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Involvement of HLA-DR/DQ, ApoE and ACE I/D Gene Polymorphisms in Development of Secondary Complication in South Indian T2DM Patients

C. Rathika[*], K. Balakrishnan[*], T. Manikandan, N. Raja[δ], V. Palani Kumaran[¥] and N. S. Prasad[#]

[*]Department of Immunology, Madurai Kamaraj University, Madurai 620 021, Tamil Nadu, India
[*]Department of Biotechnology, Bharathidasan University, Tiruchirappalli 620 024, Tamil Nadu, India
[δ]Venu Elderly Hospital, Madurai 625 014, Tamil Nadu, India
[¥]Win Diabetics Centre, Madurai 625 009, Tamil Nadu, India
[#]Indira Seshadri Nursing Home, Tiruchirappalli 620 001, Tamil Nadu, India

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ABSTRACT The gene loci such as HLA (DRB1* and DQB1*), ACE I/D and ApoE were implicated in the spectrum of type 2 diabetes mellitus (T2DM) leading to secondary complications such as hypertension, retinopathy and cardiac complications. Two hundreds T2DM patients and 151 controls for ACE and ApoE and 156 T2DM patients and 102 controls for HLA-DRB1* and DQB1* were genotyped by PCR methods. The overall frequency of D allele (ID + DD) was higher in T2DM patients than controls (P = 0.056). Consistently higher frequency of ID genotype was noticed in T2DM patients (pooled) (69.50%), T2DM- without complications (71.71%) and T2DM- with complications (68.31%) than controls (49%). The genotype II was significantly elevated in controls than T2DM (p=0.0002), T2DM-WOC (p=0.0005) and T2DM-WC (p=0.0002). Further, we observed a complete absence of genotype II in patients with retinopathy, genotype DD in patients with hypertension and retinopathy and both II and DD genotype in patients with hypertension and neuropathy, conferring longevity and/or survival benefit to T2DM patients with these complications. The ApoE carrier ε2 (P = 0.007) and ε4 (*P* = 0.003) were strongly associated with T2DM. A preferential co-occurrence of ACE-ApoE was observed for ID–2/3 combination (*P* = 0.001) and ID-3/4 (*P* = 0.269) in T2DM patients than controls. For HLA, the allele frequency was the highest for DRB1*03 in patients than the controls (15.70 vs. 5.88%) and for DRB1*07 in controls than T2DM patients (23.52 vs. 12.17%) affording a susceptible and protective roles of these alleles respectively. Heterozygote combinations were preferentially seen in T2DM patients of higher age groups than controls.

INTRODUCTION

Type 2 Diabetes Mellitus (T2DM) is a common multifactorial disease with genetic predisposition that is strongly influenced by environmental and behavioral factors such as obesity and sedentary life style (Defronzo et al. 1992). It is more prevalent in Asian, American and Canadian Indians, African-Americans, Pacific Islanders, Hispanics and Japanese (Mokdad et al. 2003). Several candidate genes have been implicated in the development of diabetes. The ACE gene maps to chromosome 17q23, spans 21kb, and comprises 26 exons and 25 introns (Mattei et al. 1989; Hubert et al. 1991). The ACE encoded by the gene catalyzes the conversion of angiotensin I to the physiologically active peptide, angiotensin II, which controls fluid –electrolyte balance and systemic blood pressure. The insertion –deletion polymorphism is located in a noncoding region of the ACE gene and several investigators have shown that it is not silent and that the D allele is associated with increased serum activity of ACE in serum (Rigat et al. 1990; Foy et al. 1996; Sakuma et al. 2004). The ACE I/D polymorphism was reported in all populations studied so far, with marked ethnic differences (Johanning et al. 1995). Variation in the Alu ACE polymor-phism has been reported to associate with number of secondary complications of diabetes (Araz et al. 2001; Hadjadj et al. 2003). The DD genotype is known as an independent risk factor in several cardiovascular diseases such as hypertrophic cardiomyopathy (Marian

[*]*Address for correspondence:*
Dr. K. Balakrishnan
Associate Professor
Department of Immunology
School of Biological Sciences
Madurai Kamaraj University
Madurai 625 021,
Tamil Nadu, India
Mobile: 9842114117;
E-mail: immunobala@rediffmail.com

et al. 1993), myocardial infarction (Cambien et al. 1992; Kario et al. 1996) and ventricular hyper-trophy (Schunkert 1997), as well as chronic renal diseases such as IgA nephropathy (Yoshida et al. 1995), diabetic nephropathy (Doria et al. 1994), renal scarring (Ohtomo et al. 2001; Erdogan et al. 2004) and congenital urological anomalies (Hohenfellner et al. 2001). Apoli-poprotein-E (ApoE) gene has been considered as a good candidate susceptibility gene for T2DM. ApoE is a 299- amino acid glycoprotein that plays a central role in lipid metabolism. The presence of three alleles leads to the formation of six different phenotypes that is, ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4 and ε4/ε4 (Utermann et al. 1977; Hatters et al. 2006). ApoE acts as a high affinity ligand for several hepatic lipoprotein receptors, including the low-density lipoprotein (LDL) receptor (Huang et al. 2004). Genetic variation at the ApoE locus in humans is an important determinant of plasma lipid levels (Tan et al. 2003) with relative risk of coronary heart disease (Wilson Schaefer et al. 1996), diabetic vascular complications and Alzheimer's disease (Strittmatter et al. 2003).

The major histocompatibility complex (MHC) includes the HLA (Human Leukocyte Antigen) genes. It is located on the short arm of chromosome 6, between 6p21.31 and 6p21.33, and is characterized by a set of highly polymorphic genes. The role of HLA in the pathogenesis of type 1 diabetes was reported by several groups (Thomson et al. 1988; Kawabata et al. 2002). Its role in T2DM is less clear and weak links were reported (Larson and Alper 2004). Previous investigation on the contribution of HLA class II in the pathogenesis of T2DM is focused on its relationship with autoimmune markers and latent autoimmune diabetes (Horton et al. 1999; Tuomi et al. 1999), possible genetic interaction between type 1 and type 2 diabetes in families (Ramachandran et al. 1999; Li et al. 2001) and its association with complications and mortality in T2DM patients (Forsblom et al. 1998; Perez-Luque et al. 2003). In the present work, we aimed to study the ACE, ApoE and HLA –DR and DQ gene polymorphism as a predisposing factor and the possible interactions of different genotypic combinations in dictating the development of secondary complications in south Indian T2DM patients.

MATERIALS AND METHODS

Biochemical profiling of TGL, HDL, LDL, VLDL and total cholesterol were performed by standard clinical protocols. ACE I/D and ApoE polymorphism were studied in 200 T2DM patients and 151 controls from south India. The patients were stratified as T2DM-with (T2DMWC; n = 101) and without (T2DMWOC; n = 99) complications. Genomic DNA was extracted from 2 ml of peripheral blood leukocytes according to a standard salting-out method (Welsh and Bunce 1999). A detailed questionnaire was filled, an informed

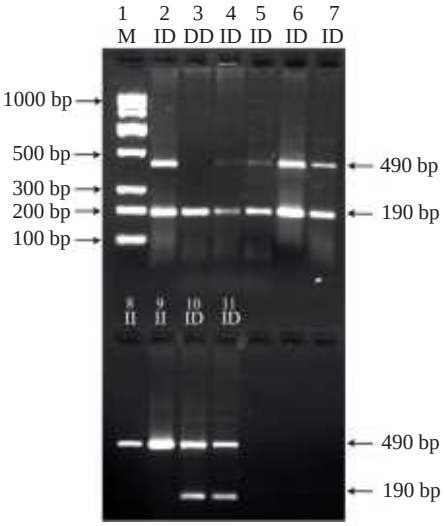
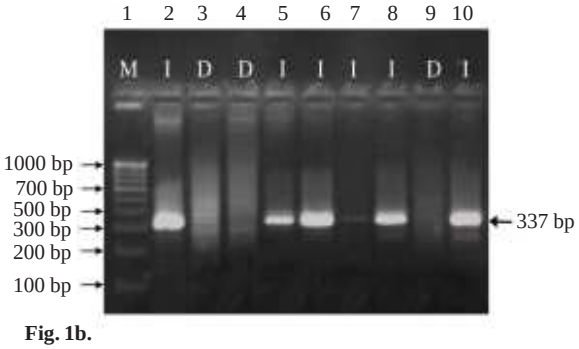


Fig. 1a.



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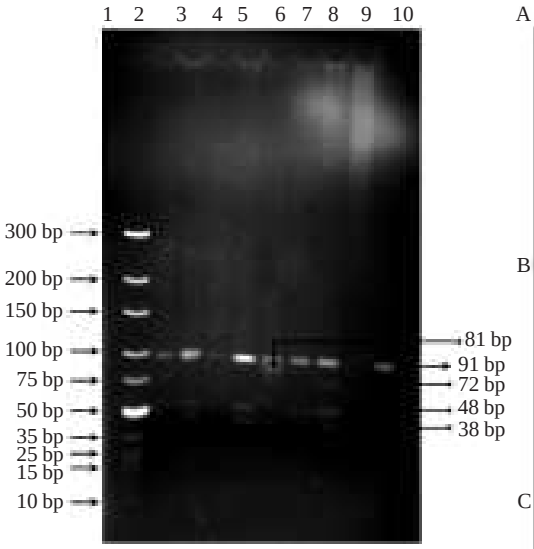

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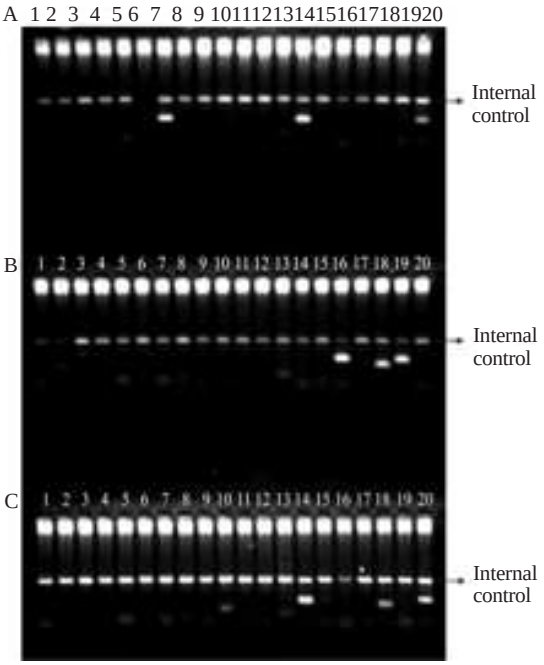

Fig. 1d.

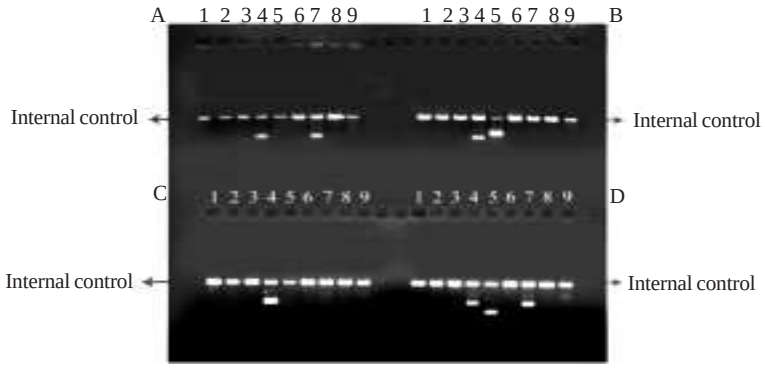

Fig. 1e.

Fig. 1a. Agarose gel electrophoresis stained with ethidium bromide, showing the initial amplification for ACE I/D polymorphism. Lane M represents the 100-1000 bp ladder. The II genotype for I allele was identified by the presence of single 490 bp product (lanes 8, 9). The DD genotype for D allele was identified by the presence of a single 190 bp product (lane 3). The DD homozygotes were reconfirmed with insertion specific primer to avoid mistyping as ID heterozygotes.

Fig. 1b. Agarose gel electrophoresis of PCR products, using insertion specific primer pair, of individuals labeled as DD homozygotes following initial amplification. Absence of the product in the lane (3,4 and 9) confirms the presence of DD genotype. Heterozygous individuals (ID genotype) were confirmed by the presence of a single 335 bp product (lane 2, 5, 6, 7, 8 and 10). Lane M represents the 100-1000 bp ladder.

Fig. 1c. Agarose gel electrophoresis for *Hha1* digested PCR product of ApoE genepolymorphism. M - Marker; Lane 3,5,8 : Apo E genotype ε3/ε3; Lane 6 : ApoE genotype ε2/ε3; Lane 8 : Apo E genotype ε3/ε4

Fig. 1d. Amplification pattern of HLA –DRB1* allele by PCR-SSP method; Lane 7: DRB1* 10;Lane 14: DRB1*15;Lane 20: DRB 5;Lane 16: DRB1*03;Lane 18: DRB 3;Lane 19: DRB4

Lane 10: DRB1*13; Lane 14: DRB1*15;Lane 18: DRB 3;Lane 20: DRB5

Fig. 1e. Amplification pattern of DQB1* by PCR –SSP method

Lane 4: DQB1*05Lane 07: DQB1*0301, 0304;Lane 4: DQB1*05;Lane 5: DQB1*06 (01/02/03);Lane 19: DRB4;Lane 4: DQB1*05;Lane 4: DQB1*05;Lane 5: DQB1*06 (01/02/03);Lane 7: DQB1* 0301, 0304

consent was obtained from the participants of the study and approved by Institutional Ethical Committee. Inclusion and exclusion criteria were followed as per ICMR guidelines. The ACE (I/D) genotype was determined by two polymerase chain reactions using the primers as described earlier (Shanmugam et al. 1993; Yoshida et al. 1996; Fig. 1a. and 1b.). The ApoE polymorphism was analysed following the method of Hixson and Vernier (1990) using restriction endonuclease enzyme *Hha1* (Fermentas, Cat No ER1851) (Fig. 1c.). HLA DRB1* and DQB1* alleles were genotyped for 156 T2DM patients and 102 controls by PCR–SSP method. DNA –typing of HLA –DR and DQ were performed using 30 and 19 sets of primers respectively following XI IHWC protocols (Olerup and Zitterquist 1992; Bunce et al. 1995). The amplified products were run on a 1.5% agarose gel stained with EtBr (Fig. 1d. and 1e.).

Statistical Analysis

The SPSS statistical software package version 11.5 was used for the statistical analyses. Genotype and allele frequencies of ACE and ApoE gene polymorphism were compared between T2DM patients and controls by using χ^2-test. Odds Ratio (OR) as estimates of relative risk (RR) for disease was calculated with 95% confidence intervals. Values are presented as mean ± S.D. Statistical significance was assumed at $P = 0.05$ level. HLA allele frequencies were calculated by direct counting. HLA–DRB1* and – DQB1* allele frequencies were calculated by using the equation [f = (n/N) × 100]: where "n" is the number and "N" is the total number of alleles. Haplotype analyses were done by using Alerquien 2.0 software. Allelic and haplotype frequencies were compared using Fisher's exact test. The PEP404X package was used for the statistics analysis.

RESULTS

Frequencies of ACE (I/D), ApoE, HLA –DR and DQ Genes

The genotype and allele frequencies for ACE I/D and ApoE are presented in Table 1a and 1b. The frequency of ID heterozygote was higher in T2DM patients (P= 0.0001; χ^2 = 14.299) than controls. The overall D allele frequency (DD + ID) was higher in patients (P=0.056; χ2 = 3.651). However, no significant difference in frequency was observed for DD genotype among patients and controls (20.50 vs. 23.17%; P = 0.617; OR = 0.85). Out of 156 T2DM patients,

Table 1a: Allele and genotype frequencies of ACE I/D polymorphism in T2DM patients, with- and without secondary complications and controls

Genotype/ Allele	*Group*	*% Frequency*	*Disease association indices*			
			OR	*95% CI*	χ^2	*p value*
II	Control[a] (151)*	27.81 (42)				
	T2DM[b] (200)	10.00 (20)	0.29	0.15 - 0.54	17.570	0.0002
	T2DMWOC [c] (99)	12.12 (12)	2.72	1.32 - 5.99	7.7941	0.0005
	T2DMWC[d] (101)	7.92 (8)	4.27	1.90 -10.92	3.835	0.0002
ID	Control	49.00 (74)				
	T2DM	69.50 (139)	2.36	1.49 - 3.77	14.299	0.0001
	T2DMWOC	71.71 (71)	0.38	0.21 - 0.67	11.745	0.006
	T2DMWC	67.32 (68)	0.48	0.27 - 0.82	7.335	0.06
DD	Control	23.17 (35)				
	T2DM	20.50 (41)	0.85	0.50 - 1.47	0.223	0.637
	T2DMWOC	16.16 (16)	1.54	0.78 - 3.18	1.407	0.236
	T2DMWC	24.75 (25)	0.99	0.53 - 1.88	0.000	1.000
I	Control	52.31 (158)				
	T2DM	44.75 (179)	0.74	0.54 - 1.01	3.651	0.056
	T2DMWOC	47.97 (95)	1.19	0.82 - 1.73	0.735	0.391
	T2DMWC	41.58 (84)	1.51	1.04 - 2.20	4.680	0.031
D	Control	47.68 (144)				
	T2DM	55.25 (221)	1.35	0.99 - 1.85	3.651	0.056
	T2DMWOC	52.02 (103)	0.84	0.58 - 1.22	0.735	0.391
	T2DMWC	58.41 (118)	0.66	0.452- 0.960	4.680	0.031

a-Controls; b-T2DM (pooled); c-T2DM-WOC; d-T2DM-WC; * - Figures in parenthesis indicates numbers (n)

Table 1b: Percentage frequencies of genotypes and alleles of ApoE gene in T2DM patients and controls

Genotype/ Alleles	*T2DM (n=200)*	*Controls (n=151)*	*Disease association indices*			
			OR	*95% CI*	χ^2	*p value*
ε2/ε2	1.00 (2)	2.64 (4)	0.41	0.05- 2.37	0.597	*0.040
ε2/ε3	15.00 (30)	11.92 (18)	1.29	0.67- 2.56	0.455	0.500
ε3/ε3	61.00 (122)	75.49 (114)	0.51	0.31- 0.83	7.563	*0.006
ε3/ε4	21.00 (42)	8.60 (13)	2.75	1.40- 5.79	9.081	*0.003
ε4/ε4	2.00 (4)	1.32 (2)	1.37	0.24-12.11	0.005	0.946
ε2	8.50 (34)	8.60 (26)	2.16	1.22- 3.89	7.272	*0.007
ε3	79.00 (316)	85.76 (259)	0.63	0.41- 0.95	4.863	*0.027
ε4	12.50 (50)	2.35 (17)	2.35	1.31- 4.42	8.631	*0.003

99 (63.46%) were showing any one form of diabetic asso-ciated secondary complications. Number of T2DMWC increases with the age of patients and the number of T2DMWOC remained high only in lower age groups i.e., up to 36-45 yrs. Thus, the transition from T2DMWOC to T2DMWC is around 50-55 yrs (Fig. 3). There observed an equal number of cases among T2DMWOC and T2DMWC in this age group. Among the age groups 56-65 yrs and above, the T2DMWC surpassed T2DMWOC. The mean age of T2DMWC patients bearing DD homozygote is lower (51.91 ± 8.61 yrs) than for those bearing II homozygote (58.00 ± 8.97 yrs) and ID het-erozygote (56.37 ± 9.51 yrs). The mean age of T2DMWOC bearing II, ID and DD geno-types are much lower (43.15 ± 7.45 yrs; 49.06 ± 12.20 and 48.73 ± 9.71 yrs respectively) than T2DMWC.

Among T2DM patients, the frequencies are 61, 21 and 15% respectively for ApoE genotypes 3/3, 3/4 and 2/3. The frequency of genotype 3/4 was higher in patients than controls (*P*=0.003; OR=2.75). The logistic regression analysis revealed a crude OR of 2.16 (*P*=0.007) for ε2 carriers, 2.35 for ε4 carriers (*P*=0.003) and 0.63 for ε3 carriers (*P*=0.027). The frequency of genotype 3/3 was the highest among controls than patients (*P*=0.0063; OR= 0.51). The distribution of HLA-DRB1* and DQB1* alleles were summarized in Table 2. The allelic frequency of DRB1*03 was significantly higher in patients than controls (*P*= 0.0005; OR=3.33). No significant difference in the frequency of DRB1*15 was observed in patients and controls (22.43 vs. 26.47%; *P* = 0.254). Interestingly, the frequency of allele DRB1*07 was higher in controls (12.17 vs. 23.52%; *P*=

Table 2: Percentage frequencies of HLA-DRB1*/DQB1* alleles in T2DM patients and controls

DRB1/DQB1* Alleles*	*Patients (n=156)*	*Controls (n=102)*	*Disease association indices*			
			OR	*95 % CI*	χ^2	*p value*
DRB1*01	1.28 (4)	0.98 (2)				
DRB1*03	15.70 (49)	5.88 (12)	3.33	1.65-7.29	12.119	**0.0005**
DRB1*04	7.05 (22)	5.88 (12)				
DRB1*07	12.17 (38)	23.52 (48)	0.37	0.20-0.64	13.298	**0.0003**
DRB1*08	2.24 (7)	2.94 (6)				
DRB1*09	1.28 (4)	0.49 (1)				
DRB1*10	14.42 (45)	11.27 (23)				
DRB1*11	5.76 (18)	3.92 (8)				
DRB1*12	2.56 (8)	2.94 (6)				
DRB1*13	5.12 (16)	3.92 (8)				
DRB1*14	3.20 (10)	2.94 (6)				
DRB1*15	22.43 (70)	26.47 (54)				
DRB1*16	0.64 (2)	0.49 (1)				
Blank	6.08 (19)	6.86 (14)				
DQB1*02	7.37 (23)	5.88 (12)				
DQB1*04	2.56 (8)	0.98 (2)				
DQB1*05	37.50 (117)	45.50 (93)	0.30	0.12-0.66	9.616	**0.002**
DQB1*06 (01/02/03)	19.23 (60)	14.20 (29)				
DQB1*06(03-09)	1.28 (4)	2.45 (5)				
DQB1*07	8.65 (27)	5.88 (12)				
DQB1*08	-	0.49 (1)				
Blank	23.39 (73)	24.50 (50)				

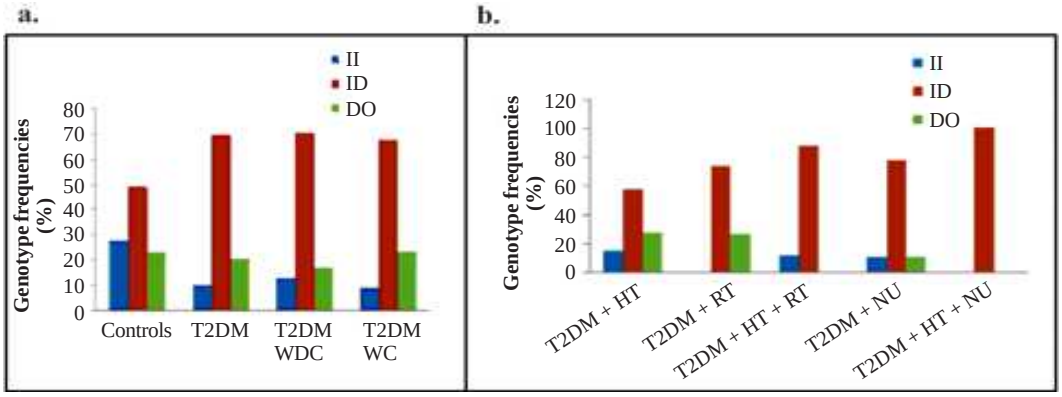

Fig. 2. Genotype frequencies of ACE I/D polymorphism in: (a) controls, T2DM patients with and without secondary complications; (b) T2DM patients with different types of secondary complications

0.0003; OR=0.37). No significant difference in distribution was noticed for any of the DQB1* alleles except the higher frequency for DQB1*05 in controls (*P*=0.002; OR=0.30) (Table 2).

Frequencies in Secondary Complications

The south Indian T2DM patients were presented with an array of micro- and macro- vascular complications such as hypertension, retinopathy, neuropathy, heart diseases and nephropathy. The data was analyzed based on with or without secondary complication (T2DMWC and T2DMWOC). The genotypic frequencies of II, ID and DD were 7.92, 67.32 and 24.75% and 12.12, 71.71 and 16.16% respectively for T2DMWC (n =101) and T2DMWOC (n =99). Among T2DMWC the hypertension (T2DM-WC–HT) was the most predominant complication (n = 40; 39.6%). The genotype frequencies were 15, 57.50 and 27.50% respectively for II, ID and DD (Table 1a). The second largest complication was retinopathy (RT), occurring in 15 patients (7.50%) exhibiting 73.33 and 26.66% respectively for genotypes ID and DD genotypes with a complete absence of genotype II. The combination of retinopathy and hypertension was observed in eight patients (4%) with genotypic frequencies of 87.50 and 12.20% respectively for ID and II with a complete absence of genotype DD. In patients with neuropathy (n=8),

Table 3: Percentage frequencies of DRB1*/ DQB1* in controls and T2DM patients with secondary complications

Allele	*Controls (n = 102)*	*T2DM patients (n = 55[a], 23[b], 15[c])*	*Disease association indices*			
			OR	*95% CI*	χ^2	*p value*
DRB1*03	**5.88 (12)**	**18.18 (20)[a]**	**4.18**	**1.77 -10.51**	**11.851**	**0.001**
		19.56 (9)[b]	**4.74**	**1.53 -15.30**	**8.193**	**0.004**
		13.33 (4)[c]				
DRB1*07	**23.52 (48)**	**11.81 (13)**	**0.36**	**0.16 - 0.77**	**7.295**	**0.007**
		13.04 (6)				
		13.33 (4)				
DRB1*09	**0.49 (1)**	1.81 (2)				
		2.17 (1)				
		16.66 (5)	**35.44**	**4.81 -1267.42**	**21.877**	**0.000**
DRB1*14	**2.94 (6)**	2.72 (3)				
		-	-	-	-	-
		16.66 (5)	**7.78**	**1.72 - 37.84**	**8.571**	**0.003**
DRB1*15	**26.47 (54)**	18.18 (20)				
		26.08 (12)				
		6.66 (2)	**0.16**	**0.02 - 0.69**	**6.710**	**0.010**
Blank	6.86 (14)	7.27 (8)				
		6.52 (3)				
		-				

a- Hypertension (HT; n=55) b- Retinopathy (RT; n=23) c- Neuropathy (NU; n=15)

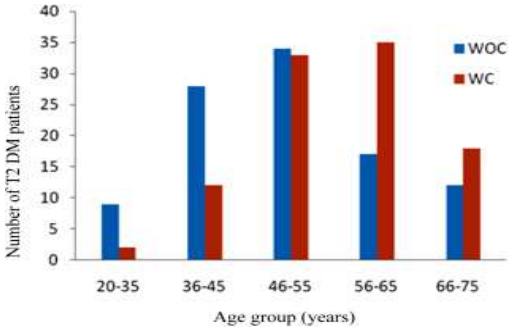

Fig. 3. Age groups in T2DM patients with (WC) and - without complications (WOC)

the frequencies were 11.11, 77.71 and 11.11% respectively for II, ID and DD genotypes. Neuropathy along with hypertension was observed only in seven (3.5%) T2DM patients and all of them were with ID genotype (100%; Fig. 2a, b). Thus, among T2DMWC, ID heterozygote selectively predominates than either of the homozygotes (II or DD). The other complications such as heart diseases were fewer in number and hence not subjected for frequency analysis (data not shown). The frequencies of ApoE genotypes were 12.50 (2/3), 65 (3/3) and 17.5% (3/4) among T2DM-HT and 20 (2/3), 67 (3/3) and 13% (3/4) among T2DM-RT and 12.50 (2/3), 62.50 (3/3) and 25 (3/4) among T2DM-HT+RT respectively (data not shown). No significant difference was observed for ApoE genotypes among T2DM patients with secondary complications. Table 3 represents the allele frequencies among T2DM patients with different secondary complications. The data for T2DM patients with hypertension and other secondary complication (s) was pooled (n = 55; HT, HT+ NU and HT+RT) for the purpose of analysis. The frequency differences were significant only for DRB1*03 (control vs. T2DM-HT; *P*=0.001; OR=4.18), DRB1*09 (control vs. T2DM-NU; *P*=0.001; OR=35.44). Further, a negative association was observed for DRB1*07 with T2DM-HT (*P*=0.007; OR=0.36). In T2DM-RT patients, DRB1*03 was significantly elevated (*P*= 0.004; OR= 4.74) and in T2DM+NU patients, the frequency of DRB1*14 (DR6) was significantly increased (*P*=0.003; OR=7.78).

ACE–ApoE-HLA Combination in T2DM

The analysis of data for the co-occurrence of ACE and ApoE genotypes revealed a preferential distribution of ID-3/3 in T2DM patients than controls (41.50 vs. 36.42%; OR=1.22 χ^2 = 0.729; *P*=0.393). This was followed by double heterozygote ID–2/3 in patients than controls (17vs.4.64%; χ^2 =11.579; P=0.001; OR=3.99) as the preferred hetero-zygote combinations in T2DM patients than controls. These observations revealed a possible role of heterozygote combination of ACE-ApoE gene loci in the development of secondary complications in T2DM patients. The genotype combinations such as DD–2/2, DD–2/3, II-2/2 and II-2/3 were poorly represented in both patients and controls. The II–3/3 combination was over–represented among controls than patients (P=0.0004; OR=0.24) (Table 5). Furthermore, there observed a preponderance of HLA–DR heterozygotes among ACE-ApoE

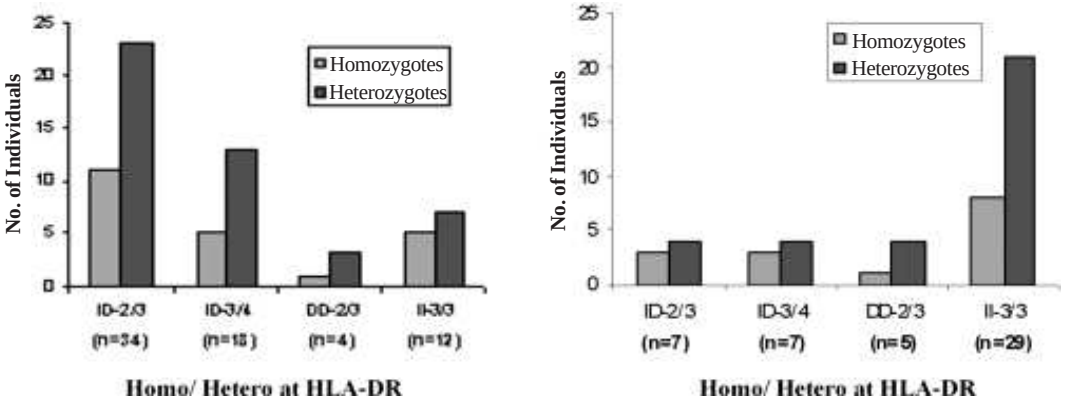

Fig. 4. Number of individuals heterozygous at HLA-DR for various ACE-ApoE genotypic combinations in (a) T2DM patients (left); and (b) controls (right)

Table 4: HLA-DRB1*-DQB1* two locus haplotype frequencies in T2DM patients and controls

Haplotype	*T2DM patients*	*Controls*	*Disease association indices*			
			OR	*95% CI*	χ^2	*p value*
DRB1*03-DQB1*05	0.0621	0.0451	1.337	(0.582-0.620)	0.637	0.0010
DRB1*07-DQB1*02	0.0250	0.0125	1.921	(3.379-3.600)	0.636	0.0006
DRB1*07-DQB1*06	0.0185	0.0173	1.410	(1.132-1.201)	0.567	0.0004
DRB1*11-DQB1*05	0.0199	0.0245	0.993	(2.134-2.263)	0.668	0.0009
DRB1*11-DQB1*0301	0.0129	0.0098	1.306	(3.548-3.771)	0.641	0.0051
DRB1*12-DQB1*0301	0.0154	0.0098	1.581	(3.871-4.113)	0.629	0.0016
DRB1*13-DQB1*05	0.0210	0.0063	2.498	(2.521-2.674)	0.691	0.0112
DRB1*15-DQB1*0301	0.0113	0.0098	1.297	(4.838-5.14)	0.659	0.0007

Table 5: Percentage frequency distribution of ACE-ApoE genotype combinations in T2DM patients and controls

ACE –Apo E combination	*T2DM patients (n=200)*	*Controls (n=151)*	*Indices of disease association*			
			OR	*95% CI*	χ^2	*p value*
ID-2/2	1.00 (2)	0.66 (1)				
ID-2/3	17.00 (34)	4.64 (7)	3.99	1.72 - 10.77	11.579	0.001
ID-3/3	41.50 (83)	36.42 (55)				
ID-3/4	9.00 (18)	5.29 (8)				
ID-4/4	0.50 (1)	1.98 (3)				
DD-2/2	0.50 (1)	0.66 (1)				
DD-2/3	2.00 (4)	4.64 (7)				
DD-3/3	12.50 (25)	14.50 (22)				
DD-3/4	5.50 (11)	3.31 (5)				
II-2/2	0.50 (1)	1.32 (2)				
II-2/3	2.00 (4)	3.31 (5)				
II-3/3	6.00 (12)	21.11 (32)	0.24	0.11 - 0.50	16.752	0.0004
II-3/4	1.50 (3)	1.32 (2)				
II-4/4	0.50 (1)	0.66 (1)				

double heterozygotes showing an inverse correlation with genotypic combination among T2DM patients than the controls (data not analyzed for any specific HLA type; Fig 4).

DISCUSSION

ACE I/D, ApoE and HLA-class II (DR and DQ) genes are implicated in T2DM and its complications (Shimizu et al. 2004; Baghai et al. 2004). In recent years a vast amount of data has been published on the association of ACE I/D dimorphism and diabetes mellitus. In the present study, the frequency of ACE ID heterozygote and D allele was significantly higher in T2DM patients than the controls. Previous studies have shown that the DD genotype was strongly associated with the increased plasma or serum ACE levels (Rigat et al. 1990; Ormezzano et al. 2005). Thus the DD genotype favors high ACE expression and activity and thus predisposes individuals to T2DM and its complications (Feng et al. 2002; Wolf and Ritz 2003). Hypertension forms the single largest type of secondary complication with ID heterozygote as the predominant genotype among T2DM-HT (57.50%), T2DM-HT+RT (87.50%) and T2DM-HT+NU (100%) compared to controls (49%). Previous studies have shown that more than 50% of T2DM patients with newly diagnosed T2DM will present with co-existing hypertension (HTN), and ~ 50% of patients with essential HTN (Stern et al. 1995; Daimon et al. 2003). A significant association of the D allele with essential hypertension was documented in the African-American (McFarlane et al. 2001), Chinese (Duru et al. 1994) and Japanese populations (Nakano et al. 1998; Nikzamir et al. 2008). Taiwanese stroke patients with DD genotype (with traditional risk factors) were reportedly at the highest risk and were the best candidates in considering the preventability of stroke by using an ACE inhibitor (Tseng et al. 2007). In south Indian T2DM patients, hypertension (HTN) is common and is a major risk factor for coronary heart disease (CHD), particularly when associated with diabetes. The DD genotype is a marker for diabetic nephropathy (Butler et al. 1997; Fujisawa et al. 1998), hypertension (Butler et al. 1997; Kennon et al. 1996), renal artery steno-

sis (Missouris et al. 1996), cardiomyopathies (Butler et al. 1997; Taniguchi et al. 2001; Jimenez et al. 2007). To the best of our knowledge, ours is the first report revealing the complete absence of II homozygote among diabetic retinopathy patients. Further, the finding that the complete absence of DD homozygote in T2DM-HT+RT (n=8) and both II and DD in T2DM-HT+NU (n=7) was noteworthy finding and needs to be confirmed in a larger cohort. These findings emphasized the detrimental role of ID heterozygote in the development of secondary complications in T2DM. Thus, the heterozygous state along with other host genetic and/or environmental factors dictates T2DM patients to develop spectrum of secondary complications. In the present study, the ACE II homozygotes were significantly higher in controls than the patients (P=0.0002; χ^2=17.570; OR= 0.29). Thus, ACE II genotype and I allele in particular may afford protection because of its threefold elevated frequency in controls than T2DM patients.

Thus, it is hypothesized that T2DM patients develop secondary complication only after the age of 50-55 yrs and preferably among those bearing ACE ID heterozygote genotype. This finding compels us to propose a possible role of ACE ID heterozygote in affording the longevity to T2DM patients, however with, debilitating secondary complications.

The present study documented a significant increase of HLA-DRB1*03 among patients than controls suggesting a strong role of this allele in the development of T2DM. However, several previous studies reported the association of this allele with T1DM (Dorman and Bunker 2000; Ganga et al. 2004). Further, analysis of our data revealed a similar frequency of HLA-DRB1*15 in both the patients and the controls. However, this allele, is found to be the commonest in South East Asia and south Indian populations (Pitchappan et al. 1984; 1986). A positive association of HLA-DRB1*1502 with anti-glutamic acid decarboxylase (GAD)-positive T2DM was reported (Fukui et al. 1998). Finnish T2DM patients had a higher frequency of HLA-B7, -DR2, -DR5, -Cw4, and -DR3/DR4 when compared with either type 1 diabetes patients or controls (Groop et al. 1988). An increased frequency of DRB1*0701 was observed in controls than T2DM patients in the Middle East (Zavala et al. 1992; Motala et al. 2005). Studies in Mexican and Turkish T2DM patients revealed a positive association of DRB1*070101 with T2DM (Groop et al. 1988; Fukui et al. 1998; Perez-Luque et al. 2003). In the present study, a significant increase of DQB1*06 (DQw1) in T2DM patients was observed. A few studies have reported the association of DQB1*0201 (Gambelunghe et al. 2000; Li et al. 2000) and DQB1* 0601 with T2DM patients from different ethnic backgrounds (Horton et al. 1999; Obayashi et al. 2000; Li et al. 2000). However, other reports failed to link HLA–DQ with T2DM (Bruno et al. 1999) and hence it would appear that the association between HLA class II (DR and DQ) with T2DM is racially and geographically restricted.

There observed a preferential occurrence of HLA–DR heterozygotes among ACE-ApoE double heterozygotes. At is point, it is highly meaningful to realize the fact that heterozygote at MHC gene loci increases the health and survival benefits in semi natural animal populations subjected for simulated epidemics. However, the evidence for the heterozygote advantage hypothesis is mixed and equivocal (Apanius et al. 1997; Penn 2002). MHC heterozygosity is associated with resistance to some infectious disease (Jeffery et al. 2000) and prolonged survival of HIV infected individuals (Carrington et al. 1999). In the present study too, the heterozygotes at multiple genetic loci (ACE-ApoE–HLA-DR) are advantageous in terms of longevity. Nonetheless, these individuals are leading an uncomfortable way of life with myriads of secondary complications. In other words, the T2DM patients with secondary complications bearing homozygotes are under high selection pressure to be eliminated. Hence, these heterozygotes that affords survival and/or protection. Given the limitations of the present study such as, number of patients in each secondary complication and non-inclusion of family members of T2DM patients, these tentative conclusions need to be reconfirmed in a larger cohort in multicentric collaborative studies.

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