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Genetic Association of DPP4 C/T and GLP1R C/A with Cytokines and Hormones in Undiagnosed Diabetic Subjects

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ABSTRACT

OBJECTIVE: Genetic relationship with biochemical, hormones and markers of inflammation.
METHODOLOGY: This collaborative study was cross-sectional and carried out by the Department of Biochemistry, Karachi University, Baqai Diabetology and Endocrinology Institute (BIDE), Karachi-Pakistan and Kansai Electric Power Institute of Medical Research (KEPIIMR), Osaka-Japan. The duration of the research was from March 2019 to May 2020. Both genders aged 30-70 (years) visiting the outpatient department of BIDE and fulfilling the inclusion criteria were recruited. An oral glucose tolerance test as diagnostic criteria was used. Subjects were categorized into healthy (glucose levels of fasting glucose (FPG) less than 100 (mg/dL), a two-hour glucose post level (2-hr PGL) below 140mg/dL). Newly diagnosed diabetics (NDD) (FPG ≥126mg/dL or 2-hr PGL ≥200mg/dL or both without any antidiabetic medicines use). Subjects with any complications were excluded. SPSS (Social Science Package for Statistical) Version 20 was used to analyze data.
RESULTS: Of 34 subjects, 17 were NDD and 17 were healthy. The mean age was 49.65±1.95, and the mean BMI was 28.12±0.93. DPP-4 C/T exhibited significant findings ($p<0.05$) compared with GLP-1R C/A among healthy and NDD subjects. Polymorphism of GLP1R C/A is associated significantly with fasting triacylglycerides and GLP1. In Comparison, polymorphism of DPP4 C/T was associated significantly in fasting with GLP1, GIP, HDL and IL-1β when compared to NDD with healthy participants.
CONCLUSION: We found significantly more association of DPP4 C/T polymorphism rs2970932 with biochemical parameters than GLP1R C/A polymorphisms rs1042044 in NDD individuals.

KEYWORDS: DPP-4; GLP-1R; hormones; cytokines; dyslipidemia

INTRODUCTION

Diabetes mellitus type 2 (T2DM) is challengeable in treatment. Low secretion of insulin from β-cells of the pancreas is the main characteristic[1]. Worldwide, 463 million people were affected by T2DM in 2019, and according to new predictions, this number may well escalate to 700 million in 2045[2]. In Pakistan, a recent survey estimates a 26.3% prevalence of T2DM[3].

Along with lifestyle, genetic predisposition contributes to T2DM development with a high risk[4]. Hormones comprised of incretin (GIP- insulinotropic polypeptide of Glucose-dependent and GLP1-glucagon-like polypeptide) enhance the secretion of glucose-stimulated insulin from beta cells of the pancreas[5]. The Gene of DPP4 (peptidase4 dipeptidyl) impairs insulin sensitivity and cleaves the dipeptides terminal N for regulating homeostasis energy[6]. Among healthy subjects, DPP4 regulate hormone incretin and T2DM pathophysiology and play a predictor role for developing insulin resistance[7]. A vital part of DPP4 was seen in the T2DM pathophysiology by controlling hormone incretin. Among the Asian population, the genetic rs2970932 variant is the sequence of the DPP4 gene and is associated with metabolic syndrome risk[8]. Consequently, inhibitors of DPP4 can be used for therapeutic purposes in managing and treating T2DM.

In humans, the association of GLP1 was observed with the pituitary hypothalamic adrenal axis (HPA axis). The gene of GLP1R genetic variants is found in membrane cell structures of cell islets of Langerhans, and it works for incretin modulation[9]. Insulin secretion also requires the involvement of GLP1. It hinders the transformational channels, which results in the depolarization of K^+ ions and Ca^{2+} ions influx raises in the inner side of membrane β-cells. This mechanism starts the exocytosis process for polypeptide insulin[10]. Hence, gene activation of GLP1R plays a pivotal role in insulin synthesis and secretion. A mutation non-synonymously (rs1042044) at GLP1R gene of exon 7 bases the cytosine to adenine (C/A) variation at the level of nucleotide and phenylalanine to leucine in GLP1R polypeptide chain at position 260 (Phe/Leu) in subjects with T2DM[11].

Moreover, biomarkers related to inflammation in T2DM subjects are connected with vascular dysfunction and the state of hypercoagulation, which increases the diseases of cardiovascular (CVDs) risk. It was also reported that during normoglycemia, progression to T2DM IL-1β with most biochemical parameters showed a significant association when compared with IL6[12]. Besides, mutually producing effects of hyperglycemia, dyslipidemia and hypertension in T2DM subjects increase the micro- and macrovascular risk complications[13].

Literature studies in our acquaintance found no such similar search presenting a mutual role of DPP4 (rs2970932) C/T and GLP1R (rs1042044) C/A Leu260Phe genetic variants with hormones and cytokines among people with newly diabetes-diagnosed (NDD). Henceforth, this study aims to determine the correlation of genetic variants with hormones and related biochemical and inflammatory markers among NDD subjects compared to healthy subjects.

METHODOLOGY

This collaborative study was cross-sectional and carried out by the Department of Biochemistry, Karachi University, Baqai Diabetology and Endocrinology Institute (BIDE), Karachi - Pakistan and Kansai Electric Power Institute of Medical Research (KEPIMR), Osaka-Japan. The duration of the research was from March 2019 to May 2020. This study is the sub-analysis of the research "Insulin, Glucagon and Incretin (GLP-1 and GIP) levels at different time intervals to 75g oral glucose tolerance tests in Newly Diagnosed Diabetes" sent for publication elsewhere. Both genders aged 30-70 (years) visiting the outpatient department of BIDE and fulfilling the inclusion criteria were recruited. Persons refusing to participate and/or having impaired plasma glucose levels, pregnancy, taking antidiabetic drugs, having any microvascular and macrovascular complications, and allergies of any type were excluded. A predesigned questionnaire was used to obtain the demographic and anthropometric details.
Regarding the American Diabetes Association test of oral glucose tolerance definition was used to identify diabetes in newly diagnosed healthy individuals without diabetes were defined as those having a plasma level of glucose in fasting (FPG) less than 100 mg/dL and a 2-hour post-meal glucose level (2-hr PGL) below 140mg/dL. NDD individuals had FPG ≥126mg/dL or 2-hr PGL ≥200mg/dL or both without any antidiabetic medicines use[14].
Following inclusion criteria, biochemical samples, including FPG, 2-hr PGL and lipid profile, were evaluated at BIDE laboratory of Pakistan. Pro-inflammatory cytokines and genetic analysis were performed at Karachi University-Pakistan, while for hormonal analysis, samples were transported to Osaka-Japan following laboratory standard operating procedure[3].

Techniques used

The DPP4 levels were analyzed by indirect immunosorbent sandwich enzyme-linked assay (ELISA) using the Human Bioassay Technology Laboratory DPP4 ELISA Kit. The salting out method performed the genomic DNA extraction[15]. The primers (allele-specific) for genetic variant DPP4 C/T (rs2970932) and genotypes GLP1R Phe260Leu (rs1042044) were designed by using an online software allele-specific PCR web-based primer designing tool (WASP)[16]. The designed primers were also confirmed using online bioinformatics matching software, namely primary search tool local alignment (BLAST) (https://blast.ncbi.nlm. nih.gov/Blast.cgi).
The allele-specific polymerase chain reaction (AS-PCR) technique optimized allele-specific concentration. For the DPP4 C/T genetic variant, the set comprised of forward primer for allele type wild (F1): 5'-AGCTGTAGCCACACCTTCC-3' or for allele mutant (F2): 5'-AGCTGTAGCCACACCTTCT-3' and a typical reverse primer: 3'-TGGGTGAATGAAAGTCTACC-5'. The initiation was conducted by denaturing at 94°C for 3 minutes, leading to the next denaturation step at 94^0C for 30 seconds. The primer annealing was carried out at 55.5°C for 30 seconds, moving at 72°C for a minute of extension and finally, elongation took place at 72°C for 5 minutes. The PCR product of 122 base pair was found on 2% gel agarose by electrophoresis and visualized at documentation gel system (FastGene®FAS V, Germany).
The primer sequences designed for GLP1R Phe260Leu were as 5'GCTGGCCTTCTCGGTCTCA-3' (forward A allele), 5'-GCTGGCCTTCTCGGTCTCC- 3' (forward C allele) and 5'-TACGGCATCTTAACAGCAC- 3' (reverse allele). The Bio-Rad Thermal Cycler was designed following denaturation at 94°C for 30 seconds, annealing at 58.1°C for 30 seconds, and an extension of 72°C for 1 minute. The cycle was completed, and the extension was finalized at 72°C for 5 minutes. The PCR product size of 232 base pairs was obtained after successful amplification. Samples for homozygosity of the C/C or A/A genotype and heterozygosity of the C/A genotype were analyzed by

agarose gel electrophoresis. Whole DNA (genomic) in study participants was determined to evaluate its reliability by gel electrophoresis.

For insulin (serum) enzyme chemiluminescent immunoassay-CLEIA technique was used quantitatively (catalogue kit number KA2801. ELISA was used for glucagon (plasma) by Glucagon Cosmic Corporation ELISA (catalogue kit ARG81293 number), GLP-1 (plasma) by ELISA V-plex Scale Meso Discovery® GLP-1 total (catalogue kit K1503PD number) and GIP (plasma) by Merck® ELISA GIP (total) Human (catalogue kit EZHGIP-54K) number. Cytokines pro-inflammatory (serum) (IL-1β and IL6) were analyzed by ELISA quantitatively (catalogue kits E0143Hu and E0090Hu numbers), respectively.

Statistical analysis

SPSS (Social Science Package for Statistical) Version 20 was used to analyze data. Mean ± SD was used to present continuous variables, and numbers (proportion) were used to show the categorical variables. A t-test was used to differentiate among groups of students. Test chi-square and ratios odd with CI 95% were used to identify the association of the variables. P value <0.05 is known as significant statistically. The analysis presents the genotypic and allelic frequencies distribution and shows an association between DPP4 and GLP1R polymorphism with the disease group. Hardy Weinberg equilibrium was used for calculating allelic and genotypic frequencies. The Odds ratio was done by MedCalc online software to test the association's role in disease pathogenicity with 95% confidence intervals (95% CI).

RESULTS

The total subjects were 34, including 17 NDD and 17 healthy individuals. Males and females were 19(55.9%) and 15(44.1%) respectively. The mean age (years) was 49.65±1.95, and the mean BMI (kg/m^2) was 28.12±0.93. Among healthy and NDD subjects, levels of total cholesterol (mg/dL) were 202.94±11.25 and 178.88±12.04, triglycerides (mg/dL) was 143.71±14.98 and 207.47±28.57, LDL (mg/dL) was 130.41±9.07 and 110.47±11.46 and HDL (mg/dL) was 33±1.82 and 32.82±5.43, respectively. Fasting levels of plasma glucose (mg/dL) were 102.29 and 147.26, serum insulin (µIU/ml) was 11.23 and 20.96, plasma glucagon (pg/ml) was 24.39 and 28.47, plasma GIP (pmol/l) were 126.29 and 129.42 and plasma GLP-1 (pg/ml) were 29.52 and 38.14 and serum DPP4 (ng/µl) were 1.52 and 0.45 among healthy and NDD subjects, respectively. IL-1β (pg/L) and IL-6 (ng/L) levels were significantly greater in NDD subjects compared to healthy.

GLP1R genetic variant rs1042044 for disease susceptibility was explored in **Table I**. Genetic dispersal of C/C, C/A, and A/A genotypes and allelic distribution of C and A alleles displayed no significant results in NDD subjects. But, we observed an increased C/A genetic variant frequency in the development of risk in T2DM.

The allele and genotype dispersal of the variant DPP4 C/T (rs2970932) is presented in **Table II**. In NDD participants, genotypes C/T and T/T were statistically more common than in healthy people. Besides, the T allele prevalence rate was higher and showed a trend towards an association with T2DM susceptibility.

Table III presented the clinical variable association with polymorphism GLP1R C/A. GLP1R C/A polymorphism was significantly associated with GLP1 and triglycerides in fasting among NDD and healthy subjects. Ratios (odd) specify that triacylglycerides were 1.54 times and IL-1β was 1.98 times had higher diabetes developing risk in subjects with genotyping GLP1R C/A.

Polymorphism DPP4 C/T in **Table IV** presents a significant correlation with GLP1, GIP, HDL, IL1β and IL6 in fasting. Ratios (odd) showing that GLP1 had 1.67 times, triglycerides had 1.35 times, IL-1β had 1.73 times and IL-6 1.01 times high diabetes developing risk in subjects having genotyping DPP4 C/T.

TABLE I: REPRESENTATION OF FREQUENCY DISTRIBUTION OF GLP1R C/A POLYMORPHISM

Genotypes N=34	Healthy subjects N = 17	Newly diagnosed diabetic subjects N = 17	Chi-square χ^2	p-value
A/A	03 (0.17)	02 (0.12)		
C/A	10 (0.59)	12 (0.70)	1.629	0.30
C/C	04 (0.24)	03 (0.18)		
Alleles N=64	**Healthy subjects N = 34**	**Newly diagnosed diabetic subjects N = 34**	**Odd ratio (95% CI)**	**p-value**
A	15 (0.44)	17 (0.50)	1.37 (0.16-5.43)	0.61
C	19 (0.56)	17 (0.50)		

Data presented as number (proportion) and odd ratio (95% CI), P>0.05 indicates non-significant. Chi-square test and odds ratios with 95% confidence intervals (95% CI) were calculated to determine the association between variables.

TABLE II: REPRESENTATION OF FREQUENCY DISTRIBUTION OF DPP4 C/T POLYMORPHISM

Genotypes N=34	Healthy subjects N = 17	Newly diagnosed diabetic subjects N = 17	Chi-square χ^2	p-value
C/C	1 (0.06)	1 (0.06)		
C/T	5 (0.29)	9 (0.53)	1.437	0.048
T/T	11 (0.65)	7 (0.41)		
Alleles N=64	**Healthy subjects N = 34**	**Newly diagnosed diabetic subjects N = 34**	**Odd ratio (95% CI)**	**p-value**
C	6 (0.18)	10 (0.29)	0.480 (0.14-0.63)	0.049
T	28 (0.82)	24 (0.71)		

Data presented as number (proportion) and odd ratio (95% CI); P<0.05 indicates significance Chi-square test and odds ratios with 95% confidence intervals (95% CI) were calculated to determine the association between variables

TABLE III: ASSOCIATION OF GLP1R C/A POLYMORPHISM WITH CLINICAL VARIABLES AMONG NDD AND HEALTHY SUBJECTS

Clinical Parameters	GLP1R C/A Polymorphism		Odd Ratios	P-value
	Healthy subjects (N=10)	Newly Diagnose Diabetic subjects (N=12)		
Fasting Insulin (µIU/ml)	10.05±0.56	17.86±1.77	0.123	0.19
Fasting GLP1 (pg/ml)	24.0±1.87	33.0±2.13	0.106	0.04
Fasting GIP (pmol/l)	119.1±1.28	121.3±3.9	0.988	0.91
DPP4 levels (ng/µl)	1.10±0.28	0.61±0.18	0.851	0.37
Fasting Glucagon (pg/ml)	21.1±1.28	23.5±3.20	0.047	0.69
Cholesterol (mg/dL)	165.07±7.32	191.23±9.78	0.932	0.765
Triglyceride (mg/dL)	131.61±6.62	186.71±5.31	1.541	0.038
Low-density lipoprotein (mg/dL)	111.32±3.61	106.16±9.33	0.025	0.471
High-density lipoprotein (mg/dL)	32.46±4.07	24.2±2.5	0.160	0.871
Interleukin 1-beta (pg/L)	171.56±7.81	1371.46±73.18	1.983	0.121
Interleukin-6 (ng/L)	49.68±3.28	101.19±4.58	0.761	0.273

Data were presented as mean ± SEM; an independent t-test was applied to investigate group differences. P-value <0.05 was considered statistically significant, and odds ratios were calculated to determine the association between variables.

Note: Of all data available, only those cases were selected having GLP1R C/A polymorphism that was newly diagnosed diabetic subjects (N=12), and they were compared to healthy subjects (N=10).

Abbreviations: GLP-1= glucagon-like peptide-1; GIP= glucose dependent insulinotropic polypeptide; DPP4 = Dipeptidyl peptidase 4.

TABLE IV: ASSOCIATION OF DPP4 C/T POLYMORPHISM WITH CLINICAL VARIABLES AMONG NDD AND HEALTHY SUBJECTS

Clinical Parameters	DPP4 C/T Polymorphism		Odd Ratios	P-value
	Healthy subjects (N=5)	Newly Diagnose Diabetic subjects (N=9)		
Fasting Insulin (µIU/ml)	13.48±1.16	09.29±0.66	0.081	0.79
Fasting GLP-1 (pg/ml)	23.74±1.63	31.09±2.66	1.672	0.031
Fasting GIP (pmol/l)	117.4±7.97	95.44±5.77	0.541	0.02
DPP4 levels (ng/µl)	0.12±0.03	0.905±0.32	0.489	0.07
Glucagon (pg/ml)	18.57±1.07	20.95±1.36	0.454	0.37
Cholesterol (mg/dL)	194.3±7.61	177.41±4.17	0.672	0.384
Triglyceride (mg/dL)	159.5±5.84	185.04±7.16	1.35	0.845
Low-density lipoprotein (mg/dL)	118.1±4.43	91.13±3.21	0.372	0.417
High-density lipoprotein (mg/dL)	29.8±1.11	23.6±0.68	0.531	0.001
Interleukin 1-beta (pg/L)	159.14±5.68	1191.34±61.7	1.736	0.037
Interleukin-6 (ng/L)	49.51±3.63	98.61±6.14	1.012	0.032

Data were presented as mean ± SEM; the independent sample T-Test was applied to investigate the differences among groups. P-value <0.05 was considered statistically significant, and the odds ratio was calculated to determine the association between variables.

Note: Of all data available, only those cases were selected having DPP4 C/T polymorphism that was newly diagnosed diabetes (N=7) and was compared to healthy subjects (N=5)

Abbreviations: GLP-1= glucagon-like peptide-1; GIP= glucose dependent insulinotropic polypeptide; DPP4 = Dipeptidyl peptidase 4.

DISCUSSION

In recent pre-clinical studies, an association between DPP4 inhibitors and incretin were observed, which is somewhat similar to our study[9]. It was previously found that the secretion of insulin is stimulated in a manner depending on glucose. Hormones (incretin) release with hyperglycemia after ingestion of food intake and stimulate insulin secretion. In addition, the action of the DPP4 inhibitor raises the incretin levels endogenously for speedy degradation of GIP and GLP1 by inhibiting DPP4 and decreasing the glucose-dropping effect of incretin circulating hormones[17]. An association was observed between fasting levels of DPP4, GLP-1 and GIP. The study of Jamaluddin JL 2016[8] showed that high gene DPP4 expression plays a significant role as a marker of tissue (visceral adipose) inflammation and is linked to metabolic disturbances. Findings are also relevant to a study by Jamaluddin JL 2016[8] showing that DPP4 (mRNA) is mostly positively associated with the total-/HDL-plasma cholesterol ratio. Improvement in glycemic indexes and lipid parameters occur by inhibitors of DPP4, similar to study findings that polymorphism DPP4 C/T had a significant correlation with healthy cholesterol HDL[9].

In the previous study, the DNA of tissues (visceral adipose) was analyzed for promoter (DPP4) island CpG (5'—C—phosphate—G—3') methylation for different gene DPP4 polymorphisms. Moreover, the plenty of mRNAs (DPP4) exhibited a negative relationship, while the HDL/total cholesterol ratio positively correlated with the methylation rate of DPP4 in Island CpG[18]. In literature, polymorphism of IL-6 has presented a relationship with inhibitor DPP4 response[19]. He X 2022[20] observed in their study the IL-1β down-regulation with inhibiting DPP4 in mice. Also this study observed a significant relationship between polymorphism DPP4 C/T and IL1β, indicating an inflammatory role in T2DM progression. According to the Diabetes Association of America and the Diabetes Study of the European Association in 2020 diabetes medical care, inhibitors of DPP4 and receptor agonists, GLP1, have the properties of a second-line drug for lowering glucose. People may need a combined treatment for achieving adequate glycemic control in case of any adverse reaction or accompanying hypoglycemia by prescribing metformin or sulfonyl urea[21]. Genetic GLP1R and DPP4 polymorphisms genes were studied previously regarding insulin secretion and diabetes pathogenesis[22]. We found no significant relationship between genetic variants GLP1R and DPP4 with glucagon and insulin. Several neighbouring polymorphisms, single Tag nucleotide (SNPs) within the gene GLP1R, were observed to be linked with the rate of gastric emptying in young, healthy Caucasian men; however, they need further investigation[23]. In the case of hyperglycemia, in GLP1R, two SNPs non-synonymously are nominally connected with improved secretory insulin response to infused GLP-1[24]. The GLP1R had a significant relationship with hypertriglyceridemia in NDD people, consistent with the Lin CH et al. study[25].

Our study was not designed as a pharmacogenetics study to give details regarding SNPs as a cause of T2DM. However, the results adequately address the effect of DPP-4 C/T and genetic GLP-1R C/A variants on hormones, parameters lipids, and cytokines (pro-inflammatory). Therefore, this data will allow us to understand better the biological causes of DPP4 C/T and GLP-1R C/A genetic variants in our population, corroborating in respective pharmacogenetics studies. These gene variations could also influence clinical decisions, for instance, regarding the use of inhibitors of DPP4 versus incretin mimetics for T2DM treatment.

CONCLUSION

We found significantly more association of DPP4 C/T polymorphism rs2970932 with biochemical parameters than GLP1R C/A polymorphisms rs1042044 in NDD individuals. Overall, polymorphism DPP4 C/T gene may contribute to the pathogenesis of T2DM in NDD subjects and might be used as a biomarker for early diagnosis of diabetes and a predictive marker for the development of diabetes.

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AUTHOR CONTRIBUTIONS

Waris N: Design and concept, data interpretation and manuscript writing.
Nangrejo R: Analysis of data, data interpretation, edited and approved the final manuscript.
Bano S: Design and concept, data interpretation, edited and approved the final manuscript.
Kamran M: Data interpretation, edited and approved the final manuscript.
Fawwad: Edited, reviewed and approved the final manuscript.
Basit A: Edited, reviewed and approved the final manuscript
All authors have read and agreed to the published version of the manuscript.

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