium, dan fosfat).

Metodologi:

Seramai 189 peserta terdiri daripada 126 pesakit T2DM (63 dengan tahap glisemik terkawal dan 63 dengan tahap glisemik tidak terkawal) dan 63 subjek sihat sebagai kawalan dalam kajian kes-kawalan ini. Semua esei biokimia diukur menggunakan analisis spektrofotometri manakala paras vitamin D dan insulin dianalisis menggunakan teknik imunoesei. Polimorfisme gen VDR FokI dan BsmI dianalisis menggunakan tindak balas rantai polimerase dan pencernaan endonuklease

Keputusan:

Penemuan kami tidak menunjukkan perbezaan yang signifikan dalam genotip VDR FokI dan BsmI antara pesakit T2DM dan subjek sihat. Selain itu, tidak ada perkaitan yang signifikan antara kedua-dua polimorfisme nukleotida tunggal dan faktor kawalan

glisemik. Peserta yang mempunyai tahap glisemik tidak terkawal mempunyai paras serum magnesium yang lebih rendah dan kerintangan insulin (HOMA-IR) yang lebih tinggi berbanding kumpulan lain.

Kesimpulan:

Kajian ini menunjukkan bahawa polimorfisme pada gen VDR BsmI dan FokI tidak mempunyai kaitan yang signifikan dengan T2DM dan tahap kawalan glisemik.

Kata kunci: reseptor vitamin D; diabetes mellitus jenis 2; BsmI; FokI; kawalan glisemik

ABSTRACT

ASSOCIATION BETWEEN VITAMIN D RECEPTOR POLYMORPHISMS (BSMI AND FOKI) AND GLYCEMIC CONTROL FACTORS AMONG PATIENTS WITH TYPE 2 DIABETES

Introduction:

Several studies have suggested that the vitamin D receptor (VDR) gene plays a role in type 2 diabetes mellitus (T2DM) susceptibility. Nonetheless, the association between T2DM and VDR polymorphisms remains inconclusive. We determined the genotype of VDR rs1544410 (BsmI) and rs2228570 (FokI) polymorphisms among Malaysian patients with T2DM and their association with glycemic control factors (vitamin D levels, calcium, magnesium, and phosphate).

Methodology:

A total of 189 participants comprising 126 patients with T2DM (63 with good glycemic control and 63 with poor glycemic control) and 63 healthy controls were enrolled in this case–control study. All biochemical assays were measured using spectrophotometric analysis while vitamin D and insulin levels were measured by immunoassay technique. VDR gene FokI and BsmI polymorphisms were analyzed using polymerase chain reaction and endonuclease digestion.

Results:

Our findings revealed no significant differences in VDR FokI and BsmI genotypes between participants with T2DM and healthy controls. Moreover, no significant association was observed between both single nucleotide polymorphisms and glycemic control factors. Participants with poor glycemic control had significantly lower serum

magnesium levels and significantly higher homeostatic model assessment for insulin resistance (HOMA-IR) compared to the other groups.

Conclusions:

The present study revealed that VDR gene BsmI and FokI polymorphisms were not significantly associated with T2DM and the glycemic control factors.

Keywords: vitamin D receptor, type 2 diabetes mellitus, BsmI, FokI, glycemic control

CHAPTER 1: INTRODUCTION

1.1 Introduction

Type 2 diabetes mellitus (T2DM) is a worrying metabolic disorder which is high in prevalence globally. It was estimated that about 451 million adults aged 18 to 99 years old were suffering from diabetes in the year 2017 worldwide. This was about 8.4% of the global population, and it is increasing rapidly in low- and middle- income countries. Diabetes prevalence is expected to increase to 693 million or about 9.9% of the same population by 2045 [1]. In Malaysia, National Health and Morbidity Survey (NHMS 2019) estimated about one out of five adults or nearly 3.9 million individuals aged 18 years and above have diabetes. The prevalence has increased from 13.4% in 2015 to 18.3% in 2019 [2].

T2DM is a complex, polygenic, and multi-factorial disease. Both genetic predisposition and environmental factors give rise to the development and pathogenesis of T2DM [3]. Accumulating evidence from human and animal studies has linked vitamin D status to insulin secretion and insulin resistance given that both vitamin D and its receptor complex play important roles in regulating the β-cell insulin secretion [4-9]. Furthermore, vitamin D deficiency has been associated with impaired insulin sensitivity, whereas vitamin D replacement in deficient individuals has been shown to improve insulin sensitivity [6, 10, 11]. Similarly, studies have shown that vitamin D receptor (VDR) knockout impaired glucose-induced insulin secretion, whereas vitamin D supplementation improved insulin secretory response [6, 12, 13].

VDRs, which are present in over 38 tissues, control vital genes related to bone metabolism, oxidative damage, chronic diseases, and inflammation [14]. The VDR gene,

located on chromosome 12q13.1, consists of 14 exons (exons 2–9) and 6 untranslated exons (exons 1a–1f), with alternative splicing sites. Four common single nucleotide polymorphisms (SNPs) of the VDR gene are rs2228570 (FokI) in exon 2, rs1544410 (BsmI) and rs7975232 (ApaI) in intron 8, and rs731236 (TaqI) in exon 9 [15]. Earlier studies have shown that several VDR gene polymorphisms, such as BsmI and FokI, alter VDR protein activity [16, 17]. All VDR polymorphisms are located between exons 8 and 9, except for FokI, which is located in exon 2 [17]. VDR polymorphisms are believed to be the primary reason for inherited VDR dysfunction.

The association between VDR polymorphisms and T2DM remains inconclusive. Studies conducted at various locations and involving a diverse group of individuals have observed various genetic VDR polymorphisms. A meta-analysis conducted by Li et al. (2013) [18] among the Asian community revealed a possible link between polymorphisms at the FokI site and the onset of T2DM. Similarly, a study by El Gendy et al. [19] observed significant differences in FokI genotypes and allele distribution between Egyptian patients with T2DM and controls, which could be a risk factor for T2DM. Another study by Ortlepp et al. [20] observed a significant association between the BsmI VDR genotype and fasting blood glucose, while Oh and Barrett-Connor [21] observed a significant association between the VDR 1544410 (BsmI) polymorphism and Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) levels among individuals with T2DM in the Rancho Bernando Cohort.

Conversely, studies by Bid et al. [22] in the North Indian population and Malecki et al. [23] in the Polish population, found no correlation between VDR gene polymorphisms and diabetes at any of the four polymorphic sites. Likewise, a meta-analysis conducted by Yu et al. [24] concluded no significant association existed between BsmI and FokI

polymorphisms and T2DM. Nonetheless, results have varied according to sample size and study population ethnicity.

Hypomagnesemia, hypophosphatemia, hypocalcemia, and vitamin D deficiency are the glycemic control factors that have linked to insulin resistance [25-27]. This condition often precedes the development of T2DM as well as its complications. The mechanism of action of vitamin D in T2DM is thought to be mediated through regulation of plasma calcium (Ca^{2+}) levels, which regulate insulin synthesis and secretion. Among the actions of vitamin D in insulin secretion arises from its effects on Ca^{2+} influx, mobilization, and buffering in pancreatic β-cells [28], independent of glucose level [29]. The effects of vitamin D on intracellular Ca^{2+} in pancreatic β-cells has been linked to plasma membrane and endoplasmic reticular membrane bound vitamin D receptors [30, 31]. Pulsatile insulin release from pancreatic β-cells is related with frequency of Ca^{2+} oscillations. By the same mechanism, insulin release oscillations are proportional to the vitamin D concentration [29, 32].

Although results from previous studies on calcium intake and risk of diabetes are inconsistent [33, 34], the results do indicate a potential link between these two factors. In a large cohort study involving 83,779 middle-aged women, they found that both vitamin D and calcium intakes were inversely associated with development of T2DM, and the benefits of the two nutrients appear to be additive. Both vitamin D and calcium supplements from diet were significantly associated with a lower risk of T2DM [33]. In another cohort study involving 41,186 women, however, higher dietary calcium intake was not associated with a lower risk of T2DM [34].

Hypophosphatemia is thought to be the cause of altered glucose uptake and metabolism, and impaired responsiveness to insulin [35]. However, there is also evidence, that phosphate supplementation in glucose intolerant hypophosphatemic patients

improves glucose tolerance significantly [36]. Phosphate levels influences insulin-dependent glucose uptake and metabolism by several possible mechanisms. The insulin-dependent glucose uptake into target cells is accompanied by translocation of phosphate from the extracellular into the intracellular space [37]. Phosphate is involved in energy balance and ATP generation. Earlier study by Jaedig et al. [37] revealed an infusion of several electrolytes or minerals including phosphate in obese individuals results in increase postprandial thermogenesis [38, 39]. Moreover, *in vitro* studies demonstrated low ATP levels, elevated cytosolic calcium and impaired glycolytic activity in pancreatic islets of phosphate-depleted rats. The phosphate depletion leads to impaired insulin secretion by pancreatic islets. These effects were normalized after phosphate supplementation [38, 40]. In addition, hypophosphatemia may also induce glucose intolerance and resistance to the action of insulin [35].

Despite the widespread clinical evidence of the association of hypomagnesemia and T2DM, the molecular mechanisms of magnesium (Mg^{2+}) on insulin secretion and insulin resistance are still not well understood [41]. Current available evidence supports the effect of Mg^{2+} on insulin sensitivity and insulin receptor phosphorylation. It was shown that insulin resistance associates with reduced serum Mg^{2+} levels in a cross-sectional study of patients with Metabolic Syndrome [42]. Additionally, a cohort study of adult black Americans reported that Mg^{2+} deficiency contributes to an insulin-resistant state [42]. Similar results found induced Mg^{2+} deficiency reduced insulin action and secretion in healthy human subjects [43]. It is generally accepted that intracellular Mg^{2+} plays a role in autophosphorylation of the β-subunits of the insulin receptor and other downstream signal kinases of the target cells [44]. Mg^{2+} enhances tyrosine kinase activity by increasing the receptor's affinity for ATP [45]. Indeed, hypomagnesemia results in a defective tyrosine kinase activity, post-receptor impairment in insulin action, altered

cellular glucose transport, and decreased cellular glucose utilization, which promotes peripheral insulin resistance in T2DM [44].

To date, only one study in the central region of Malaysia has explored the association between the VDR BsmI (rs1544410) polymorphism and vitamin D deficiency, obesity, and insulin resistance among participants without diabetes across different age groups [46]. Accordingly, the aforementioned study had found that the BsmI (rs1544410) polymorphism was associated with increased risk for vitamin D deficiency and insulin resistance among the Malaysian population [18]. However, insufficient studies have investigated the effects of VDR polymorphisms on the modulation of glycemic control factors (i.e., vitamin D, calcium, magnesium, and phosphate levels) in Malaysian patients with T2DM. The current study therefore aimed to determine the possible association between VDR polymorphisms and diabetic phenotype, and obesity, among Malaysian patients with T2DM.

CHAPTER 2: OBJECTIVES OF THE STUDY

2.1 General objective

To determine the genotype of VDR polymorphism among type 2 diabetic patients and non-diabetic control and its association between VDR polymorphism and glycemic control factors in T2DM patients in Hospital Universiti Sains Malaysia (HUSM).

2.2 Specific objectives

1. To determine the association between VDR BsmI (rs1544410) and FokI (rs2228570) polymorphism and insulin resistance among T2DM patients in HUSM.
2. To determine the association between VDR BsmI (rs1544410) and FokI (rs2228570) polymorphism and glycemic controls factors (magnesium level, calcium level, phosphate level and vitamin D status) among T2DM patients in HUSM.
3. To determine the association between VDR BsmI (rs1544410) and FokI (rs2228570) polymorphisms with obesity among T2DM patients in HUSM.

CHAPTER 3: MANUSCRIPT

3.1 Title page

Association between Vitamin D Receptor Polymorphisms (BsmI and FokI) and glycemic control factors among patients with Type 2 diabetes

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3.2 Abstract

ABSTRACT

Aims: Several studies have suggested that the vitamin D receptor (VDR) gene plays a role in type 2 diabetes mellitus (T2DM) susceptibility. Nonetheless, the association between T2DM and VDR polymorphisms remains inconclusive. We determined the genotype of VDR rs1544410 (BsmI) and rs2228570 (FokI) polymorphisms among Malaysian patients with T2DM and their association with glycemic control factors (vitamin D levels, calcium, magnesium, and phosphate).

Methods: A total of 189 participants comprising 126 patients with T2DM (63 with good glycemic control and 63 with poor glycemic control) and 63 healthy controls were enrolled in this case–control study. All biochemical assays were measured using spectrophotometric analysis while vitamin D and insulin level were measured by immunoassay technique. VDR gene FokI and BsmI polymorphisms were analyzed using polymerase chain reaction and endonuclease digestion.

Results: Our findings revealed no significant differences in VDR FokI and BsmI genotypes between participants with T2DM and healthy controls. Moreover, no significant association was observed between both single nucleotide polymorphisms and glycemic control factors. Participants with poor glycemic control had significantly lower serum magnesium levels and significantly higher HOMA-IR compared to the other groups.

Conclusions: The present study revealed that VDR gene BsmI and FokI polymorphisms were not significantly associated with T2DM and the glycemic control factors.

Keywords: vitamin D receptor; type 2 diabetes mellitus; BsmI; FokI; glycemic control

3.3 Introduction

Approximately 50% of the population worldwide still suffers from vitamin D deficiency despite adequate sunlight exposure in Asian regions [1]. Accordingly, the prevalence of vitamin D deficiency has been attributed to weather, clothes, lifestyle, dietary intake, age, gender, predisposing of metabolic syndromes, and genetic heredity, all of which influence the bioavailability of vitamin D [2].

Accumulating evidence from human and animal studies has linked vitamin D status to insulin secretion and insulin resistance given that both vitamin D and its receptor complex play important roles in regulating the β-cell insulin secretion [3–8]. Furthermore, vitamin D deficiency has been associated with impaired insulin sensitivity, whereas vitamin D replacement in deficient individuals has been shown to improve insulin sensitivity [5,9,10]. Similarly, studies on animals and humans have shown that vitamin D receptor (VDR) knockout impaired glucose-induced insulin secretion, whereas vitamin D supplementation improved insulin secretory response [5,11,12].

VDRs, which are present in over 38 tissues, control vital genes related to bone metabolism, oxidative damage, chronic diseases, and inflammation [13]. The VDR gene, located on chromosome 12q13.1, consists of 14 exons (exons 2–9) and 6 untranslated exons (exons 1a–1f), with alternative splicing sites. Four common single nucleotide polymorphisms (SNPs) of the VDR gene are rs2228570 (FokI) in exon 2, rs1544410 (BsmI) and rs7975232 (ApaI) in intron 8, and rs731236 (TaqI) in exon 9 [14]. Earlier studies have shown that several VDR gene polymorphisms, such as BsmI and FokI, alter VDR protein activity [15,16]. All VDR polymorphisms are located between exons 8 and 9, except for FokI, which is located in exon 2 [17]. VDR polymorphisms are believed to be the primary reason for inherited VDR dysfunction.

The association between VDR polymorphisms and type 2 diabetes mellitus (T2DM) remains inconclusive. Studies conducted at various locations and involving a diverse group of individuals have observed various genetic VDR polymorphisms. To date, only one study in the central region of Malaysia has explored the association between the VDR BsmI (rs1544410) polymorphism and vitamin D deficiency, obesity, and insulin resistance among participants without diabetes across different age groups [18]. Accordingly, the aforementioned study had found that the BsmI (rs1544410) polymorphism was associated with increased risk for vitamin D deficiency and insulin resistance among the Malaysian population [18]. However, insufficient studies have investigated the effects of VDR polymorphisms on the modulation of glycemic control factors (i.e., vitamin D, calcium, magnesium, and phosphate levels) in Malaysian patients with T2DM. The current study therefore aimed to determine the possible association between VDR polymorphisms and diabetic phenotype, and obesity, among Malaysian patients with T2DM.

3.4 Methodology

3.4.1 Study participants

This case–control study recruited participants from the outpatient clinic and medical specialist clinic at Hospital Universiti Sains Malaysia (USM), Kelantan. Ethics approval was obtained from the Human Research Ethics Committee of USM (USM/JEPeM/18100498). Written informed consent was obtained from each study participant after explaining the objectives and potential benefits of the study. Cases were defined as patients aged between 35 and 65 years old with confirmed T2DM based on the American Diabetes Association, 2015. Individuals presenting with factors that could

potentially alter vitamin D metabolism, such as severe hepatocellular disease (e.g., liver cirrhosis), history of bone disease including recent fractures (within 6 months), chronic gastrointestinal disorder (loose stool or diarrhea for more than 3 months), gastric and small bowel resection, drugs that increase vitamin D metabolism (e.g., ritoglicazone, rifampicin, phenobarbital, and phenytoin), and vitamin D supplementation, were excluded from this study. Controls were selected from relatives accompanying the patients and USM staff who volunteered. Enrollment of participants was carried out from February 2019 to January 2020.

3.4.2 Power calculation

The largest sample size obtained was 57 participants with an F/f FokI genotype polymorphism proportion of 18.4% [19] among individuals with diabetes at a significance level (α) of 0.05 and precision of 0.1. After accounting for a dropout rate of 10%, the required sample size was 63 participants per group. Considering that analysis will be conducted according to group, a total of 189 participants were required, among whom 63 had good glycemic control, 63 had poor glycemic control, and 63 had no diabetes.

Participants were classified into three main groups: (i) healthy controls (HbA1c less than 6.5% (48mmol/L) and random blood sugar less than 11.1 mmol/L), (ii) good glycemic control (good DM) (HbA1c less than or equal to 7.0% (53mmol/L) for at least two consecutive measurements or HbA1c less than 6.5% (48mmol/L) for a single measurement), and (iii) poor glycemic control (poor DM) (HbA1c more than 7.0% (53mmol/L) for at least two consecutive measurements).

3.4.3 Biochemical measurements

A total of 10 mL of fasting venous blood samples was obtained from each study participant. Blood was collected into ethylenediaminetetraacetic acid tubes, plain bottles, and sodium fluoride tubes and centrifuged at 3500 rpm for 8 min at 25°C. Thereafter, the collected plasma was aliquoted into microcentrifuge tubes (100 µL each). All blood and plasma samples were stored at −80°C until assayed.

All biochemical assays, including fasting plasma glucose, serum calcium, serum magnesium, serum phosphate, and serum creatinine levels, were measured via spectrophotometric analysis using the ARCHITECT C800 analyzer (Abbott Diagnostics, Abbott Park, IL, USA). Serum 25(OH)D and fasting insulin levels were measured using the Elecsys® Vitamin D total assay (Cobas, Roche Diagnostic Limited, Switzerland) and Elecsys® Insulin kit (Roche Diagnostics Co, Indiana, USA), respectively.

Vitamin D status was categorized into three groups according to World Health Organization and Institute of Medicine. Vitamin D deficiency, insufficiency, and sufficiency were defined as 25(OH)D levels <12 ng/mL (30 nmol/L), between 12–20 ng/mL (30–50 nmol/L), and ≥20 ng/mL (50 nmol/L), respectively [20,21]. Insulin resistance was calculated using the homeostatic model assessment of insulin resistance (HOMA-IR): FI (µU/mL) × FBG (mmol/L)/22.5. HOMA-IR value of >2.5 indicates insulin resistance in the general population [22].

3.4.4 Genotyping

Genomic DNA was extracted from the participants' peripheral blood using the EXGENE Blood SV Mini Kit (GENEALL Biotechnology, Korea) according to the manufacturer's protocol with slight modification. The extracted DNA was used for amplifying target

sequences of rs1544410 (BsmI) and rs2228570 (FokI) using sequence-specific primers. Primer sequences and conditions are presented in Table 1.

Polymerase chain reaction (PCR) amplification for rs1544410 (BsmI) and rs2228570 (FokI) was conducted separately in a 25 µL reaction mixture containing 2 µL of 25ng DNA sample, 0.5 µL of 10mM dNTP mix, 2 µL of 25mM $MgCl_2$, 1 µL of 10µM forward and reverse primers, 0.25 µL of 2.5U/ µL Taq Polymerase, 5 µL of 10X buffer, 0.25 µL of 100% dimethyl sulfoxide, and 13 µL of double-distilled water (Tables 10 -11). PCR cycling conditions consisted of initial pre-denaturation at 95°C (2 minutes), denaturation at 95°C (30 seconds), annealing (variable temperature based on SNPs), and extension at 72°C for 30 seconds, followed by a final extension at 72°C for 5 min. The process comprised of 35 cycles for each SNP (Table 12). The amplified products were digested with *MVA12691 (Bsm1)* and *Fok1* restriction enzymes at 37°C, followed by genotyping on 3% agarose gel electrophoresis. Few randomly selected representative samples were sent for sequencing (Apical Scientific Sdn. Bhd. Labs, Kuala Lumpur, Malaysia), with results being in concordance with RFLP genotyping.

3.4.5 Statistical analysis

Data are expressed as means ± standard deviations. Group comparisons for continuous and categorical variables were conducted using one-way analysis of variance with Scheffe post-hoc test and Chi-square test, respectively. Haplotype analysis was performed using Haploview version 4.2. Genotypic distribution was assessed for compatibility with Hardy–Weinberg equilibrium (HWE), with a p value of more than 0.05 indicating agreement with HWE. Associations between genetic VDR polymorphism with insulin resistance (HOMA-IR), glycemic control factors (magnesium level, calcium level, phosphate level, vitamin D status), and obesity were analyzed using the Pearson

correlation. Risk prediction was analyzed using simple and multiple logistic regression analyses. A p value of less than 0.05 indicated statistical significance.

3.5 Results

3.5.1 Demographic and biochemical parameters

Majority of the study participants were Malay. The healthy control group was significantly younger than the DM groups ($p = 0.002$). Participants with poor DM had significantly higher mean body mass index (BMI) compared to the healthy control group ($p = 0.008$). Participants with poor DM had significantly lower mean vitamin D level compared to those with good DM ($p = 0.041$). Majority of the study participants had normal serum calcium, magnesium, and phosphate levels. Nevertheless, participants with poor DM had significantly lower serum magnesium levels ($p < 0.001$) and significantly higher HOMA-IR compared to healthy controls, with insulin resistance having been observed in 90.5% of those with poor DM, 84.1% of those with good DM, and only 58.7% of healthy control participants (Table 2).

3.5.2 Distribution of VDR 2228570 C>T (FokI) and VDR 1544410 G>A (BsmI) gene polymorphisms among patients with T2DM having good and poor glycemic control

The allele and genotype frequency distribution, and carriage rate of VDR (FokI and BsmI) genes, among patients with T2DM and healthy controls are summarized in Table 3. Among the participants with T2DM, majority (53.2%) demonstrated the heterozygous CT genotype of the FokI polymorphism, 13.5% showed the variant TT genotype, and 33.3% had the homozygous wild-type CC genotype of the FokI polymorphism. Among the 63 healthy controls, 29 (46%) demonstrated the heterozygous CT genotype, 18

(28.6%) had the homozygous wild-type CC genotype, and 16 (25.4%) had the variant TT genotype of the FokI polymorphism.

Among the participants with T2DM, 73% had the homozygous wild-type GG genotype, 24.6% had the heterozygous GA genotype, and 2.4% had variant AA genotype of the BsmI polymorphism. Among the control group, 71.4% showed the homozygous wild-type GG genotype, 23.8% showed the heterozygous GA genotype, and 4.8% showed the variant AA genotype of the BsmI polymorphism. However, no significant differences in genotype and allele distributions of the VDR 2228570 C>T (FokI) and VDR 1544410 G>A (BsmI) polymorphisms were observed between participants with T2DM and healthy controls.

Among the participants with T2DM who had good and poor DM, majority (54% and 52.4%) appeared to have the heterozygous CT genotype, 30.2% and 36.5% exhibited the homozygous wild-type CC genotype, and 15.9% and 11.1% had the variant TT genotype of the FokI polymorphism, respectively. However, no significant difference in genotype and allele frequencies of the FokI polymorphism was observed between participants with good and poor DM.

Likewise, no significant differences in genotype and allele distributions of the VDR 1544410 G>A (BsmI) polymorphism was observed between participants with good and poor DM. Homozygous wild-type GG genotype was predominant in both groups, followed by heterozygous GA and variant AA genotypes in 4.8% of those with poor DM and none of those with good DM. Furthermore, we compared the VDR (FokI and BsmI) genotypes according to the different clinical parameters of all studied groups.

Genotypic distribution of VDR 2228570 C>T (FokI) and VDR 1544410 G>A (BsmI) were observed to be consistent with Hardy–Weinberg equilibrium in both cases and controls. Evaluation of linkage disequilibrium between FokI and BsmI based on r^2 values

showed that both SNPs were not in linkage disequilibrium (Supplementary Figure 1). Haplotype analysis showed no significant difference between the studied groups (Supplementary Tables 1–2).

3.5.3. Association between FokI (VDR 2228570 C>T) and BsmI (VDR 1544410 G>A) and risk of insulin resistance

Table 4 shows the association between VDR 2228570 C>T (FokI) and VDR1544410 G>A (BsmI) polymorphisms and the risk of insulin resistance among healthy controls and participants with good DM. Accordingly, FokI and BsmI polymorphisms were found to have no association with the risk of insulin resistance among healthy controls and participants with good DM. Among healthy controls, those with BsmI, both heterozygous GA and variant AA genotypes, showed higher risk values [odds ratio (OR) 2.406, confidence interval (CI), 0.665–8.702 and OR 1.750, CI 0.148–20.707, respectively], although differences were not significant (p = 0.181 and 0.657, respectively).

The same analyses among participants with poor DM (Table 4) showed that the homozygous variant AA genotype of BsmI (VDR 1544410 G>A) was significantly associated with insulin resistance (p = 0.025) such that those with the AA genotype had a 95% lower likelihood of having insulin resistance. Meanwhile, no significant association was observed between FokI (VDR 2228570 C>T) and insulin resistance among participants with poor DM.

3.5.4 Association between FokI (VDR 2228570 C>T) and BsmI (VDR 1544410 G>A) and glycemic control factors

Identical analyses were conducted to determine the association between VDR 2228570 C>T (FokI) and VDR1544410 G>A (BsmI) polymorphisms and glycemic control factors

(vitamin D, calcium, magnesium, and phosphate levels) (Tables 5–8). Accordingly, VDR 2228570 C>T (FokI) and VDR 1544410 G>A (BsmI) polymorphisms showed no significant association with all biochemical parameters in all groups.

3.5.5 Association between FokI (VDR 2228570 C>T) and BsmI (VDR 1544410 G>A) and risk of obesity

As shown in Table 9, a significant association was found between heterozygous CT genotype of FokI (VDR 2228570 C>T) and risk of obesity among healthy controls ($p = 0.035$), such that healthy participants with a heterozygous CT genotype had a 92% lower likelihood of becoming obese. However, no significant association was observed between BsmI (VDR 1544410 G>A) and risk of obesity among healthy controls ($p > 0.05$). Likewise, no significant association had been identified between VDR 2228570 C>T (FokI) and VDR 1544410 G>A (BsmI) polymorphisms and the risk of obesity among participants with good and poor DM.

3.6 Discussion

Studies have shown that vitamin D plays an essential role in insulin synthesis, secretion, and function, and elements of inflammation, which may affect the development of T2DM [23]. A meta-analysis of 11 studies by Shen et al. [24] found that patients with T2DM had lower vitamin D levels than controls, while Errouagui et al. [25] documented that a higher prevalence of vitamin D deficiency among those with T2DM (40%) than among controls without diabetes (20%) in the Moroccan population. The current study found that among participants with T2DM, those having poor glycemic control exhibited significantly lower vitamin D levels compared to those having good glycemic control and healthy controls.

Similarly, Mackawy and Badawi [26] revealed that Egyptian patients with diabetes, especially those with metabolic syndrome, had decreased vitamin D levels.

Previous studies had found that VDR gene polymorphisms affect VDR protein activity. Genetic variations in the VDR, which altered calcium metabolism, adipocyte function, insulin release, and cytokine expression, played a significant role in the pathogenesis of T2DM [9]. However, previous studies had presented inconsistent, inconclusive, and variable results according to study populations and ethnic groups.

The present study evaluated the association between two potentially functional VDR gene variants (BsmI and FokI) and glycemic control factors among healthy controls and patients with T2DM who had good and poor glycemic control. Accordingly, our findings showed that both VDR (FokI and BsmI) genotypes were not significantly associated with diabetes risk among the Malaysian population.

Moreover, haplotype analysis conducted herein showed no significant association between both SNPs and diabetes. This result was consistent with findings presented by Bid et al. [27] in the North Indian population and Malecki et al. [28] in the Polish population, both of whom found no correlation between VDR gene polymorphisms and diabetes at any of the four polymorphic sites. Likewise, a meta-analysis conducted by Yu et al. [29] concluded that no significant association existed between BsmI and FokI polymorphisms and T2DM. Nonetheless, results have varied according to sample size and study population ethnicity.

However, a study by Li et al. [19] among the Asian community revealed a possible link between polymorphisms at the FokI site and the onset of T2DM. Similarly, a study by El Gendy et al. [30] observed significant differences in FokI genotypes and allele distribution between Egyptian patients with T2DM and controls, which could be a risk factor for T2DM. Another study by Ortlepp et al. [31] observed a significant association between

the BsmI VDR genotype and fasting glucose, while Oh and Barrett-Connor [32] observed a significant association between the VDR 1544410 (BsmI) polymorphism and HOMA-IR levels among individuals with T2DM in the Rancho Bernando Cohort.

The present study showed a significant association between insulin resistance and VDR 1544410 G>A (BsmI) polymorphism among patients with T2DM who had poor glycemic control. Moreover, our results showed that GG and GA genotype carriers had higher HOMA-IR levels compared to homozygous variant AA genotype carriers, suggesting that the homozygous variant AA genotype appeared to be protective against insulin resistance. Furthermore, we noticed significant associations between the VDR 2228570 C>T (FokI) polymorphism and risk of obesity among healthy participants without diabetes. Participants carrying the heterozygous CT genotype of the FokI polymorphism had lower BMI levels compared to those carrying the homozygous wild-type (CC) and variant (TT) genotypes.

These contradictory findings may be related to the divergent genetic backgrounds of the populations studied. Non-identical ethnic groups may have varying numbers of susceptibility alleles. T2DM has a complicated etiology involving polygenic heredity. Accordingly, various allele integrations may exist among patients with diabetes. Subsequently, abnormalities in insulin secretion associated with VDR polymorphisms might play an important role only in specific environmental or genetic backgrounds. Moreover, reported VDR polymorphisms may possibly be just markers of linkage disequilibrium with another gene, which may be responsible for the associations observed with type 2 diabetes mellitus. Nonetheless, more polymorphisms likely remain to be discovered [9].

Growing evidence has revealed that individuals with diabetes have impaired cellular calcium homeostasis. Accordingly, investigations on cellular calcium regulation defects

in multiple cells, including cardiac muscle, skeletal muscle, kidneys, adipocytes, liver, osteoblasts, retinal tissue, and pancreatic beta cells, have confirmed that such defects are an underlying pathology associated with a diabetic state [33]. Hypocalcemia has been considered to be related to uncontrolled hyperglycemia among patients with T2DM, the correction of which may promote better glycemic control [34]. However, the present study found no significant association between calcium status and glycemic control given that none of our participants with our T2DM were hypocalcemic. The average calcium level observed among our study population may be attributed to the possible calcium rich-diet among our community, and increased physical activity considering that most of our study participants were younger than 60 [34].

Hypophosphatemia has been generally associated with poor glycemic control. Accordingly, studies have shown that insulin, which increases the extracellular-to-intracellular transfer of phosphate, mediates the relationship between serum phosphate and glucose [35]. Moreover, hypophosphatemia has been implicated in the pathogenesis of diabetes mellitus given that low serum phosphate levels promote insulin resistance and glucose intolerance. Considering the importance of phosphate in carbohydrate metabolism, reduced phosphate levels may decrease peripheral glucose utilization, leading to insulin resistance. The resulting compensatory hyperinsulinemia can further decrease phosphate concentrations, leading to the development of a vicious cycle that may contribute to the pathogenesis of metabolic syndrome [36]. Indeed, a previous study by De Fronzo and Lang agreed that chronic hypophosphatemia resulted in decreased tissue insulin sensitivity [37], while subsequent studies found that phosphorus supplementation for patients with hypophosphatemia who had glucose intolerance improved glucose tolerance [38, 39]. Nonetheless, the current study found no significant mean difference in serum phosphate levels among our participants regardless their diabetic status.

Hypomagnesemia is the most frequent electrolyte abnormality among ambulatory patient with diabetes and is frequently observed among patients with diabetic ketoacidosis. The most critical factor for the onset of hypomagnesemia among patient with diabetes is glycosuria-induced excessive urinary magnesium loss. The clinical consequences of hypomagnesemia include impaired insulin secretion, insulin resistance, and increased macrovascular risk. However, the role of magnesium deficiency in microvascular complications has yet to be clearly established [40]. The aforementioned mechanism can explain our findings of hypomagnesemia only among participants with T2DM who had poor glycemic control [41]. None of the healthy controls and cases with good glycemic control included herein exhibited hypomagnesemia. Appropriate magnesium supplementation might prove beneficial for normalizing low plasma and tissue magnesium levels, subsequently preventing or hindering the development of vascular complications among patients with diabetes [42].

The current study found that among participants with T2DM, those with poor glycemic control had significantly higher BMI compared to those with good glycemic control and healthy controls. This is consistent with the findings of Daousi et al. [43] who reported that 86% of adults with T2DM were overweight or obese, with at least 52% having obesity and 8.1% having morbid obesity. Insulin resistance is one of the vital factors in the etiopathogenesis of T2DM. Accordingly, HOMA-IR and certain obesity indices have been identified as significant independent determinants of glucose intolerance. Indeed, a study by Lawal et al. [44] proposed the periodic use of HOMA-IR assessment on high-risk individuals, such as obese individuals and those whose first-degree relatives had diabetes, to identify those on the pathogenetic ladder toward glucose intolerance for early T2DM intervention. Our study observed that those with poor glycemic control had a significantly higher mean difference in HOMA-IR compared to those with good glycemic

diabetic, with healthy controls having the lowest mean difference in HOMA-IR. Such findings are consistent with those presented in previous studies that suggested HOMA-IR as an established index of insulin resistance for the assessment of patients with T2DM [45].

One limitation of the current study is that our small population, which only included participants from the Kelantan region mainly consisting of the Malay community, may not be representative of the actual genetic polymorphisms among all Malaysians. Hence, more genetic epidemiological studies including larger populations ideally from all main ethnicities, including Malay, Chinese, and Indians, are required for a better understanding of the relationship between VDR variations and various phenotypes for insulin sensitivity, glycemic control factors, anthropometric data, and potential clinical implications.

Given the aforementioned findings, the current study concluded no significant association existed between VDR FokI and BsmI polymorphisms and T2DM. Nevertheless, our results suggested that the BsmI polymorphism was associated with insulin resistance among participants with T2DM who had poor glycemic control and that the VDR FokI polymorphism was associated with obesity risk among participants without diabetes. Moreover, those with poor DM had significantly lower serum magnesium levels and significantly higher HOMA-IR compared to the other two groups.

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Declarations of interest

None.

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Ethics approval

Ethics approval was obtained from the Human Research Ethics Committee of USM (USM/JEPeM/18100498).

Consent to participate

Written informed consent was obtained from each study participant after explaining the objectives and potential benefits of the study.

Consent for publication

Informed consent for publication was obtained from all individual participants.

Data and code availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Wan Nur Amalina Zakaria, Nazihah Mohd Yunus, Najib Majdi Yaacob, Wan Mohd Izani Wan Mohamed, K.N.S. Sirajudeen, and Tuan Salwani Tuan Ismail. Wan Nur Amalina Zakaria drafted the initial manuscript, and all authors read, critically revised, and approved the final manuscript.

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