INTRODUCTION

Prematurity represents major social and economic public health problem. It has an immense impact on individual family and healthcare system. Gestational age with <37 weeks is considered to be premature birth [1]. Preterm birth (PTB) is one of the largest causes of neonatal death globally. 35% of neonatal deaths are due to prematurity, an important contributor to child morbidity and long-term disability [2,3]. The prevalence of PTB varies among different ethnic groups. PTB rate is 16.3% in black women, 13% in American Indian, 11.3% in Hispanic, and 10% in Asian and Indian women [4,5]. The effect of genetic factors on PTB is difficult to understand due to the involvement of various environmental factors. It is needed to identify causes and effective way to prevent prematurity which could save countless infant lives. The development of nutritional status in children is strongly depending on maternal nutritional status. Maternal diet can alter gene expression during prenatal life and can increase susceptibility to individual to different disorder such as diabetes and hypertension [6]. Genetic polymorphism could increase the risk for the development of diseases in human population, which modify metabolic response to diet. Dietary Vitamin D is efficiently absorbed in small intestine and transport to lymphatic system in chylomicrons. Vitamin D is a steroid hormone that maintains mineral homeostasis in the gastrointestinal tract and kidney. It modulates about 10% of the whole human genome and involved in developmental processes. Vitamin D homeostasis during pregnancy is an important for mother's health and progeny across a range of possible health outcomes [7,8]. Verity of adverse maternal outcomes [9] and offspring birth weight [10] demonstrated in pregnant women due to deficiency of vitamin D. The previous studies reported that a pregnant woman with lower level of vitamin D increases the risk of PTB in African-Americans and Caucasians population [11,12]. 1, 25-dihydroxyvitamin D (calcitriol) deficiency may be an independent risk factor for pregnancy and birth complications needed to study. Vitamin D produces its biological effects through binding with vitamin D receptor (VDR) present in bone, kidney, small intestine and neuronal cell which is a steroid hormone receptor and mediates gene expression [13,14].

VDR possesses both ligand and DNA binding domains and it is located in the cytosol. VDR gene expression in placenta regulates various genes linked with implantation, maturity of fetus, and bone formation. Vitamin D status may influence intratubular mineralization and growth during pregnancy [15]. VDR gene polymorphism may affect its expression, activity and subsequently downstream biological activity of vitamin D. Therefore, it could be a potent genetic marker for prematurity in pregnant women [16]. Several polymorphic sites have been described within VDR gene. Polymorphism near the 3’ end of VDR gene identified by restriction enzyme BsmI, TaqI, ApaI, and FokI associated with osteoporosis [17]. Type 1 diabetes mellitus [18], polycystic ovary syndrome [19], end-stage renal disease [20], several types of carcinoma such as breast, prostate, and colon carcinoma [21]. Therefore, genetic disparities of prematurity with regards to VDR gene polymorphism need to be investigated. This study was conducted to explore the genetic inference of VDR gene polymorphism on risk of PTB in West Indian women.

METHODS

Study population

The randomized cross-sectional study involved a total 210 women at the Department of Obstetrics and Gynaecology of Akanksha and Garima Hospital at Anand and Dr. Padma Gynaecology Hospital at Vadodara from October 2015 to May 2016. Written informed consent form and data collection sheets were obtained from all participated subjects. Subjects were classified into two groups; preterm group consisted of mothers who delivered their babies between 24 and 36 weeks and control group consisted mothers with uncomplicated normal delivery.
between 38 and 41 weeks. Anthropometric measurements of subject such as weight, height, body mass index (BMI), obstetrics history, and ethnicity were obtained from data collection sheet. Human Ethnic Committee of Shri A. N. Patel PG Institute, Anand, has approved this study. Gynecologist calculated gestational age from last menstrual period and confirmed by ultrasonography. The inclusion criteria including healthy mother between the age of 20 and 35 years old, without any metabolic disordered with normal and healthy fetus were used to select subject. Pregnant women with a history of hyperuricemia, tuberculosis, diabetes, renal disease, and hypertension were excluded from the study. Abnormal fetus, preclampsia, maternal age >35 years and stillbirth were also considered as exclusion criteria.

Biochemical measurements
Blood was drawn in anticoagulant-coated vacutainer blood collection tube and centrifuged at 5000 rpm to separate serum. Serum was stored in deep freezer at -20°C until analysis was performed. Total albumin was determined by bromocresol green kit method from serum. 250H vitamin D was measured using a commercially available enzyme-linked immunosorbent assay (DIA source Immunoaassays, Belgium) kit on microtiter plates and expressed in ng/dl.

Isolation of genomic DNA
Genomic DNA was isolated from the collected fresh blood by G-Biosciences DNA extraction kit (OmniPre™ Himeda Kit) and used for genotyping. Isolated genomic DNA was confirmed for quality by electrophoresis in 0.8% agarose gel.

VDR genotyping
Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) were performed for genotyping of single nucleotide polymorphisms (SNPs): FokI (rs10735810) and TaqI (rs731236) of VDR gene. Amplification of selected gene was carried out by PCR using thermocycler machine (Thermo Scientific). Primers and restriction enzymes were purchased for Eurofine, Europe. FokI polymorphic site was amplified using following specified primer set described previously [22].

FP: 5'-AGCTGGCCCTGGCAGACAGGCGTTAGC-3'
RP: 5'-ATGGAAACACCTTGCTTCTTCTCCCTC-3'
The following primer set was used to amplify TaqI polymorphic site according to [23].

FW: 5'-CAGACGATGACAGGGGAGCAA-3'
RP: 5'-CCTCAGGAGCACAAGGGGCGTTAGC-3'
PCR condition and pattern of RFLP is mentioned in Table 1. PCR product was resolved on 2-3% agarose gel and visualized under UV light and further used for RFLP. PCR product (265 bp) contains FokI polymorphic site was digested for overnight at 37°C with FokI restriction enzyme. Amplified TaqI polymorphic segment (740 bp) was digested for 4 hrs at 55°C with TaqI restriction enzyme. Restriction digested products were resolved by electrophoresis in 12% polyacrylamide gel stained with silver or by 2-3% agarose gel contained 0.5 µg/ml ethidium bromide using electrophoresis system (Bio-Rad) and visualized under UV transilluminator system.

Statistical analysis
Unpaired two-tailed Student’s t-test was utilized to determine comparison between means. Pearson Chi-square (χ²) test was used to compare categorical variables and genotypic frequencies from Hardy-Weinberg equilibrium for each SNP. Odds ratios (ORs) and 95% confidence interval (95% CI) were obtained using χ² unbiased risk estimation. The statistical analyses were performed with the help of SPSS statistical software. A p<0.05 was considered statistically significant.

RESULTS
A total of 210 subjects including 72 preterm and 138 healthy controls belonging to the same ethnicity were analyzed for VDR gene polymorphism and its association with maternal vitamin D deficiency. Studied polymorphisms were found in Hardy-Weinberg equilibrium. The demographic and clinical characteristics of 210 participated women were summarized in Table 2. The mean age 29.01±4.92 years of the control group is nonsignificantly differ from preterm group with mean age 28.4±5.6 years. The percentage of previous miscarriage cases was higher (18%) in women with premature compared to control group (7%). Women with PTB had significantly (p<0.05) lower level of vitamin D (18.7±5.2 ng/ml) in serum compared to control subjects (29.3±6.1 ng/ml). The prevalence of vitamin D deficiency is significantly higher in women with PTB than in control women with full-term birth (Table 2).

The allele and genotype frequency distribution of VDR (FokI) gene among women with preterm delivery and control women with full-term delivery is shown in Table 3. The genotype frequency of VDR FokI and TaqI was agreement with Hardy-Weinberg equilibrium in all groups in present study. FF, Ff, and ff genotypes were observed after RFLP of VDR (FokI) PCR products (Fig. 1). In preterm group, FF, Ff, and ff genotypes accounts for 30.55%, 40.28%, and 29.17% compared to 39.86%, 49.27%, and 1.87% in control group, respectively. Preterm women showed significantly (p<0.05) higher frequency of ff genotypes compared to control group. The frequency of f allele was higher in preterm women than full-term women (49.31%) vs. 35.51%; OR=0.57; 95% CI=0.37-0.87; p=0.006) compared to wild-type F allele. Preterm women carrying ff genotypes (FokI gene) had significantly higher risk for vitamin D deficiency than women with FF and Ff genotypes.

In women with PTR, the TT, Tt and tt genotypes of VDR TaqI polymorphism accounts for 35 (48.61%), 31 (43.06%) and 06 (8.33%) compared to 72 (52.17%), 54 (39.13%) and 12 (8.7%) in control subjects, respectively (Table 4). There was no significant difference in all genetic model (TT vs. Tt, p=0.59 and Tt vs. tt, p=0.96) between preterm women and control group for VDR TaqI polymorphism. Fig. 2 exhibits PCR-RFLP pattern obtained in VDR TaqI polymorphism. The frequency of T alleles (70.14%) vs. 71.71%) and t alleles (29.86% vs. 28.26%) of PCR-RFLP pattern of VDR was observed to be nonsignificantly differ between PTB group and control group, respectively.

Table 1: PCR-RFLP protocol for FokI and TaqI polymorphism of VDR gene

| Polymorphism | Set of primers | Annealing temperature (°C) | Size of PCR product | RFLP pattern |
|--------------|----------------|---------------------------|---------------------|--------------|
| FokI (T/C)   | Forward primer: 5'-AGCTGGCCCTGGCAGACAGGCGTTAGC-3’ Reverse primer: 5'-ATGGAAACACCTTGCTTCTTCTCCCTC-3' | 61°C | 265 bp | FF-265 |
|              |                |                           |                     | FF-196, 69   |
|              |                |                           |                     | ff-196, 69   |
| TaqI (C/T)   | Forward primer: 5'-AGCTGGCCCTGGCAGACAGGCGTTAGC-3’ Reverse primer: 5'-CCTCAGGAGCACAAGGGGCGTTAGC-3’ | 64°C | 740 bp | TT-495, 245 |
|              |                |                           |                     | Tt-495, 245, 205, 290 |
|              |                |                           |                     | tt-205, 245, 290 |
The genotype frequency did not differ significantly by age and BMI between PTB and control women. We observed significantly (p<0.01) lower level of serum vitamin D in women with ff genotype (15.24±2.31 ng/ml) than in women with FF (28.65±7.23 ng/ml) and Ff (24.36±4.51 ng/ml) genotypes, respectively (Table 5). The relationship between FokI polymorphism in VDR gene and onset pattern of prematurity was observed significantly but not shown significance in TaqI polymorphism. In this study, FokI variant (ff genotypes) of VDR gene was identified genotype associated with maternal vitamin D deficiency in Indian women with PTB.

**DISCUSSION**

Maternal or fetal genetic predisposition has been suggested as one of the strongest risk factors for individual risk of prematurity. It also

### Table 2: Demographic characteristics of subjects

| Characteristics                  | Control   | Preterm   |
|----------------------------------|-----------|-----------|
| Sample (n)                       | 138       | 72        |
| Mean age±SD (year)               | 29.0±4.92 | 28.4±5.6  |
| Married status (%)               | 100       | 100       |
| Previous miscarriage (%)         | 12        | 21*       |
| BMI (kg/m²)                      | 27.8±1.5  | 23.4±2.7  |
| SBP (mmHg)                       | 135±12.11 | 135.2±15.11|
| DBP (mmHg)                       | 77.0±6.84 | 76.8±8.68 |
| 25OH Vitamin D (ng/ml)           | 29.3±8.1  | 18.7±5.2* |

*p*<0.05 is considered as statistically significant. BMI: Body mass index, SBP: Systolic blood pressure, DBP: Diastolic blood pressure

### Table 3: Genotyping and allele frequency of FokI SNP in VDR gene between control and preterm cases

| Polymorphic genotype | n (%)    | p value | OR (95% CI) |
|----------------------|----------|---------|-------------|
| FF                   | 55 (39.86) | 22 (30.55) |            |
| FF                   | 68 (49.27) | 29 (40.28) | 0.849       |
| ff                   | 15 (10.87) | 22 (30.55) | 0.849       |
| Alleles              |          |         | 0.002**     |
| **F**                | 178 (64.49) | 73 (50.69) | 0.006**     |
| **f**                | 98 (35.51) | 71 (49.31) | 0.006**     |

*p*<0.01 is considered as statistically significant. VDR: Vitamin D receptor, SNP: Single nucleotide polymorphism, OR: Odds ratio, CI: Confidence interval

### Table 4: Genotyping and allele frequency of TaqI SNP in VDR gene between control and preterm cases

| Polymorphic genotype | n (%)    | p value | OR (95% CI) |
|----------------------|----------|---------|-------------|
| TT                   | 72 (52.17) | 35 (48.61) |            |
| Tt                   | 54 (39.13) | 31 (43.06) | 0.59        |
| tt                   | 12 (8.7)  | 6 (8.33)  | 0.96        |
| Alleles              |          |         | 0.97 (0.30-3.20) |
| **T**                | 198 (71.74) | 101 (70.14) | 0.73        |
| **t**                | 78 (28.26) | 43 (29.86) | 0.73        |

VDR: Vitamin D receptor, SNP: Single nucleotide polymorphism, OR: Odds ratio, CI: Confidence interval

### Table 5: Heterogeneity in mean±SD values of different biochemical traits according to the genotypes of VDR polymorphism in participated women subjects

| FokI genotype | Biochemical traits | FF     | Ff     | ff     | p value | OR (95% CI) |
|---------------|--------------------|--------|--------|--------|---------|-------------|
|                | Age (year)         | 27.4±4.87 | 26.34±4.98 | 26.39±5.39 | 0.49 | 0.63 |
|                | BMI (kg/m²)        | 26.12±2.34 | 25.64±2.87 | 23.21±2.47 | 0.55 | 0.42 |
|                | 25OH Vitamin D (ng/ml) | 28.65±7.23 | 24.36±4.51 | 15.24±2.31 | 0.18 | <0.01** |

| TaqI genotype | Biochemical traits | TT     | Tt     | tt     | p value | OR (95% CI) |
|---------------|--------------------|--------|--------|--------|---------|-------------|
|                | Age                | 28.3±3.74 | 29.12±4.76 | 26.74±5.02 | 0.41 | 0.50 |
|                | BMI (kg/m²)        | 25.45±1.85 | 26.47±2.31 | 24.74±1.89 | 0.39 | 0.62 |
|                | 25OH Vitamin D (ng/ml) | 25.74±4.56 | 22.34±9.34 | 23.45±8.63 | 0.27 | 0.42 |

**p**<0.01 is considered as statistically significant. BMI: Body mass index, VDR: Vitamin D receptor, SD: Standard deviation
caused by infection and inflammation. Preterm labor and PTBs are still one of the leading causes of perinatal morbidity and mortality in the developed world [24]. In the current study, we investigated the possible contribution of VDR gene polymorphism in susceptibility to prematurity among West Indian pregnant women. Our results revealed that serum levels of 25 (OH) D were significantly (p<0.05) lower in women with PTB than in control subjects which confirm that vitamin D deficiency is a risk factor for prematurity.

The relationship of vitamin D insufficiency with PTB during pregnancy has been attracted by public health attention. PTB in women could increase by 59% with vitamin D level at or below 20 ng/mL [25]. The previous meta-analysis studies also confirmed the association between vitamin D insufficiencies and PTB [12,26]. This is the first study carried out to analyze the prevalence of vitamin D deficiency in West Indian women and its impact on prematurity. This study revealed that prevalence of vitamin D deficiency was considerably higher in women with PTB compared to control group. Adequate vitamin D status during pregnancy could reduce the risk of preterm delivery due to its decreasing placental colonization by bacterial vaginosis species [27]. PTB in women with vitamin D deficiency could be due to it's contribution in activating innate immune response in placenta [28] and increased bacterial infection in maternal and fetal cell [27].

Transcriptional activity of the VDR gene regulates the transcription of about 3% of the human genome including genes that are crucial for preterm delivery. Vitamin D participates in the regulation of bone remodeling and mineral homeostasis. Due to involvement in fetomaternal interface (site of PTB) and also in ameliorates function, VDR system may serve as risk markers for PTB. Placental tissue obtained with PTB exhibited reduced miRNA expression of VDR gene compared to normal placental tissue [29]. Allele F in VDR produce shorter protein by three amino-acids which are more active form in trans-activation of the VDR and producing 1, 25 (OH)D effect compared to f allele of VDR gene [30]. Results of the present study indicated that subjects with ff genotype have significantly more risk to develop PTB compared with the reference genotype FF. Our results are consistent with the previous study which established an association between VDR FokI polymorphism and prematurity in a Saudi population [31]. Therefore, VDR FokI gene polymorphism showed an association with the occurrence of PTB in studied population. Spontaneous idiopathic PTB in an Israeli population was associated with FokI polymorphism in VDR receptor [15].

Contradictory results were found in study carried out by Cai et al. [32] which showed FF genotype of VDR FokI gene was associated with increased risk of preterm delivery. A new Danish study indicates that genetic variations are associated with low levels of vitamin D in the blood leads to premature death [33]. In the present study, it is observed that pregnant women with lower level (>20 ng/mL) of vitamin D in serum have increasing risk of PTB.

TaqI located in 3′ untranslated region should be related to VDR activity and expression [13]. Our results are also consistent with previous studies in which no relationship between VDR (TaqI) polymorphisms and PTB was found. TaqI polymorphism is located in the non-coding region of the VDR gene, and they do not have any effect on the final protein product. The TaqI genotype/allele distribution pattern in our study did not yield any significant results. A number of other potential candidate gene variants have been reported that predispose women for PTB. Subjects with deficient in vitamin D level are more susceptible to microbial infection and have a major risk factor for PTB [34]. Immunomodulating activity of vitamin D on cytokine production by endometrial cells of women plays an important role in etiology of PTB [35]. This study helps scientists to understand the complex genetic factor associated with an increased risk of PTB and valuable for effective ways to prevent or delay PTBs.

CONCLUSION

Our results indicated that FokI polymorphism of VDR gene may contribute to maternal vitamin D deficiency and increases PTB in Indian Women. Further, studies involving other genetic linkage assessment are required to evaluate the direct effect of VDR gene polymorphism in the development of PTB.

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