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SNP Screening of PC-1 Gene And Biochemical Analysis In Type-II Diabetic Patients From Jaipur (Rajasthan, India)

KEYWORDS: PC-1, SNP, Diabetes Mellitus-2. ENPP1, K121Q, India.

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ABSTRACT *The type II diabetes mellitus (T2DM) is characterized by insulin resistance but the genes responsible for it are poorly defined. Ectonucleotide pyrophosphatase-1 (ENPP1) also known as plasma cell membrane glycoprotein-1 (PC-1) is known to influence insulin signal transduction pathway. Previous studies have demonstrated that the K121Q variant of ENPP1 gene has a significant role in determining susceptibility to insulin resistance resulting in T2DM. To assess whether the K121Q polymorphism in ENPP1 gene has any impact on T2DM and other atherogenic factors in Indian subjects, a case-control association study was done on 28 T2DM and 27 control subjects from Jaipur, Rajasthan, India. Blood samples were collected under fasting condition to assess the relevant biochemical parameters. PCR-RFLP analysis revealed the KK, KQ and QQ genotypic frequencies (at the 121 position of the ENPP1) to be 71.4%, 28.6%, 0% and 70.4%, 29.6%, 0% in diabetic and control subjects, respectively. However between T2DM and control subjects fasting blood glucose (FBG) was found to vary significantly within and between different genotypic groups. Clinical parameters and atherogenic factors among genotypes were found to have no significant differences. There was significant positive correlation between systolic and diastolic blood pressure- 0.707**, total cholesterol (TC) and triglycerides 0.354**, TC and high density lipoprotein (HDL-C) 0.407**, TC and low density lipoprotein (LDL-C) 0.958** and TC and very low density lipoprotein (VLDL-C) 0.354** and HDL-C and LDL-C 0.226* while there was non-significant negative correlation between BMI and FBG -0.084. $\chi 2$ -test revealed significant association of genotypes with FBG ($\chi 2$- value 9.655, df= 3, sig= 0.022). We conclude that the K121Q polymorphism in the present population from Jaipur, Rajasthan, India is moderate ranging from 25-30% that is having significant positive correlation with FBG.*

Introduction

Type-II Diabetes Mellitus (T2DM) is the most common form of diabetes accounting for more than 90 percent of all diabetes cases (Ramachandran et al., 2002) and is closely associated with Coronary Heart Disease (CHD) and Obesity (Fontbonne & Eschwege, 1991, Pyorala et al., 1998, Pyorala et al., 1985, Despres et al., 1996, Howard et al., 1996, Reaven, 1988, Lillioja et al., 1993, Haffner et al., 1990, Bergstrom et al., 1990, Charles et al., 1991, Bogardus et al., 1985, Abate et al., 1995, & Frittitta et al., 2001). It has been reported that Asian Indians are highly susceptible to this disease making India infamous diabetic capital of the world (Mohan et al., 2007). An International Diabetes Federation (IDF) estimate and other studies put the total number of diabetic patients to be around 40.9 million in India in 2007 that is projected to rise to ~70 million by the year 2025 (Mohan et al., 2007, McKeigue et al., 1991, Chandalia et al., 1999, & Sicree et al., 2006). Genetic factors have been implicated to play an important role in the development of this disease by development of insulin resistance, though the genes responsible for it are poorly defined (Pizzutti et al., 1999: Frittitta et al., 1996). The causative factors become more complex as the gene may be elicited by the non-genetic factors resulting from rapid lifestyle changes (Kommoju & Reddy, 2011).

Ectonucleotide pyrophosphatase-1(ENPP-1) also called plasma cell membrane glycoprotein-1 (PC-1) is known to affect insulin signaling (by direct interaction with α-subunit of the insulin receptor) inhibiting it either at the level of the insulin receptor or downstream at a post-receptor site (Maddux et al., 1995, Maddux & Goldfine, 2000, & Kumakura et al., 1998). It is a class II transmembrane glycoprotein, having broad specificity of cleaving variety of substrates including phosphodiester bonds, which is involved in various physiological processes ranging from bone mineralization to the insulin singling pathway (Maddux et al., 1995, Maddux & Goldfine, 2000, Kumakura et al., 1998, & Youngren et al., 1996). Out of the several polymorphisms located in this gene, the K121Q single nucleotide polymorphism (SNP) has been implicated to play a role in insulin resistance due to the strong interaction of the Q allele protein with the insulin receptor, reducing or inhibiting the receptor tyrosine kinase activity and subsequent signaling pathways (Frittitta et al., 2001, Frittitta et al., 1996, Kommoju & Reddy, 2011, Maddux et al., 1995, Maddux & Goldfine, 2000, Kumakura et al., 1998, Youngren et al., 1996, Frittitta et al., 1997, Frittitta et al., 1998, & Costanzo et al., 2001). The clinical manifestation of this polymorphism, which is documented to have allelic frequency variation among different ethnic groups, is reported to have conflicting results for its association with T2DM and associated atherogenic factors (Abate, 2003).

Hence, the present study was designed to assess the genotypic frequency of K121Q SNP in ENPP1 from Jaipur (Rajasthan, India) and its association with diabetogenic and atherogenic factors.

Volume : 4 | Issue : 10 | October 2014 | ISSN - 2249-555X

Material and Methods

Subjects

A total 55 diabetic and control subjects from Jaipur were recruited from the local clinic of Jaipur (Rajasthan, India) by offering free lipid profiling and fasting blood glucose (FBG) assessment. As this study was conducted in accordance with the tenets of the declaration of Helsinki, the study was cleared by the institutional ethics committee and informed consent was obtained from each subject. All the participants were administered health questionnaire. Subjects were divided into two categories (a) Case Group- Patients with diabetes were identified based on their history, use of hypoglycemic agents and FBG ≥110 mg/dl, (b) Control Group- Subjects were identified based on FBG level less than <110 mg/dl and no history of diabetes. T2DM patients on insulin treatment were excluded from this study.

Basic Biochemical Parameters:

Weight and Height were taken by standard technique for body mass index (BMI) calculation; Blood pressure was measured by a trained person with Hicks-N800- digital blood pressure monitor after 5 min rest in seated position. Fasting blood samples (3ml) were drawn from vein of patients into an EDTA containing vaccutainer. Plasma was separated by centrifugation and stored at 4ºC until analyzed. FBG, total-Cholesterol (TC), and triglycerides (TGL) were estimated by standard enzymatic methods; High density lipoprotein cholesterol (HDL-C) was determined in supernatant by precipitating apolipoprotein B containing lipoproteins using phosphotungstate in presence of magnesium ion; very low density lipoprotein cholesterol (VLDL-C) and low density lipoprotein cholesterol (LDL-C) were calculated by Friedewald's equation (Friedewald et al., 1972).

PCR-RFLP:

Genomic DNA was isolated from whole blood by standard phenol- chloroform method. Quantity as well as quality of isolated genomic DNA was analyzed by using Nanodrop 1000 (Thermofisher). The integrity of isolated genomic DNA was checked by resolving on 0.8% Agarose gels. For amplification of ENPP-1 gene, forward primer 5'-CTGTGTTCACTTTGGACATGTTG-3', and a reverse primer 5'-GACGTTGGAAGATACCAGGTTG-3' (Frittitta et al., 1996) were used to perform PCR in a thermal cycler (Quanta Biotech Ltd., England). The reactions were performed in a total volume of 30 µl containing 100ng of genomic DNA, forward and reverse primer, 1 U Taq DNA polymerase (Promega) and 100 µM/ liter dNTPs. The PCR program conditions were- initial denaturation at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 40 Sec, annealing at 52°C for 40 Sec, and elongation at 72°C for 40 Sec, and a final elongation step at 72°C for 5 Min.

K121Q polymorphism was screened by digestion of amplified PCR product with AvaII restriction enzyme at 37°C for 1:30 hours followed by electrophoresis on 2% Agarose gel (Fig. 1). The absence of AvaII restriction site resulted in a single 238bp band while its presence resulted in two bands of 148bp and 90bp. Some of the amplicons were validated by commercial sequencing and Blast search to confirm the SNP.

Statistical Analysis

All the analysis was performed using SPSS 17.0 (For windows) statistical analysis software. Independent sample t-test was conducted within and between groups for both diabetic and control subjects to compare means of various biochemical and atherogenic factors. Bivariate correlation was performed to see any co-variability of these factors. Association were considered statistical significant at the P value of 0.05 or 0.01. χ^2 goodness of fit test was also performed between the T2DM and control groups to infer any association between genotypes and FBG levels.

Results

The frequency of KK genotype was 71.4% in diabetic and 70.4% in control subjects, while the frequency of KQ variant was 28.6% and 29.6% for diabetic and control subjects respectively. The QQ genotype was not recovered during the present study. When the differences in age were investigated the mean age of control subjects (45.59 ± 16.15) was less than mean age for diabetic subjects (56.21 ± 9.29).

Table 1: Clinical and biochemical characteristics according to the K121Q polymorphism of the PC-1 gene For The Subjects Of Present Study From Jaipur (Rajasthan, India).

Group	Heterozygous diabetics KQ variant (N=8)	Homozygous diabetics KK variant (N = 20)	Heterozygous control KQ variant (N=8)	Homozygous control KK variant (N= 19)	TOTAL N=55
Factor	MEAN ± S.E.	MEAN ± S.E.	MEAN ± S.E.	MEAN ± S.E.	MEAN ± S.E.
%	28.6%	71.4%	29.6%	70.4%	
Age (Year)	61.25 ± 11.0	54.20 ±7.75	42.88 ± 13.75	46.74 ± 17.28	
BMI (kg/m^2)	26.2 ± 1.62	26.80 ±1.20	26.49 ± 2.55	24.84 ± 1.25	25.99 ± 0.74
Systolic blood pressure (mmHg)	130.00 ± 5.00	126.6 ± 1.86	128.12 ± 4.62	130.00 ± 3.67	128.49 ± 1.71
Diastolic blood pressure (mmHg)	85 ± 1.89	84 ±1.12	83.75 ± 1.83	85.78 ± 1.92	84.72 ± 0.85
Fasting Blood Glucose (mg/dl)	166.25 ± 12.03	154.9 ± 4.13	103.12 ± 1.48	71.36 ± 1.58	120.16 ±5.92
Total cholesterol (mg/dl)	185.25 ±10.4	175.1 ± 9.29	204.62 ± 16.79	184.94 ± 11.89	184.27 ± 6.03
Triglycerides (mg/dl)	174.5 ± 16.15	173.75 ±10.10	181.87 ± 15.95	159.21 ± 12.70	170.01 ± 6.53
HDL-cholesterol (mg/dl)	43.75 ± 2.10	46.9 ± 1.87	47.5 ± 1.63	49.52 ± 1.68	47.43 ± 0.99
LDL-cholesterol (mg/dl)	106.6 ± 8.99	93.45 ± 9.01	120.75 ± 14.54	103.57 ± 10.09	102.83 ± 5.40
VLDL-cholesterol (mg/dl)	34.9 ± 3.23	34.75 ± 2.02	36.37 ± 3.19	31.84 ± 2.54	34.00 ± 1.31

When the clinical characteristics and genotype frequen-

Volume : 4 | Issue : 10 | October 2014 | ISSN - 2249-555X

cies of control and diabetic subjects were compared; the FBG values were found to vary significantly between diabetic and control groups. The independent-samples t-test results for comparison of FBG levels between various groups (Table-2) revealed a significant difference in the FBG levels between Heterozygous diabetics (M=166.25, SE=12.03) and heterozygous controls (M=103.12, SE=1.48); t(14) = 5.21, P = 0.001, Homozygous diabetics (M=154.9, SE=4.13) and Homozygous controls (M= 71.36, SE=1.58); t(37)= 18.495, P = 0.001, Heterozygous diabetics (M=166.25, SE=12.03) and Homozygous diabetics (M=154.9, SE=4.13); t(26)= 1.15, P = 0.263, heterozygous controls (M= 103.12, SE=1.48); and Homozygous controls (M= 71.36, SE=1.58); t(25)=12.027, P = 0.001, and total diabetics (M=133.96, SE=9.22) and total controls (M=77.22, SE=2.46); t(53)= 14.081, P = 0.001, respectively.

Table 2:
Independent sample t-test between heterozygote diabetic and Control (T1), homozygous diabetic and Control (T2), and between total diabetics and control (T3) for the subjects of present study from Jaipur (Rajasthan, India).

	T1 (df = 14)	Sig. (2-tailed)	T2 (df = 37)	Sig. (2-tailed)	T3 (df = 53)	Sig. (2-tailed)
BMI (kg/m^2)	-.095	.926	1.124	.268	.868	.389
Systolic blood pressure (mmHg)	.275	.787	-.840	.407	-.545	.588
Diastolic blood pressure (mmHg)	.475	.642	-.814	.421	-.522	.604
Fasting Blood Glucose (mg/dl)	5.209	.000	18.495	.000	14.081	.000
Total cholesterol (mg/dl)	-.981	.343	-.656	.516	-1.060	.294
Triglycerides (mg/dl)	-.325	.750	.901	.373	.612	.543
HDL- cholesterol (mg/dl)	-1.411	.180	-1.042	.304	-1.502	.139
LDL- cholesterol (mg/dl)	-.828	.422	-.751	.458	-1.063	.293
VLDL- cholesterol (mg/dl)	-.325	.750	.901	.373	.612	.543

Table 3: Bivariate correlation between various diabetogenic and atherogenic factors for the present subjects from Jaipur (Rajasthan, India)

	BMI	SBP	DBP	FBG	CHL	TGL	HDL-C	LDL-C	VLDL-C
BMI	1	.157	.092	.129	.076	.090	-.084	.078	.090
P value		.127	.252	.174	.292	.258	.271	.285	.258
SBP	.157	1	.707**	-.055	.099	.080	.102	.073	.080
P value	.127		.000	.346	.235	.282	.229	.297	.282
DBP	.092	.707**	1	-.099	.094	.174	.095	.046	.174
P value	.252	.000		.236	.247	.102	.246	.370	.102
FBG	.129	-.055	-.099	1	.022	.103	-.169	.030	.103
P value	.174	.346	.236		.437	.228	.109	.413	.228
CHL	.076	.099	.094	.022	1	.354**	.407**	.958**	.354**
P value	.292	.235	.247	.437		.004	.001	.000	.004
TGL	.090	.080	.174	.103	.354**	1	.190	.119	1.000**
P value	.258	.282	.102	.228	.004		.082	.193	.000
HDL-C	-.084	.102	.095	-.169	.407**	.190	1	.226*	.190
P value	.271	.229	.246	.109	.001	.082		.049	.082
LDL-C	.078	.073	.046	.030	.958**	.119	.226*	1	.119
P value	.285	.297	.370	.413	.000	.193	.049		.193
VLDL-C	.090	.080	.174	.103	.354**	1.000**	.190	.119	1
P value	.258	.282	.102	.228	.004	.000	.082	.193	

A close look at the table-1 reveals that, KK genotype variants of T2DM patients had remarkably lower blood pressure (SBP=126.6 ± 1.86 and DBP= 84.0 ± 1.12) than KQ variants (SBP= 130.0 ± 5.00 and DBP= 85.0 ± 1.89), opposite of the control subjects where KK variant had higher blood pressure (SBP= 130.0 ± 3.67 and DBP= 85.78 ± 1.92) than KQ variant (SBP= 128.12 ± 4.62 and DBP= 83.75 ± 1.83). The atherogenic factors showed a similar trend of high TC, TGL, LDL-C, VLDL-C, and low HDL-C in KQ variant of both diabetic and control subjects, though it was not significant.

The KK genotypic controls had significantly lower BMI (24.84±1.25) than KQ variant (26.49 ± 2.55) in controls, while there was very small non-significant difference in BMI of T2DM subjects (KK= 26.8 ± 1.20, and KQ= 26.2 ± 1.62). There was significant positive correlation between SBP and DBP - 0.707**, TC and TGL 0.354**, TC and HDL-C 0.407**, TC and LDL-C 0.958** and TC and VLDL-C 0.354** and HDL-C and LDL-C 0.226*. While there were non-significant negative correlation between BMI and FBG -0.084. χ test was performed to analyze association between the genotypes and FBG levels reveals that there is sufficient evidence to conclude that the genotypic groups (KK and KQ genotype) are significantly different (χ^2- value 9.655, df= 3, sig.= 0.022).

Discussion

The involvement of ENPP1 gene in insulin signaling pathway due to its role as a potential inhibitor of insulin receptor tyrosine kinase has resulted in its implication in insulin resistance making it an important target for screening of SNPs within and between different populations. Baratta et al. (Baratta, 2008) studied the association between ENPP1 121Q variant and its correlation with obesity and atherogenic factors and reported that QQ carriers have impaired first phase insulin secretion, while KQ carriers had the diabetes disposition index entirely due to insulin resistance. Further, they described significant BMI interaction for modulating hyperinsulinemia, atherosclerosis and T2DM but with conflicting results that varied with ethnicity and geographic location. According to Kubaszek (2004) positive association between hypertension and T2DM may be because of insulin resistance and it may play a role in the development of hypertension. These conflicting reports and implication of its association with atherogenic factors prompted us to initiate the present study with the objective to assess the association of the K121Q variant with diabetogenic and atherogenic factors in a small population from Jaipur (Rajasthan, India).

Genotypic frequency variation within and between groups

The reported prevalence of the Q121 allele has been variable in different ethnic and geographic group subjects. In the Caucasian populations it has been reported to have low frequency of 10% in Finns (Kubaszek, 2004), 13.8% in Finnish–Swedish mixed (Gu, 2000), 12.3% in Spanish (Gonzàlez-Sànchez, 2003), 14.3% in a European American (Rasmussen, 2000), 16.1% in Danish (Bacci et al., 2005), 16.9% in French (Meyre et al., 2005) and 17.8% in Sicilian (Frittitta et al., 1996). Similarly, the allele frequency in South Asian immigrants living in the United States has been reported to be 17.9%, i.e., comparable to that in the Sicilian (Abate et al., 2005). In contrast, a significantly higher 121Q allele frequency of 54.2% was reported in the Dominican Republic population with a mixed genetic background of indigenous Caribbean, African and Hispanic subjects (Seo, 2008). In this context a relatively average 121Q allele frequency of 28.6% in the T2DM group and 29.6% in the control group was found for the subjects under study from Jaipur, Rajasthan (India).

ENPP1 SNP association with T2DM

Moderate but non-significant association was found in Sicilian population (Frittitta et al., 1996), while no association was reported from Spanish (Gonzàlez-Sànchez, 2003), Finnish (Kubaszek, 2004), Finnish and Swedish mixed population (Gu, 2000), Danish (Bacci et al., 2005), Italian

Volume : 4 | Issue : 10 | October 2014 | ISSN - 2249-555X

and United States Caucasian population (Matsuoka et al., 2006), Korean population (Hamaguchi et al., 2004), Chinese population (Chen et al., 2006), and Japanese population (Keshavarz et al., 2006).

In contrast present study, reveals positive association of K121Q SNP with T2DM which is in consistency with those of United States, Caucasians and South Asians (Abate et al., 2005), Dominican Republic population (Seo, 2008) and French population (Meyre et al., 2005) but inconsistent with a similar study from north India of Punjabi population (Bhatti et al., 2010).

BMI and T2DM

Obesity is one of the main risk factor for the development of T2DM. Previous studies of association between obesity and K121Q polymorphism in Chinese Han population (BMI of obesity group ≥27 kg/m2) (Wan et al., 2006), Caucasians (BMI of obesity group >90th percentile), African-American (BMI of obesity group >80th percentile) (Matsuoka et al., 2006), French (BMI of obesity group ≥95th percentile) (Meyre et al., 2005), and Dominican (BMI of obesity group ≥30 kg/m2) (Seo, 2008) showed positive association. However, the present study (BMI of obesity group ≥25 kg/m2); reveals no association between obesity and K121Q SNP in Jaipur (Rajasthan, India) subjects.

Conclusion

In conclusion, the present study suggests that ENPP1 K121Q polymorphism is associated with T2DM and atherogenic factors but not with BMI in the subjects from Jaipur (Rajasthan, India). Further to substantiate the present finding, more studies from neighboring states are needed.

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Figure 1: Photograph showing restriction digestion products using AvaII to screen K121Q polymorphism resolved on 2% agarose gels. Lane M shows 100 bp DNA ladder, lane 1,2,3,6,7,9,10 and 11 shows wild type fragment (238 bp). Lane 4,5 and 8 shows the heterozygous fragments (238, 148 and 90 bp).

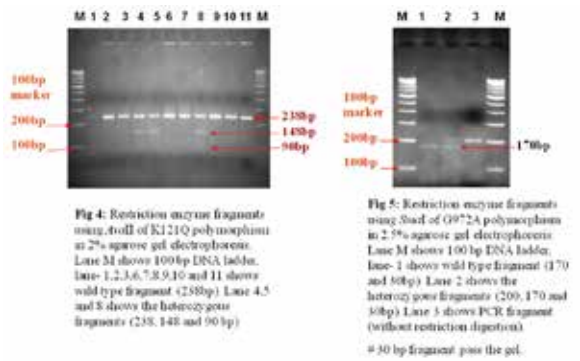

Fig 4: Restriction enzyme fragments using AvaII of K121Q polymorphism in 2% agarose gel electrophoresis. Lane M shows 100bp DNA ladder. lane- 1,2,3,6,7,8,9,10 and 11 shows wild type fragment (238bp). Lane 4,5 and 8 shows the heterozygous fragments (238, 148 and 90 bp)

Fig 5: Restriction enzyme fragments using StuI of G972A polymorphism in 2.5% agarose gel electrophoresis. Lane M shows 100 bp DNA ladder. lane- 1 shows wild type fragment (170 and 30bp) Lane 2 shows the heterozygous fragments (200, 170 and 30bp) Lane 3 shows PCR fragment (without restriction digestion).

30 bp fragment pass the gel.

Volume : 4 | Issue : 10 | October 2014 | ISSN - 2249-555X

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