Original Article

DOI: 10.5582/irdr.2015.01008

Validation of the association of *TCF7L2* and *SLC30A8* gene polymorphisms with post-transplant diabetes mellitus in Asian Indian population

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Summary **The rs7903146 and rs13266634 polymorphisms in the *TCF7L2* and *SLC30A8* genes, respectively, have been reported to be associated with type 2 diabetes. However, little is known about the association of these polymorphisms with post-transplant diabetes mellitus (PTDM). To study this linkage, we determined a distribution of allele and genotype frequencies in Asian Indians. 42 PTDM and 98 non-PTDM subjects were recruited. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis was performed to detect for rs7903146 and rs13266634 polymorphisms. The clinical details and statistical analysis for PTDM and non-PTDM subjects were recorded. Our results observed higher frequencies of the minor alleles in rs7903146 and rs13266634 polymorphisms in the PTDM group compared to the non-PTDM subjects. The allele frequencies also found to be significantly associated with PTDM (rs7903146: T *vs* C: OR-2.6; (95%CI: 1.2-5.6); p = 0.01; rs13266634: T *vs* C: OR-2.0; (95%CI: 1.1-3.4); p = 0.01). These findings suggest that rs7903146 and rs13266634 polymorphisms are associated with PTDM in the Asian Indian population despite a relatively small study group.**

Keywords: PTDM, non-PTDM, rs7903146, *TCF7L2*, rs13266634, *SLC30A8* and Asian Indians

1. Introduction

New onset diabetes after transplant (NODAT), or Post-transplant diabetes mellitus (PTDM), has become a common complication in renal transplant (RT) subjects (*1*). Tacrolimus (FK506, Tac) and cyclosporine A (CsA) are immunosuppressive drugs commonly used in the treatment after organ transplantation (*2*). Both PTDM and type 2 diabetes mellitus (T2DM) are multifactorial metabolic disorders (*3*). T2DM is a chronic disease, characterized by insulin resistance and impaired insulin secretion from pancreatic β-cells (*4*). PTDM incidence has been increasing dramatically throughout the globe, in parallel to T2DM (*5*). The pathophysiology of PTDM is characterized by a drug-induced deficiency in insulin secretion, rather than deteriorating of insulin resistance over time (*6*).

The appearance of family history of diabetes in predicting PTDM is still query and the relation for the risk factors of PTDM in the general population both from a common mechanistic viewpoint and from a practical viewpoint is unclear (*7*).Genome-wide studies have identified diabetes susceptibility loci on chromosomes 10q25.3 and 8q24.11, where transcription factor 7 like 2 (*TCF7L2*) and solute carrier member 3 zinc transporter member 8 (*SLC30A8*) genes are located (*3*). Earlier studies at the same genetic polymorphisms (*TCF7L2*-rs7903146 and *SLC30A8*-rs13266634) in PTDM subjects (*8,9*). Similar to T2DM, PTDM is a polygenic disease, and inconsistencies in genetic associations have been reported. In many of these studies, hypotheses were originated based on previously reported genetic associations of the predisposition to T2DM in general population, however no clear rationale

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Table 1. Details of the selection of snips for this study

Gene	region	Chr Region	SNP	rs No.	Forward Primer	Reverse Primer	Band Size	Enzyme
TCF7L2	Intron 3	10q25	C>**T**	rs7903146	ACAATTAGAGAGCTA AGCACTTTTTAGGTA	GTGAAGTGCCC AAGCTTCTC	C: 158/30 bp T: 188 bp	*Rsa*I
SLC30A8	Exon 8	8q24.11	C>**T**	rs13266634	GAAGTTGGAGTCA GAGCAGTC	TGGCCTGTCAAAT TTGGGAA	C: 176/80 bp T: 256 bp	*Hpa*II

was provided for the selection of the investigated genetic variants. Therefore, a well-defined candidate-gene approach is attractive, because it lowers the number of research hypotheses and reduces a chance of finding false genetic associations (*10*). Here we studied the association of rs7903146 (*TCF7L2*) and rs13266634 (*SLC30A8*) gene polymorphisms with the risk of developing PTDM after RT in Asian Indian population.

2. Materials and Methods

2.1. *Patient enrolment*

The study was carried out in Kamineni Hospitals, Hyderabad, India. First, the signed informed consent was obtained from the patients during the recruitment. In total, 140 subjects were recruited. Inclusion and exclusion criteria for selection of subjects were defined previously (*3*). Recruited subjects had undergone renal transplant and on immunosuppressive therapy for more than 3 months. Among them, 42 subjects developed PTDM and 98 were remained as euglycemic subjects, termed non-PTDM as per the American Diabetes Association. Patient information was documented, and the blood was collected to perform polymorphism.

2.2. *DNA and PCR-RFLP analysis*

Two mL of the peripheral blood were collected in tubes containing EDTA. Genomic DNA was extracted using a salting-out technique (*11*). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay was performed to detect rs7903146 and rs13266634 variations in *TCF7L2* and *SLC30A8* genes. The details of the selected primers, restriction enzymes, and band sizes for digested and undigested PCR products are shown in Table 1. Primers were synthesized by Bio-serve technologies limited, Hyderabad, India. Genotyping was performed using a 25-µL-reaction mix using the Bangalore Genei kit (Bangalore Genei Pvt. Ltd., Bangalore, Karnataka, India), and Applied Biosystems thermal cycler machine (Life Technologies, Carlsbad, CA, USA). *Rsa*I (GT^AC) enzyme was used to detect the rs7903146 polymorphism and *Hpa*II (C^CGG) enzyme for rs13266634 polymorphism. The protocol for PCR-RFLP analysis was described previously (*3*). The digested PCR products were separated by 3.5% agarose gel electrophoresis.

2.3. *Statistical analysis*

Genotype and allele frequencies were calculated using gene-counting method. Assessment of PTDM and non-PTDM parameters with alleles was performed using chi-square and Student's *t* tests. The difference in the occurrence of *TCF7L2*-rs7903146 and *SLC30A8*-rs13266634 genotypes in the PTDM and non-PTDM subjects was evaluated with the chi-square test. The odds ratios and 95% confidence intervals were used in order to determine the relative risks. Data on PTDM and non-PTDM individuals were statistically evaluated using the Openepi software. Hardy-Weinberg Equilibrium (HWE) was tested using the chi-square test with one degree of freedom. $p < 0.05$ was considered statistically significant.

3. Results

3.1. *Clinical investigation*

Current study has enrolled 140 subjects. Among the subjects, 42 had a case of PTDM, and 98 were non-PTDM controls. The clinical details of the PTDM and non-PTDM subjects are presented in Table 2. When we performed the *t*-test between PTDM and non-PTDM subjects, gender, weight, subjects with cyclosporine drugs (*i.e*. CsA, Tac) and along with the dosage were found statistically significant ($p < 0.05$).

3.2. *Genotypes and allele distribution*

Genotype distribution of *TCF7L2* (rs7903146) and *SLC30A8* (rs13266634) polymorphisms in cases and control subjects were in accordance with the HWE. The allele and genotype distribution in the PTDM and non-PTDM subjects are shown in Table 3. The distribution of rs7903146 genotype in PTDM subjects is 31% CC, 42.8% CT and 26.2% TT, while the non-PTDM group consists of 45.9% CC, 33.7% CT and 12.2% TT. The C and T allele frequencies in PTDM are 0.52% and 0.48%, and non-PTDM subjects are 0.75% and 0.25%, respectively. The genotype and allele frequencies were found to be significantly associated when we compared PTDM and non-PTDM subjects (for T *vs* C: OR-2.6; (95%CI: 1.2-5.6); $p = 0.01$ and for CT *vs* CC: OR-2.7; (95%CI: 1.5-4.6); $p = 0.0001$). Simultaneously, the dominant model also positive associated *i.e*. CT + TT *vs* CC: (OR-2.9; (95%CI: 1.3-6.4); $p = 0.006$). The PTDM

Table 2. Baseline characteristics for PTDM and non-PTDM subjects

S. No.	Characteristics	PTDM (n = 42)	non-PTDM (n = 98)	p Value
1	Males/Females	30/12	75/23	0.001
2	Age			
	a) Males (Mean ± S.D.)	39.39 ± 12.12	39.55 ± 10.58	0.27
	b) Females (Mean ± S.D.)	40.01 ± 11.63	39.26 ± 10.87	0.58
3	Weight			
	a) Males (Mean ± S.D.)	62.73±15.81	66.03 ± 12.73	0.08
	b) Females (Mean ± S.D.)	61.71 ± 16.93	65.49 ± 13.68	0.09
4	*a*) On CsA therapy	22	58	0.01
	b) On Tac therapy	20	40	0.02
5	*a*) CsA Dose (mg)	163.88 ± 57.4	201.29 ± 76.86	0.03
	b) Tac Dose (mg)	3.15 ± 1.24	3.11 ± 1.62	0.05
6	*a*) C2 levels (ng/mL) CsA	750 ± 299.03	1024.8 ± 353.42	0.23
	b) Trough levels (ng/mL) Tac	9.55 ± 3.38	8.0 ± 3.32	0.86
7	*a*) C2 levels/dose of CsA	5.24 ± 2.59	5.52 ± 1.97	0.02
	b) Trough levels/dose of Tac	3.62 ± 1.96	2.98 ± 1.49	0.02

Table 3. Genotype and Allele frequencies for rs7903146 and rs13266634 polymorphisms with T2DM and PTDM subjects with their specific control subjects

Genotypes/ Alleles	PTDM cases (n = 42)	Non-PTDM (n = 98)	OR (95% CI)	χ^2	p Value
TCF7L2					
CC	13 (31%)	57 (45.9%)	*Reference*		
CT	18 (42.8%)	33 (33.7%)	2.7 (1.5, 4.6)	13.8	0.0001
TT	11 (26.2%)	8 (12.2%)	3.1 (1.4, 6.6)	8.6	0.003
CT + TT	29 (69%)	41 (54.1%)	2.9 (1.3, 6.4)	7.3	0.006
C	44 (0.52)	147 (0.75)	*Reference*		
T	40 (0.48)	49 (0.25)	2.6 (1.2, 5.6)	6.2	0.01
SLC30A8					
CC	16 (38.1%)	59 (60.2%)	*Reference*		
CT	20 (47.6%)	32 (32.6%)	2.3 (1.0, 5.0)	4.4	0.03
TT	6 (14.3%)	7 (7.1%)	3.1 (0.9, 10.7)	3.5	0.06
CT + TT	26 (61.9%)	39 (39.7%)	2.4 (1.1, 5.1)	5.7	0.01
C	52 (0.62)	150 (0.76)	*Reference*		
T	32 (0.38)	46 (0.23)	2.0 (1.1, 3.4)	6.2	0.01

group consists of 38.1% CC, 47.6% CT and 14.3% TT genotypes in rs13266634 polymorphism, and non-PTDM group consists of 60.2% CC, 32.6% CT and 7.1% TT genotypes. The PTDM group consists of 0.62% of C and 0.38% of T alleles, while the non-PTDM consists of 0.76% C alleles and 0.24% of T alleles. The dominant model, genotype and allele frequencies were significantly associated with PTDM and non-PTDM subjects, *i.e.* for CT + TT *vs* CC: OR-2.4; (95%CI: 1.1-5.1); p = 0.01; T *vs* C: OR-2.0; (95%CI: 1.1-3.4); p = 0.01 and for CT *vs* CC: OR-2.3; (95%CI: 1.0-5.0); p = 0.03).

3.3. *Distribution between calcineurin inhibitors dosage in PTDM and non-PTDM subjects*

The genotype distribution was studied in PTDM and non-PTDM subjects who received treatments with CsA and Tac. Calculations of genotype and allele frequencies were performed using odds ratio and 95% confidence intervals, and are shown in Table 4. Treatment with CsA was significantly associated with rs7903146 genotypes in PTDM and non-PTDM subjects (T *vs* C: OR-3.3 (95%CI: 1.5-6.9); p = 0.001 and CT + TT *vs* CC: OR-4.3 (95%CI: 1.4-12.8); p = 0.005). However, none of the rs7903146 genotypes were significantly associated with Tac treatment in rs7903146 polymorphism (T *vs* C: OR-2.1 (95%CI: 0.9-4.6); p = 0.05 and CT + TT *vs* CC: OR-2.0 (95%CI: 0.6-6.2); p = 0.20). There was no association of treatment with CsA or Tac and rs13266634 polymorphism in both PTDM and non-PTDM subjects ($p > 0.05$).

4. Discussion

Currently, India is the second populous country in the World, after China. A high prevalence of endogamy and relatively low admixture distinguishes Asian Indians from most of other populations presently used in genetic studies (*12*). Life style and pharmacological intervention are important in prevention of diabetes in high-risk patients. Family history and genetic information are the practical tools in the identification of high-risk subjects (*13*). In the present study, we inspected the positive association of *TCF7L2* (rs7903146) and *SLC30A8* (rs13266634) polymorphisms with PTDM in Asian Indians. Allele and genotype frequencies were

Table 4. Genotype distribution between CsA and Tac dosage in PTDM and non-PTDM subjects

Genotypes	CsA (n = 80)				Tac (n = 60)			
	PTDM (n = 22)	non-PTDM (n = 58)	OR (95% CI)	p Value	PTDM (n = 20)	non-PTDM (n = 40)	OR (95% CI)	p Value
rs7903146								
CC	6 (27.3%)	36 (62%)	*Reference*		7 (35%)	21 (52.5%)	*Reference*	
CT	11 (50%)	19 (32.8%)	3.4 (1.1-10.8)	0.02	7 (35%)	14 (35%)	1.5 (0.4-5.2)	0.52
TT	5 (22.7%)	3 (5.1%)	10 (1.8-53.2)	0.002	6 (30%)	5 (12.5%)	3.6 (0.8-15.5)	0.07
CT + TT	16 (72.7%)	22 (37.9%)	4.3 (1.4-12.8)	0.005	13 (65%)	19 (47.5%)	2.0 (0.6-6.2)	0.20
C	23 (0.52)	91 (0.78)	*Reference*		21 (52.5)	56 (0.70)	*Reference*	
T	21 (0.48)	25 (0.24)	3.3 (1.5-6.9)	0.001	19 (47.5)	24 (0.30)	2.1 (0.9-4.6)	0.05
rs13266634								
CC	9 (40.9%)	35 (60.3%)	*Reference*		7 (35%)	24 (60%)	*Reference*	
CT	9 (40.9%)	19 (32.8%)	2.3 (1.0, 5.0)	0.26	11 (55%)	13 (32.5%)	2.9 (0.9-9.2)	0.06
TT	4 (18.2%)	4 (6.9%)	3.1 (0.9, 10.7)	0.07	2 (10%)	3 (7.5%)	2.2 (0.3-16.5)	0.40
CT + TT	13 (59.1%)	23 (39.7%)	2.4 (1.1, 5.1)	0.11	13 (65%)	16 (40%)	2.7 (0.9-8.4)	0.06
C	27 (0.61)	89 (0.77)	*Reference*		25 (62.5)	61 (0.76)	*Reference*	
T	17 (0.39)	27 (0.23)	2.0 (1.1, 3.4)	0.05	15 (37.5)	19 (0.24)	1.9 (0.8-4.3)	0.11

significantly associated with both polymorphisms ($p < 0.05$). This is a preliminary study, and our data is in accordance with earlier studies in other ethnicities looking at rs7903146 polymorphism (*8,14*), but not the rs13266634 polymorphism in PTDM subjects (*9*). The molecular mechanism underlying the progression of diabetes in PTDM is not fully understood (*15*). There are different forms of diabetes, such as type 1 diabetes mellitus (latent autoimmune diabetes of adults), T2DM, gestational diabetes, PTDM/NODAT, maturity onset diabetes of the young. These types have been ascribed to genetic defects in β-cell function or insulin action, endocrinopathies, diseases of the exocrine pancreas, and drug- or chemical induced and transient diabetes. However, there is no consensus on molecular diagnosis and screening in identification of diabetes patients. Current diagnosis and screening are based on biochemical analysis of coagulated blood serum (*4*). The results of the present study reveal that there is an association between *TCF7L2* and *SLC30A8* gene polymorphisms in PTDM in Asian Indians. In the previous study we have shown this association with PTDM compared to normal controls. We found a significant association of PTDM with both the alleles and genotypes ($p < 0.05$) (*14*). Present study was carried out comparing PTDM and non-PTDM subjects.

Several studies have observed at T2DM susceptibility loci. For instance, the associations were established between PTDM and genetic mutations (*16*) and T2DM genetic polymorphisms, such as *PPARG*, *POR*, *IGFBP2*, *ADIPONECTIN*, *KCNQ1*, *CALPAIN*, and *INTERLEUKIN* genes (*8,10,17-20*). Few genetic polymorphisms appeared as negative association, such as *SLC30A8*, *IGFBP2*, *HHEX*, *CDKN2A/B*, *PPARG*, *KCNJ11*, and *TCF7L2* (*9,21*). Our studies showed positive association towards rs7903146 and rs13266634 polymorphisms and not in accordance with the prior studies carried out in PTDM subjects in different ethnicities (*9,15,21*). The associations of diabetic diseases with rs7903146 and rs13266634 polymorphisms have been carried in Indian population (*14,22-29*). Our results showed the positive association between rs7903146 polymorphism and T2DM (submitted for publication) and GDM (unpublished). We also showed a varying association of rs13266634 polymorphism with T2DM (submitted for publication), and a significant association with GDM (unpublished). Meta-Analysis studies showed positive and negative associations of rs7903146 and rs13266634 polymorphisms and T2DM (*13,22,30*).

Earlier genetic studies have suggested the involvement of multiple genes in the pathogenesis of T2DM (*13*). GWAS studies identified hundred, thousands to million single nucleotide polymorphisms (SNPs) in various genes. Among them, *TCF7L2* and *SLC30A8* have been identified on 10q25.3 and 8q24.11 chromosomes containing rs7903146 and rs13266634 polymorphisms in intron 3 and exon 8 regions, respectively. These polymorphisms in the genes have been shown to be associated with impaired β-cell function (*31-32*). Sequence variations in introns or exons may affect the correct processing of the mRNA by disrupting the splice site or altering the secondary structure of the mRNA. Therefore, intronic variations can cause a blockage of translation and decrease in the protein expression levels (*33*). The initial GWAS reports discovered the diabetic genes in T2DM patients in Iceland and French populations (*34,35*). *TCF7L2* is a key component of the Wnt signaling pathway involved in the regulation of pancreatic β-cell proliferation, differentiation and insulin secretion, which regulates glucose metabolism, and rs7903146 polymorphism may operate *via* impaired glucagon-like peptide 1 secretion, stimulated more by fat than by carbohydrate ingestion. Association between *TCF7L2* variants and T2DM is supported by several prospective mechanisms, such

as decreased β-cell mass, liver insulin resistance, and altered chromatin state in 'T' allele carriers (*22,36*). The rs13266634 is an amino acid substitution (Tryptophan-Arginine) polymorphism most commonly associated with T2DM. The C325T missense variant is present in *SLC30A8* gene, product of which belongs to zinc transporter family involved in Insulin secretion (*25*). It plays a major role in transporting zinc from the cytoplasm to intracellular insulin-containing vesicles for insulin maturation, storage, and secretion from pancreatic β-cells (*37*). Zinc plays an important role in all processes of insulin trafficking, *i.e*. synthesis, storage, and secretion (*14*). Previous studies showed no association of rs13266634 polymorphism with PTDM in different ethnicities (*9,15,21*), as well with T2DM in Indian population (*25,28*). Chauhan *et al*., (*27*) and our earlier study (*14*) showed a positive association of rs13266634 polymorphism with PTDM. These results are consisted with our current finding in PTDM and non-PTDM. The results from the same population may vary due to the small sample size. Therefore, we calculated the power value of each analysis, and found it to be 76% and 78%. Thus, we are confident that our results are reliable. Transplant recipients on immunosuppressive therapy are at a particularly high risk of developing PTDM, which is a major adverse effect of immunosuppressive drugs used in the RT patients. The diabetogenicity of calcineurin inhibitors such as CsA and Tac have enabled this treatment to obtain its current place to demonstrate in humans to be mediated through suppression of pancreatic insulin secretion (*38*). These drugs are used to prevent rejection in patients with organ transplant. The role of immunosuppressive agents is to suppress the synthesis of DNA containing the blueprint of genetic information.

The major limitation of our current study was a lack of the information about glucose values, clinical data, selected the single SNP from each gene and small sample size. In conclusion, diabetes risk alleles in *TCF7L2* (rs7903146) and *SLC30A8* (rs13266634) are associated with PTDM subjects in Asian Indians, despite a relatively small study group. PTDM is an important health issue, and it is important to find biomarkers that can predict the risk of developing PTDM (*16*). Our findings could be used in finding and treatment of the high-risk renal transplant subjects with immunosuppressive drugs. Future experimental and mechanistic studies with a larger sample size, accurate clinical data in different ethnicities are warranted.

Acknowledgements

Our team is thankful to Kiran Kumar Vattam for providing the genomic DNA for PTDM and non-PTDM subjects. Our team is grateful to ICMR for providing the project funding and SRF to Imran Ali Khan Mohammed (Sanction no. 5-3-8-39-2007; RHN).

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(Received March 1, 2015; Revised April 11, 2015; Accepted April 16, 2015)