Evaluating the association between type-2 diabetes and polymorphisms at +1127 *INS–Pst*I and +3580 *IGF-II–Msp*I locus

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Type-2 diabetes is a heterogenous, multifactorial disease with a strong genetic background. The objective of the study was to analyse the relationship between polymorphisms present in the human insulin gene (*INS*) because of its impact on glucose homeostasis and its immediate neighbouring *IGF-II* gene and genetic susceptibility to type-2 diabetes. This is a study on the Indian population involving screening of *INS* and *IGF-II* candidate genes for nucleotide variation associated with type-2 diabetes. Our study population comprised fifty diabetic subjects and forty age-matched nondiabetic controls. Restriction fragment length polymorphism was employed to study +1127T/C polymorphism in *INS* gene and +3580G/A polymorphism in *IGF-II* gene. Biochemical analysis revealed hypertriglyceridaemia in diabetic group. It was observed that *T*-allele frequency in *INS + 1127* gene was significantly high in normals having high triglyceride level. No correlation was observed between susceptibility to type-2 diabetes and +3580 *IGF-II* and +1127 *INS* polymorphism. Moreover, homozygous TT was found to be the predominant *INS* genotype in Indian population, in contrast to CC which was predominant in African American, Caucasian and Hispanic populations. We propose here that our pilot study be used as a base for future comprehensive studies exploring the inter-relationships among different genetic variations at VNTRs–*INS-IGF-II* and predisposition to diabetes.

Keywords: IGF-II polymorphism, INS polymorphism, type-2 diabetes.

TYPE-2 diabetes is the most common form of diabetes mellitus constituting 90% of the diabetic population. The prevalence of diabetes for all age-groups worldwide was estimated to be 2.8% in 2000 and 4.4% in 2030. The total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030. India, China and the United States are among the top three countries estimated[1] to have the highest number of people with diabetes (i.e. 31.7, 20.8 and 17.7 millions respectively) in 2000 and (79.4, 42.3 and 30.3 millions respectively) in 2030. Epidemiological studies among migrant Asian Indians in many countries showed higher prevalence of type-2 diabetes compared with the host populations and other migrant ethnic groups[2]. A national survey[3] of diabetes conducted in six major cities in India in the year 2000 showed that the prevalence of diabetes in urban adults was 12.1%. Type-2 diabetes is a multifactorial disease with both a genetic component and important non-genetic component(s), which interact in order to precipitate the diabetic phenotype[4]. Although much is known about environmental factors contributing to type-2 diabetes, such as obesity, sedentary lifestyle, smoking and certain drugs, much less is known about the genetic predisposition which is required for the onset of the more common form(s) of the disease. Identification of genes predisposing individuals to develop type-2 diabetes will facilitate early diagnosis and thereby lead to effective treatment and intervention strategies[5]. An absolute insulin deficiency in type-1 diabetes and a relative (rather than absolute) insulin deficiency in type-2 diabetes could be the result of defects in the insulin gene. Cloning of insulin gene has facilitated molecular–genetic studies, which include identification of multiple DNA sequence polymorphisms detected with restriction fragment length polymorphisms (RFLPs) and sequencing. The insulin gene (*INS*) has been mapped to the short arm of chromosome-11 adjacent to the insulin-like growth factor II (*IGF-II*) and tyrosine hydroxylase genes. The combined RFLPs for the insulin, *IGF-II* and tyrosine hydroxylase (*TH*) genes make this a highly informative locus for genetic studies in diabetes[6].

The variable number of tandem repeats (VNTRs) that lie immediately adjacent to the 5′ promoter region of *INS* are believed to have a direct effect on *INS* regulation. In addition to VNTRs, there are 19 non-coding SNP markers (polymorphisms) in the *TH–INS–IGF-II* region on chromosome 11p15.5, which are in tight linkage disequilibrium with each other and with the VNTRs, such that they constitute two major haplotypes. In such a region of tight linkage disequilibrium, assaying for one marker would generally provide genotype information of all the others. We assayed for two of these SNP markers, +1127 T/C polymorphism of *INS* and +3580 G/A polymorphism of *IGF-II* in type-2 diabetes. The +1127 *INS-Pst*I marker is located in the 3′ untranslated region (UTR) of *INS*; the UTR regions of the preproinsulin mRNA have recently been demonstrated to play a crucial role in regulating insulin production. The 3′-UTR of *INS* acts cooperatively with the 5′-UTR and markedly increases glucose-induced proinsulin biosynthesis. Therefore, polymorphisms at +1127 *INS–Pst*I, although located in an untranslated region[7], may have functional effect on the expression of *INS*. IGF-I and IGF-II are single-chain polypeptide having 62% homology with proinsulin. IGFs are regulators of processes like growth and metabolism[8–10]. IGF-1 and IGF-II also contribute to pancreatic β-cell growth and development by regulating β-cell replication, renewal and apoptosis[11,12]. Deregulation of the balance between β-cell renewal and apoptosis due to alterations in IGF levels is potentially of

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great importance in the development of glucose intolerance. Polymorphisms in *INS* and *IGF-II* have been extensively studied and shown to be implicated in type-1 diabetes[13–17]. But few studies have been done evaluating their significance in type-2 diabetes[18–20]. In the present study we have examined the physiological significance, if any, of these two polymorphisms in type-2 diabetic patients of India.

A total of fifty diabetic subjects (equal number of men and women) with mean age 49.82 yrs, who came for clinical testing at SRL-Ranbaxy, Mumbai were included in the present study. The criterion for diagnosis of diabetes mellitus was based on the recommendations of the American Diabetes Association's modified form of the WHO report, 1985 (fasting glucose ≥126 mg/dl and 2 h post load glucose value ≥200 mg/dl after 75 g oral glucose intake)[21]. Forty age-matched subjects with no clinical evidence of diabetes mellitus and biochemically showing normal glucose tolerance (fasting plasma glucose <110 mg/dl and 2 h post load glucose <140 mg/dl) were selected randomly from corporate health check-up samples by filling up questionnaires. Biochemical evaluation included determination of fasting plasma glucose, 2 h post glucose load after 75 g oral glucose intake and lipid profile [total cholesterol, triglycerides, high density lipoprotein (HDL) and low density lipoprotein (LDL)]. The fasting glucose and 2 h post glucose load determination were carried out using fluoridated plasma, while for lipid estimation 12 h fasting serum sample was used. Plasma glucose levels were determined by hexokinase-glucose-6-phosphate dehydrogenase method on Dade Dimension AR Analyser (Dade Behring, USA). A lipid profile (total cholesterol, triglycerides and HDL) was measured spectrophotometrically on Dade Dimension AR Analyser, while LDL was calculated using Friedwald formula.

Genomic DNA was extracted from EDTA anticoagulated whole peripheral blood using Flexi Gene DNA system (Qiagen, GmbH, Germany) and 10 µl of this was used for PCR amplification. PCR amplification was performed in 50 µl volumes, containing 1 µg genomic DNA, in a reaction mix containing 1X PCR buffer, 200 µM dNTPs, 2.5 mM $MgCl_2$, 50 mM KCl, 1 µM each of sense and antisense primers and 1 unit of Taq DNA polymerase (Invitrogen, Carlsbad, CA). Primers used to amplify the genomic regions were *INS*+1035 5′–GGG TCC CCT GCA GAA GCG TGG CA–3′ and *INS*+1597 5′–CTC CCT CCA CAG GGA CTC CAT C–3′ for insulin gene and *IGF-II-Msp*F 5′–CCA CCC CTT CTG GGA AGC TAA AAG–3′ and *IGF-II-Msp*R 5′–CCC TCG GTC CTC CAG GAA TGG ACA–3′ for *IGF-II* gene. PCR amplification involved an initial denaturation step at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 55°C for 45 s and 72°C for 45 s, with final elongation step at 72°C for 7 min. The *INS* amplicon was more refractory to amplification, being a GC-rich region. Hence, we used Hot Star Taq DNA Polymerase (Qiagen, GmbH, Germany), with proof-reading activity to obtain reliable amplification. Amplified DNA (15 µl) was subjected to overnight digestion with 10 U of enzyme using the manufacturer's recommendations. Digested products were size fractionated on 3% agarose gel and visualized by UV-induced ethidium fluorescence. The restriction fragments of the alleles are as follows:

INS T allele – 562 bp
C allele – 470 bp + 92 bp

IGF-II A allele – 122 bp + 118 bp
G allele – 122 bp + 84 bp + 34 bp.

Data were represented as mean ± SD in case of continuous variables. Hardy–Weinberg equilibrium was assessed by comparison of observed genotype frequencies, with expected genotype frequencies inferred from observed allele frequencies. Standard contingency tables were used to calculate odds ratio and χ^2 analysis was performed to generate *P* values. The odds ratio (95% confidence interval, CI), was used as a measure of association between genotypes and diabetes. Unpaired *t*-test was used in case of continuous variable.

The age-matched diabetic group (50 cases) and normal group (40 controls) were compared for several biochemical parameters (Table 1). Three subjects in the diabetic group had been excluded for calculation of mean LDL levels, as their triglyceride levels were above 400 mg/dl. RFLP analysis was performed to determine the distribution of genotypes at +3580 *IGF-II-Msp*I locus (Figure 1 *a*) and +1127 *INS-Pst*I locus (Figure 1 *b*). The percentage distribution of the three *IGF-II* genotypes AA, AG and GG in diabetic group was 20, 50 and 30% respectively, whereas in the normal group these frequencies were 20, 42.5 and 37.5% (Table 2). According to the Hardy–Weinberg equilibrium, the frequency for A allele and G allele was observed to be 0.45 and 0.55 respectively, in the diabetic group in comparison to 0.41 and 0.59 in the normal group (Table 2). When the biochemical parameter of triglyceride level was taken into consideration (cut-off value of 170 mg/dl)[22,23], there was no significant difference in the frequency of A and G alleles in the diabetic group having triglyceride level >170 mg/dl as well as <170 mg/dl. The frequency of A allele was found to be significantally higher in normal groups having triglyceride values >170 mg/dl (0.58) compared to normal groups having triglycerides values <170 mg/dl (0.34). However, the odds ratio of 0.36 indicates that A allele of *IGF-II* is not a risk factor for high triglyceride levels (Table 3).

The frequencies of the three *INS* genotypes CT, TT and CC in the diabetic group were 24, 70 and 6% respectively, whereas in the normal group were 25, 72.5 and 2.5% (Table 4). Hardy–Weinberg equilibrium gives the frequency of C and T alleles to be 0.18 and 0.82 in diabetic group compared to 0.15 and 0.85 in normal group (Table 4). With reference to triglyceride levels (cut-off value of 170 mg/dl), the frequency of C and T alleles in the diabetic group

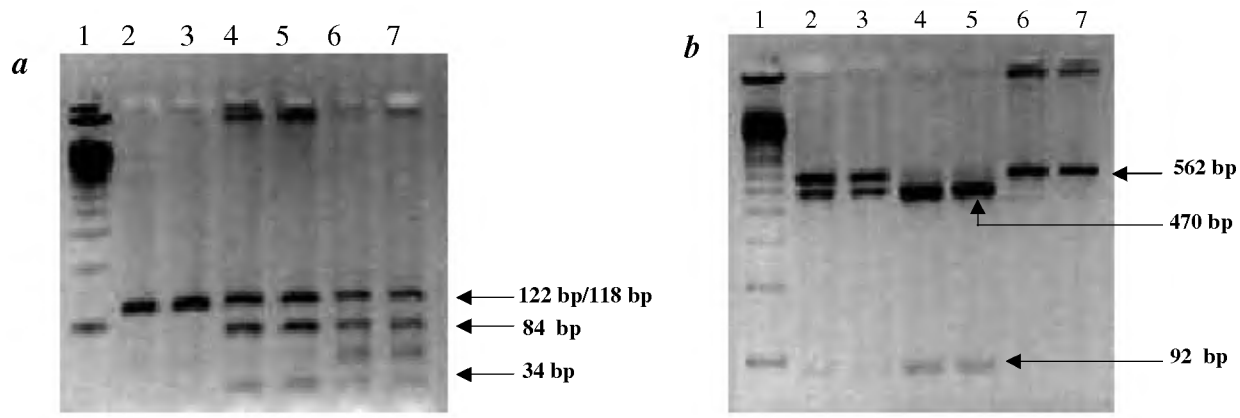

Figure 1. Representative gels depicting RFLP patterns of (***a***) *IGF-II* and (***b***) *INS* genes. Lane 1, 100 bp ladder; lanes 2–7, Specimens showing various alleles of *IGF-II* and *INS*. ***a***, Genotype distribution at *IGF-II* locus. Lanes 2, 3, AA homozygous genotype showing 122/118 bp bands; lanes 4, 5, AG heterozygous genotype showing 122/118/84/34 bp bands. Lanes 6, 7, GG homozygous genotype showing 122/84/34 bp bands. ***b***, Genotype distribution at *INS* locus. Lanes 2, 3, CT heterozygous genotype showing 562/470/92 bp bands; lanes 4, 5, CC homozygous genotype showing 470/92 bp bands; lanes 6, 7, TT homozygous genotype showing 562 bp bands.

Table 1. Clinical and biochemical parameters for diabetic patients and normals

Clinical and biochemical parameters	Diabetic group	Normal group	*P* value
Age (yrs)	49.82 ± 9.11	45.52 ± 10.92	0.05
Fasting glucose (mg/dl)	190.84 ± 58.76	91.7 ± 7.64	< 0.0001
2 h post glucose load (mg/dl)	288.52 ± 83.88	101.4 ± 15.77	< 0.0001
Triglycerides (mg/dl)	187.16 ± 97.3	130.2 ± 46.69	0.001
Total cholesterol (mg/dl)	195.89 ± 48.93	192.33 ± 35.50	0.7009
LDL* (mg/dl)	138.38 ± 78.58	124.66 ± 30.67	0.3007
HDL (mg/dl)	40.56 ± 8.78	40.25 ± 10.14	0.1443

Data are presented in arithmetic mean ± SD.
*Three subjects in diabetic group had been excluded for calculation of mean LDL levels as triglycerides levels were > 400 mg/dl.

Table 2. Distribution of *IGF-II* genotypes and alleles in diabetic patients and normals

Group	Sample size	AA (%)	AG (%)	GG (%)	Frequency of G allele	Frequency of A allele	Odds ratio (confidence interval)
Diabetic patients	50	10 (20)	25 (50)	15 (30)	0.55	0.45	0.86
Normals	40	8 (20)	17 (42.5)	15 (37.5)	0.59	0.41	(0.48–1.55)

having triglyceride levels >170 mg/dl was 0.24 and 0.76 in comparison to allele frequency of 0.3 and 0.7 in diabetic group having triglyceride levels <170 mg/dl (Table 5). When the normal group was categorized with respect to high level (>170 mg/dl) and low triglyceride levels (<170 mg/dl), there was significant difference in the frequencies of T (0.88 vs 0.68) and C alleles (0.13 vs 0.32). Odds ratio of 3.3 indicates that T allele may be a major risk factor for elevated triglyceride levels in normals, but this needs to be confirmed by increasing sample size in the normal group (Table 5).

Despite several epidemiological studies in India showing a rising prevalence of diabetes, there is a striking lack of genetic studies pertaining to type-2 diabetic Indian patients. A comprehensive national study by Ramachandran *et al.*[3] indicates towards a large pool of subjects with impaired glucose tolerance that has a high risk of conversion to diabetes. The present pilot project attempts to understand the involvement of SNPs in *IGF-II* and *INS* promoters and its implications in predisposition and diagnosis. After reviewing the literature it was observed that various SNPs exist in the *INS–IGF-II* region having implications in obesity, type-1 diabetes, polycystic ovary syndrome, hypertension and recently, various cancers also. We selected two SNP markers of +3580 *IGF-II–Msp*I and +1127 *INS–Pst*I that have shown significant role in susceptibility to few cancers like prostate[24]; colorectal cancer[25], etc. for evaluating their use as susceptibility markers in type-2

Table 3. Distribution of *IGF-II* genotypes with respect to triglyceride (TG) levels in diabetic patients and normals

Group	Sample size	AA (%)	AG (%)	GG (%)	Frequency of G allele	Frequency of A allele	Odds ratio (confidence interval)
Diabetic							
TG value > 170 (mg/dl)	23	3 (13)	14 (60.9)	6 (26.1)	0.57	0.43	1.12
TG value < 170 (mg/dl)	27	7 (25.9)	11 (40.7)	9 (33.3)	0.54	0.46	(0.51–2.45)
Normal							
TG values > 170 (mg/dl)	12	4 (33.3)	6 (50)	2 (16.6)	0.42	0.58	0.36
TG values < 170 (mg/dl)	28	4 (14.3)	11(39.3)	13 (46.4)	0.66	0.34	(0.13-0.96)

Table 4. Distribution of *INS* genotypes and alleles in diabetic patients and normals

Group	Sample size	CC (%)	CT (%)	TT (%)	Frequency of T allele	Frequency of C allele	Odds ratio (confidence interval)
Diabetic patients	50	3 (6)	12 (24)	35 (70)	0.82	0.18	0.80
Normals	40	1 (2.5)	10 (25)	29 (72.5)	0.85	0.15	(0.37–1.69)

Table 5. Distribution of *INS* genotypes with respect to triglyceride (TG) levels in diabetic patients and normals

Group	Sample size	CC (%)	CT (%)	TT (%)	Frequency of T allele	Frequency of C allele	Odds ratio (confidence interval)
Diabetic							
TG value > 170 (mg/dl)	27	6 (22.2)	1 (3.7)	20 (74.1)	0.76	0.24	1.38
TG value < 170 (mg/dl)	23	6 (26.1)	2 (8.7)	15 (65.2)	0.70	0.30	(0.57–3.34)
Normal							
TG values > 170 (mg/dl)	12	1 (8.3)	1 (8.3)	10 (83.3)	0.88	0.13	3.3
TG values < 170 (mg/dl)	28	9 (32.1)	0 (0)	19 (67.9)	0.68	0.32	(0.87–12.4)

diabetes. A recent study by Sanchez-Corona *et al.*[20] analysed eight different polymorphisms in *INS*, *INSR* and *IRS1* genes in the Mexican population. Among the eight polymorphisms analysed, the *Pst*I polymorphism in *INS* was significantly associated with hypertriglyceridaemia (>169.5 mg/dl). However, there are few reports showing that a locus at 11p18.5 confers susceptibility to insulin-dependent diabetes mellitus[17,26]. In a recent study, Rani *et al.*[27] showed that the loci DRB*0301 and *INS*–VNTR class I have the potential to predict predisposition to develop type-1 diabetes mellitus in North Indians. Here, we have evaluated the significance of VNTR–*INS*–*IGF-II* locus as a surrogate marker to determine individuals susceptibility to develop type-2 diabetes. We clarify that this was a pilot project carried out with comparatively small sample size. The objective of the project was to screen two SNPs showing relevance to type-2 diabetes risk, to get some idea of the genotype distribution pattern for these polymorphisms, before replicating them in large population studies.

Several new findings emerged from our study exploring the biochemical parameters and genotype distribution in *IGF-II* and *INS* genes in type-2 diabetes patients. Comparison of biochemical parameter revealed that fasting glucose and 2 h post glucose load were quite high in the diabetic group ($P < 0.05$) than the normal group. The high triglycerides in patient group confirmed diabetic triglyceridaemia in this group. It was observed that T allele at +1127 *INS–Pst*I locus was associated with high triglyceride accumulation risk in normal group. These observations need to replicate in large sample size in order to use T allele at +1127 *INS–Pst*I as a surrogate marker to individuals susceptibility for elevated triglycerides in Indian ethnic group. Similar observations were made by Sanchez-Corona *et al.*[20], where *Pst*I polymorphism in the *INS* gene was shown to be associated with hypertriglyceridaemia (triglycerides >169.5 mg/dl). Thus, apparently the lipid metabolism is hampered and affected in diabetic people compared to normals. We observed no significant association between +3580 *IGF-II* and +1127 *INS* polymorphism with type-2 diabetes in our study population. In case of *IGF-II* polymorphisms, genotype frequencies (AA, AG, GG) were comparable to other populations, viz. African Americans, Caucasians and Hispanic. However, homozygous TT was the predominant *INS* genotype (70%) in the Indian population compared to African American, Caucasian and Hispanic population, where the predominant genotype was CC (72, 61 and 79% respectively)[24].

The screening of various candidate genes for nucleotide variations associated with type-2 diabetes is a key component of diabetes genetic research. The significance of the genotype distribution within the important SNPs in the Indian population cannot be overemphasized. Hence,

more comprehensive studies covering other important SNPs in key genes pertaining to glucose metabolism are planned to be carried out.

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Received 10 August 2004; revised accepted 3 March 2005

Molecular characterization of a *Pao*-like long terminal repeat retrotransposon, *Tamy* in saturniid silkworm *Antheraea mylitta*

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A long terminal repeat (LTR) retrotransposon named *Tamy*, was obtained by screening *Antheraea mylitta* sub-genomic DNA library with PCR amplified partial fibroin gene sequence as probe. *Tamy* was 8387 nucleotides long with 1305 nucleotides of long LTRs at its 5′- and 3′-ends having features characteristic of a functional LTR retrotransposon. Starting from its N-terminus, nucleic acid binding motif (Cys), protease, reverse transcriptase (RT), RNAaseH and integrase domains were present in sequential order. RT domain in *Tamy* showed high homology with *Pao*-like retrotransposable elements. This is a report of a *Pao*-like LTR retrotransposon in *A. mylitta* genome.

Keywords: *Antheraea mylitta*, *Pao*-like retrotransposons, reverse transcriptase, Tasar silkworm.

THE genomes of eukaryotic organisms that display several multi-copy DNAs dispersed randomly in the genome, is referred to as mobile genetic elements or transposable ele-

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