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Research Article

Association between Vitamin D Receptor FokI and BsmI Gene Polymorphism and Diabetes Mellitus in Nepalese Population

Anil Kumar Sah[1], Melina Dahal[2], Sandip Baniya[2], Santoshi Pyakurel[2], Sonu Rai[2], Lasta Maharjan[1], Susmita Bista[1], Keshab Raj Budha[2], Biraj Lohani[3], Prakash Poudel[3], Pragati Poudyel[4], Bajrangi Rauniyar[5]

[1] Annapurna Research Center, Maitighar, Kathmandu, Nepal
[2] SANN International College, Gairidhara, Kathmandu, Nepal
[3] Agilus Diagnostics Nepal Pvt. Ltd., Maharajgunj, Kathmandu, Nepal
[4] People's Dental College and Hospital, Kathmandu, Nepal
[5] Om Hospital and Research Center, Chabahil, Kathmandu, Nepal

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CORRESPONDENCE

Anil Kumar Sah; Santoshi Pyakurel

Annapurna Research Center; SANN International College, Kathmandu, Nepal

Email: anilshah4in@gmail.com; ssantoshipyakurell@gmail.com

0000-0001-5428-8503

Abstract

Vitamin D being involved in the secretion of insulin is a known fact. Moreover, studies have shown that steroids might be a factor in influencing insulin sensitivity. Vitamin D receptor (VDR), a factor required for genetic regulation involving vitamin D, thus can be regarded as a good candidate for Diabetes Mellitus (DM). Several studies have been conducted on the association between VDR polymorphism and the risk of DM but did not provide clear-cut answers. This study was conducted to search for the involvement of FokI and BsmI polymorphisms of the VDR gene with DM in a Nepalese population. A total of 200 blood samples were collected; 100 from clinically diagnosed DM patients and 100 from healthy controls. DNA was extracted from blood by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis, where FokI and BsmI primers as well as restriction enzymes were used. After restriction digestion, SNPs of FokI (T/C) [rs2228570] and BsmI (A/G) [rs1544410] were assayed using agarose gel electrophoresis. As patients and controls were likened for genotype distribution and allelic frequencies, it was found that the frequency of VDR gene BsmI rs1544410 differed significantly ($p < 0.05$, each) between cases and control whereas A allele was dominant (91%) in healthy controls with Odd ratio (OR) of 0.55, unlike VDR FokI were not significantly associated between subjects and control. The data obtained from this research suggests that the VDR gene (especially BsmI) is associated with the risk of DM.

Keywords: Diabetes Mellitus, PCR-RFLP, Vitamin D Receptor

Introduction

Diabetes is a chronic, metabolic disease characterized by elevated levels of blood glucose (or blood sugar), which leads over time to serious damage to the heart, blood vessels, eyes, kidneys, and nerves. It is not limited to a single factor, studies have revealed genetics, obesity, heredity, lifestyle, high blood pressure, autoantibodies, age, abnormal cholesterol, triglyceride level, race, etc. as

contributing factors for Diabetes Mellitus (DM) (Angel et al., 2018; Bonakdaran et al., 2012). Recently Vitamin D has gained popularity as a risk modifier for DM. Vitamin D acts by stimulating insulin receptor expression. It is involved in the Transcription activation of the Insulin receptor gene along with the vitamin D receptor (VDR) as a transcription factor. VDR is a nuclear, ligand-dependent transcription factor that is in complex with active Vitamin D, $1,25(OH)_2D_3$, controlling the synthesis of numerous different proteins involved in calcium transport and utilization (Kjalarsdottir, 2012). More than 25 polymorphisms of VDR have been reported to date. The most common polymorphisms of the VDR gene are the BsmI, FokI, TaqI and ApaI. FokI is located in exon 2 of the VDR gene and its polymorphism involves alteration in the start codon leading to the shortening of the VDR protein (van Etten et al., 2007). BsmI polymorphism is located in intron 8 at the 3′ end of the VDR gene and does not change the amino acid sequence of the VDR protein. However, there might be changes in gene expression through changes in intronic regulatory elements, alteration of mRNA stability, or disruption of splice sites (Vogel et al., 2002; Wang et al., 2017). Single nucleotide polymorphisms (SNP) in the vitamin D receptor (VDR) gene affect vitamin D synthesis, transportation and action and might hinder insulin secretion, causing insulin resistance (Sung et al., 2012). Different studies have described the significance of BsmI and/or FokI polymorphism association with DM (Angel et al., 2018; Sarma et al., 2018; Wang et al., 2017; El Gendy et al., 2019; Jia et al., 2013; Mukhtar et al., 2017; Iyer et al., 2017; Mohammadnejad et al., 2012).

It is a known fact that Nepal shares a central origin with India as well as China. Even lifestyle of the people of Nepal has been more or less similar to these countries for a very long period; it was relevant to check the occurrence of similar mutation in the diabetic population of Nepal. Moreover, no such molecular-level studies have been performed in Nepal which is why the study was designed to investigate the association between VDR gene polymorphism (Bsml and Fokl) with the occurrence of diabetes in Nepal. Furthermore, knowing the genetic aetiology of the problem to predict the progression of mutation, screening of family members and genetic counselling regarding the risk was the objective of the study. Diabetes is both hereditary as well as lifestyle-based disease but the exact connection of the disease with a gene is still unknown. Thus, this study takes limitations to identify whether VDR Bsml and VDR fokl were associated with diabetes or not to take a step further to relate diabetes with DNA.

Materials and Methods

A cross-sectional study was conducted at Annapurna Research Center, Annapurna Neurological Institute and Allied Sciences (ANIAS), and Om Hospital and Research Center, Kathmandu from July 2019 to February 2020. The study was undertaken only after obtaining approval from the Institutional Ethics Committee of ANIAS and written informed consent was obtained from all the participants. The study populations were both inpatients and outpatients with DM cases from different hospitals in Kathmandu Valley as well as healthy people not attending any hospitals. A total number of 200 people were enrolled in the study; among them 100 were diabetic patients and 100 were healthy controls.

Inclusion and exclusion factors for sample selection

Diabetes mellitus patients with elevated glucose levels or those with normal levels of glucose but were under medication were taken as DM cases. Similarly, healthy age and sex-matched controls with no history of T2DM and normal oral glucose tolerance test as per ADA criteria of diabetes and without any family history of diabetes mellitus in first-degree relatives were enrolled as controls. People with vitamin D supplementation within the past 3 months, patients on drugs altering Vitamin D levels (anticonvulsants, estrogen, cholestyramine, and orlistat), chronic liver disease and chronic kidney disease stages 3-5 were excluded.

DNA extraction and thrombophilic mutation

Blood samples from both normal and DM subjects were collected and DNA was extracted using a Promega kit (Promega Corporation, USA). Spectrophotometric analysis was carried out to check both the quality and quantity of DNA samples. Genotype screening was performed for the identification of the VDR gene among 200 patients and healthy controls. The VDR Bsml (A/G)

polymorphism (rs1544410) gene of 825bp was amplified by polymerase chain reaction (PCR). During PCR, 25 µl reaction mixture contained 0.5 µl of oligonucleotide forward primer: 5′-CAACCAAGACTACAAGTACCGCGTCAGTGA-3′, 0.5 µl of oligonucleotides reverse primer: 5′-AACCAGCGGGGAAGAGGTCAAGGG-3′ (Macrogen, Korea), 12.5 µl master mix (Solis Bio Dyne, Estonia), 3 µl of DNA template and the rest volume was adjusted by PCR grade water. As for negative control, a similar mixture was prepared but DNA was replaced by PCR grade water. A similar method was applied for the amplification of 265bp VDR Fokl (T/C) polymorphism (rs2228570) gene using forward primer: 5′-AGCTGGCCCTGGCACTGACTCTTGCTCT-3′ and reverse primer: 5′-ATGGAAACACCTTGCTTCTTCTCCCTC-3′ (Macrogen, Korea).

The PCR tubes were placed on the tube holder of the thermo-cycler (ProFlex PCR System, Lifescience, Thermofisher, USA). For BsmI gene initial denaturation was performed at 94˚C for 3 minutes followed by 35 cycles at 94˚C for 20 seconds, 62˚C for 40 seconds, 72˚C for 1 minute and a final extension at 72˚C for 6 minutes (ProFlex PCR System, Thermofisher, USA). Likewise, for Fokl gene initial denaturation was carried out at 94˚C for 10 minutes followed by 35 cycles at 94˚C for 30 seconds, 60˚C for 30 seconds, 72˚C for 30 seconds and final extension at 72˚C for 10 minutes (ProFlex PCR System, Thermofisher, USA). For the confirmation of PCR amplification, 1.5% agarose gel was prepared in TBE (1X) buffer by adding ethidium bromide (EtBr-10mg/ml). The agarose gel was run at 60V for 90 min. Then, agarose gel was visualized under the gel doc system (UV Cambridge, USA).

Restriction fragment length polymorphism (RFLP)

RFLP of Bsm1 (rs1544410) gene: Restriction fragment length polymorphism (RFLP-PCR) was used to identify VDR genotypes. The amplified PCR product using Bsml primers was subjected to restriction digestion using Mva1269I (Thermofisher, USA) restriction endonuclease in 13 µl reaction volume contained: PCR product (1 µg/µl) 5 µl, buffer R (10X) 2 µl, restriction enzyme (Mva1269I) (100U/µl) 1 µl, nuclease-free water 5 µl. The mixture was incubated at 37˚C for 75 minutes followed by incubation at room temperature for 10 minutes. The digested DNA fragments were separated by agarose gel-electrophoresis in 2% agarose gel, run at 60 volts for 1 hour and 30 minutes and the bands were then examined under a UV light of gel documentation system (UV Cambridge, USA) as shown in Figure 1. Genotype was determined according to fragment length i.e. homozygote GG (BB) subjects = 650 and 172bp product; heterozygote GA (Bb) subjects = 825, 650 and 172bp products and homozygote AA (bb) subjects = 825bp product. SNP resulting in G to A substitution in VDR gene intron 8 leads to degeneration of a BsmI restriction site. Homozygous subjects with alleles containing nucleotide A show one band at 825bp, designated as having AA BsmI genotype. Homozygous subjects with alleles containing G show 2 bands of 650 and 172 bp were designated as GG. Subjects with heterozygote status showing 3 bands: 825, 650 and 172bp were denoted as GA (Tobón-Arroyave et al., 2017).

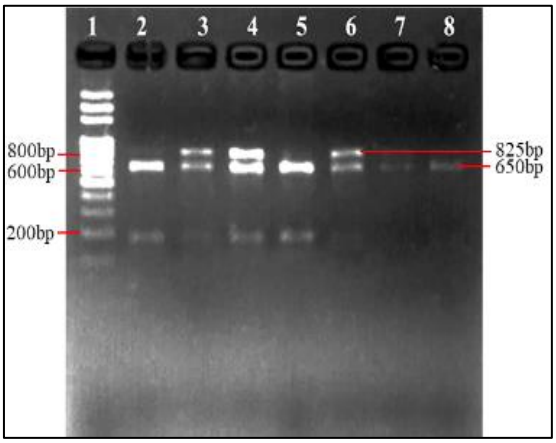

Figure 1: PCR reaction for VDR gene SNP BsmI with gene products size 825bp and the ladder marker were resolved on 2% agarose gel. RFLP analysis after digestion with BsmI restriction enzyme lane 1 in gel shows DNA ladder, lane 8 shows homozygous GG (B/B) wild type which is normal, lanes 2,3,4,5,6,7 showing heterozygous GA (B/b) mutation.

RFLP of Fok1 (rs2228570) gene: The PCR product after amplification using Fokl primers was digested by fast digest restriction enzyme (FOKL) (Thermofisher, USA) in 14.5 µl reaction mixture containing PCR product (1 µg/µl) 5 µl, green buffer (10X) 2 µl, FOKL 1 µl, nuclease-free water 7.5 µl. The mixture was incubated at 37˚C for 90 minutes followed by incubation at room temperature for 10 minutes. The digested DNA fragments were separated by agarose gel-electrophoresis in 2% agarose gel, run at 60 volts for 1 hour and 30 minutes

and the bands were examined under a UV light of gel-doc system (Figure 2).

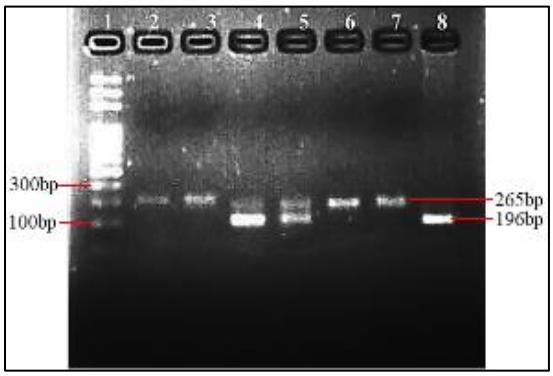

Figure 2: PCR reaction for VDR gene SNP FokI with gene products size 265bp and the ladder marker were resolved on 2% Agarose gel. RFLP analysis after digestion with FokI restriction enzyme lane 1 in gel shows DNA ladder, lane 8 shows homozygous TT (F/F) wild type, lanes 2,3,6,7 show homozygous CC (f/f) mutant, lanes 4,5 shows heterozygous TC (F/f) mutation.

VDR FokI site is located in exon 2. After RFLP using Fok1 endonuclease fragments, 3 different lengths can be obtained. Fok1 genotype was determined according to fragment length, homozygote TT (FF) subject = 196, 69bp product; heterozygote TC (Ff) = 265, 196 and 69bp product and homozygote CC (ff) subject = 265bp product. SNP resulting in T-C substitution in exon 2 of the VDR gene leads to the degeneration of a Fok1 restriction site. Homozygous subjects with alleles containing nucleotide T at this position showed an intact 265bp band and were designated as having CC Fok1 genotype. Likewise, homozygous subjects with alleles containing C at this position show 2 bands of 196 and 69bp (TT subjects). Similarly, products with all 3 bands: 265, 196 and 69bp were assigned as TC (Vandevyver et al., 1997).

Statistical analysis

The percentage genotype distribution of polymorphism and frequency of heterozygous and homozygous were compared between cases and control with RFLP using SPSS version 20. The chi-square ($\chi 2$) test was used to evaluate the Hardy-Weinberg equilibrium for the genotype distribution of patients and controls. Allele and genotype frequencies were compared between different study groups using the $\chi 2$, Odds ratio (OR) and the Z-test were calculated using Statcalc program (Epi info version 6.0.4, Atlanta, GA, July 1996).

Results and Discussion

The descriptive characteristics of the 100 diabetic mellites (DM) patients and 100 healthy controls are shown in Table 1. The gender-wise distribution of the case participants included 54 (54%) males, and 46 (46%) females and the control were 58 (58%) and 42 (42%) (p=>0.050). The overall mean age of participants was 57.6 and 55.49 years old in the case and control groups (p=<0.05). There was no significant difference between mean age, weight, BMI, systolic, diastolic, hypertension, glucose (F), glucose (PP), cholesterol, LDL, HDL, serum TG and Vitamin D ($p<0.05$), whereas height and cholesterol were significantly difference with the subjects taken for cases and control ($p>0.05$). Similarly, the occupation and alcohol consumption were not significantly different whereas age group and sex were significantly different with case and control groups (Table 1). Since the mean weight for cases were higher BMI than that of controls. In the case of blood pressure, mean systolic pressure case was high, controls were found to have normal systolic pressure. The mean diastolic pressure however was at normal range for both cases and controls. The variants like glucose level (F/PP), Vitamin D level, serum Triglycerides level, and LDL were in the normal range for controls and higher than the normal range for cases. However, other variants like cholesterol level and HDL were in the normal range for both cases and controls (Table 1).

Out of 100 cases, 36% of DM subjects were found to have the GG genotype, 57% carried heterozygous GA genotype and only 7% had mutant AA genome with 13.51, 11.93 and 0.012 Odds ratio (OR) respectively. Furthermore, allele-A was dominant (91%) in healthy control and 35.5% in DM with an OR of 0.55 whereas the G allele was dominant (64.5%) in DM and 9% in healthy control with an OR of 18.37. The frequency of VDR gene BsmI rs1544410 was not significant ($p < 0.05$, each) between cases and control (Table 2). Similarly, 64% of DM subjects were found to have the TT genotype, 27% carried heterozygous TC genotype and only 9% had mutant CC genome with 1.39, 0.60 and 1.54 Odds ratio (OR) respectively. Furthermore, the T allele was dominant (77.5%) in DM and 75% in healthy controls with an OR of 0.56. The frequency of VDR gene FokI rs2228570 was significantly different between cases and control ($p >0.05$, each) (Table 3).

Table 1: Socio-demographic and biochemical indices of diabetic Mellitus and healthy subjects.

Characteristics	Group	Case-control study		Z-test / χ^2-test	*p*-value
		Diabetes Mellitus	Control		
Age (Mean±**SD,**Years)	Age (Years)	57.6±12.93	55.49±17.68	3.81	0.00
Age Groups	30-49	25	28		
	50-69	51	46	1.03	0.79
	70-89	22	24		
	>90	2	2		
Sex	Male	54	58	0.32	0.56
	Female	46	42		
Height (Mean±**SD,** ft)	Height	5.26±0.41	5.19±0.33	0.81	0.41
Weight (Mean±**SD,** Kg)	Weight	66.49±11.40	48.29±5.85	43.82	0.00
BMI (Mean±**SD,** Kg/m^2)	BMI	26.88±4.30	20.06±2.62	25.92	0.00
Occupation	Housewife	32	24		
	Government officer	8	14		
	Non-Government officer	16	16	34.62	0.00
	Retired	28	14		
	Farmer	9	6		
	Businessman	7	26		
Alcohol Consumption	Yes	3	63		
	No	69	15	272.48	0.00
	Occasional	13	17		
	Stopped	15	5		
Systolic (Mean±**SD,** mmHg)	Systolic	129.85±17.07	121.29±5.9	17.86	0.00
Diastolic (Mean±**SD,** mmHg)	Diastolic	81.40±11.04	80.51±4.09	0.28	0.02
Hypertension	Yes	59	5	67.0	0.00
	No	41	95		
Glucose (F) (Mean±**SD,** mg/dl)	Glucose (F)	141.91±55.01	89.70±12.07	63.74	0.00
Glucose (PP) (Mean±**SD,** mg/dl)	Glucose (PP)	201.03±79.48	90.30±6.5	119.41	0.00
Family Diabetes History	Yes	24	4	16.61	0.00
	No	76	96		
Cholesterol (Mean±**SD,** mg/dl)	Cholesterol	157.53 ±34.19	158.4±31.61	1.07	0.28
LDL (Mean±**SD,** mg/dl)	LDL	93.40±38.60	86.72±6.97	9.89	0.00
HDL (Mean±**SD,** mg/dl)	HDL	41.90±19	39.94±2.11	4.26	0.00
Serum TG (Mean±SD, mg/dl)	Serum	175.52±62.73	133.4±24.70	45.04	0.00
Vitamin D (Mean±SD, ng/ml)	Vitamin D	17.54±2.66	24.40 ±9.41	19.74	0.00

Diabetes is associated with numerous factors like age, heredity, viruses, obesity, blood pressure, cholesterol, physical activity etc. Our study also linked diabetes with hypertension, serum triglyceride level and low-density lipoprotein. These parameters were found to be normal in non-diabetic subjects, but their mean was higher than the normal range in DM subjects. DM is also linked with high alcohol consumption, but we found that a greater number of non-diabetic controls were involved in the activity rather than cases. This was probably due to the reason that alcohol consumption may lead to severe consequences for DM patients rather than healthy ones. Moreover, the stoppage rate of alcohol consumption was high in some cases.

Despite the various factors involved in the onset of DM, the VDR gene plays one of the major parts as the gene is involved in the secretion, sensitivity as well as protection of insulin-producing B-cell from inflammation (Issa, 2017). Bsml polymorphism of the VDR gene occurs in intron 8 where removal of the restriction site occurs as the G allele gets replaced by A creating 3 different lengths of gene (825bp, 650 bp and 172 bp) (Tobón-Arroyave et al., 2017) after restriction digestion of the product with the longest size indicating the mutation, AA genotype.

Table 2: Vitamin D Receptor (VDR) genotype and alleles at BSML rs1544410 position in T2DM patients and healthy control.

Genotype	DM	Control	OR	95% Cl	p-value
GG	36	4	13.71	4.65-40.41	0.0001
GA	57	10	11.93	5.55-25.61	0.0001
AA	7	86	0.012	0.007-0.03	0.0001
Alleles					
G	129	18	18.37	10.45-32.30	0.0001
A	71	182	0.55	0.03-0.11	0.0001

The AA genotype is associated with increased production of VDR protein leading to a decrease in the risk of DM whereas the presence of the GG genotype is associated with lower lever Vitamin D as well as the different affiliated diseases (Sinharay et al., 2018; Ferrara et al., 2002; Morrison et al., 1994).

Table 3: Vitamin D Receptor (VDR) genotype and alleles at Fok1 rs2228570 position in T2DM patients and healthy control.

Genotype	DM	Control	OR	95% Cl	p-value
TT	64	56	1.39	0.79-2.46	0.24
TC	27	38	0.60	0.33-1.09	0.09
CC	9	6	1.54	0.53-4.52	0.42
Alleles					
T	155	150	1.19	0.73-1.82	0.56
C	45	50	0.87	0.55-1.38	0.56

Our study showed that for the VDR-Bsml polymorphism, the A allele was dominant (91%) in healthy controls, and the VDR-Bsml genotype distribution in this group was 4% with GG 10% with GA and 86% with AA. For DM subjects G allele was dominant (64.5%) and VDR-Bsml genotype distribution was 36% with GG, 57% with GA and 7% with AA. The odds ratio was 13.71, 11.93 and 0.012 with respective GG, GA and AA genotypes between cases and controls. These results were consistent with other research as reported by a meta-analysis conducted by Zhang et al. (2012) where populations of different regions were analyzed including Asian, European and Latino populations indicating that the subjects included in the current study represented the group well.

In comparison with the controls, the frequency of the GG and GA genotypes was significantly higher in subjects with T2D, suggesting an association between the VDR-FokI genotypes "GG and GA" and T2D patients of the Nepalese population. A similar result was obtained in the study conducted in Northeast India (Sarma et al., 2018) and China as well (Xu et al., 2014). However, Santos et al. observed conflicting results in the Brazilian population (Santos et al., 2012). Similar results were obtained for VDR-BsmI, a report by García et al., where three polymorphisms in the VDR gene were studied, focusing on their influence on the immune response in Chilean children with type 1 diabetes. As in the publication by García et al., the data obtained in this investigation do not suggest an association of VDR-BsmI with T2D (García et al., 2007). Likewise, Fokl is located in exon 2 of the VDR gene and its polymorphism involves alteration in the start codon leading to the shortening of the VDR protein (van Etten et al., 2007). In the N-terminal region of the VDR gene, a mutation when occurs at the 1st of the two start codons, ATG changes the nucleotide sequence to ACG leading to the translation of 3 additional codons downstream, resulting in a protein with an additional 3 amino acid chain (Harris et al., 1997). However, the reason for the difference in the activity of the two proteins, for example, if they bind to 1,25-hydroxyvitamin D differently, is not yet clear; therefore, the molecular mechanism that marks the association between individuals carrying the T allele of VDR-Fokl and T2D has not yet been defined.

Like Bsml, Fokl polymorphism, the change from T to C allele, leads to the disappearance of the restriction site. However, the formed longer mutant genotype CC (625bp) is found to decrease the secretion of VDR compared to wild-type TT (196bp, 69bp) (Tobón-Arroyave et al., 2017). Specific signs of disc generation in Turkish (Eser et al., 2010), Brazilian (Nunes et al., 2007), and Finnish (Videman et al., 1998) population subjects with TC and CC genotypes showed a predisposition towards worst genotype. Our study showed that for the VDR-FokI polymorphism, the distribution of both T and C alleles was similar for cases and control. The VDR-FokI genotype distribution in case and control was 65% and 56% with TT, 27% and 38% with TC and 9% and 6% with CC respectively. The OR ratio for respective TT, TC and CC genotypes were 1.39, 0.60 and 1.64. This proves that Fokl Polymorphism resulted to be insignificant ($p<0.05$) with DM for Nepalese population. Similar to our results, the findings on two subpopulations of Spain and Iran reported no association of Fokl ($p<0.05$) polymorphism with T1D. Moreover, the frequency of the CT allele was reported to be higher in cases as compared to controls in the Iranian population (Bonakdaran et al., 2012; Audi et al., 2004). In contrast, the current study reported that Fokl polymorphism significantly increased the chances of disease development in the Pakistani population. Further, the frequency of the Fokl "C" allele was higher in cases in Hungary (Maahs et al., 2010). The findings on Dalmatian as well as Japanese populations were in line with the results of our study that the Fokl restriction site was not significantly associated with T1D (Ban et al., 2001).

Conclusion

The findings of this study showed a significant association ($p \leq 0.05$) of the GG+GA genotype with DM cases compared to controls but the case is not the same for the FokI genotype. Furthermore, other variables such as gender, age, occupation, BMI, family history, blood pressure, alcohol consumption and serum triglyceride level were also not associated with VDR gene polymorphism except for LDL level. Thus, BsmI polymorphism can be used as a risk marker for DM in the Nepalese population.

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