"Regenerating" Iα Gene Variants g. -385T>C and g. -243T>G in Type 2 Diabetes

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ABSTRACT

Background: Restoration of the body's ability to produce insulin by regeneration of β-cells is an essential step toward the management of diabetes. *REG Iα* is released from damaged β-cell and facilitates their renewal. However, genetic variants of *REG Iα* and their clinical associations are overlooked areas. This study was designed to identify polymorphisms of *REG Iα* gene exon 1 and its association with type 2 diabetes.

Methods: This was a case-control study conducted on individuals n=51 both diabetics (n=36) (aged, 52±9 years; males 25, females 11) (group II) and age-related controls (n=15) (aged, 50±3 years; males 9, female 6) (group I) recruited from PNS Shifa Hospital, Karachi. The samples were amplified, and the gene was sequenced to identify polymorphisms. Chi-square (γ2) test was used to determine whether each polymorphism obeys Hardy-Weinberg equilibrium (HWE). Odds ratios, 95% confidence interval along with corresponding p values were calculated through logistic regression analysis. $p < 0.05$ was considered statistically significant.

Results: A set of two single nucleotide polymorphisms (SNPs) were detected in the *REG Iα* gene exon1 in the subjects under study. SNPs studied were: g.-243T>G [rs 283890], and g.-385T>C [rs10165462]). Both variants g-243T>G, and g-385T>C showed significant association (γ2=12.8, p=0.0003, γ2=13.8, p=0.0002) with smoking. The allele G showed an eight-fold increased risk of disease in smokers while allele C had less chance of the disease in smokers as compared to non-smokers.

Conclusion: Among the SNPs detected in exon 1 of the gene for *REG Iα*, the g. -385T>C and g. -243T>G variants showed an association with smoking in type 2 diabetes and the SNP -243G (rs 283890) was found to increase the risk of disease in smokers.

Keywords: *REG Iα* gene; Polymorphisms; Type 2 Diabetes; β-cells Regeneration.

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Doi: https://doi.org/10.36283/PJMD11-4/003

INTRODUCTION

Diabetes management is a continuous challenge for medical science. Researchers around the world are striving hard to discover novel molecular markers that would carry the capability of increasing long-term survival as well as functional efficacy of β-cells[1,2]. It is suggested that human insulin-secreting cells can proliferate and induce the synthesis of several progenitor/stem cells. Many genes and transcription factors take part in this process like Reg islets derived proteins, NeuroD1, Sox 9, Netrin 1, and Neurogenin-3 [3]. In humans, *REG* gene family members have been reported and some of them have been associated with regeneration and neogenesis of β-cells[4]. The *REG* gene expression in the pancreas is induced during morphogenesis of islets or by damage to β-cells[5,6]. It rejuvenates the β-cell population and improves experimentally induced diabetes[7,8]. The β-cell DNA replication occurs in an autocrine or paracrine mode. A supposed Reg protein receptor was recognized that transmits the signals for β-cells growth and regeneration[9].

Reg I the first member of the family is produced by exocrine pancreas acinar cells and resides in β-cells secretory granules along with insulin[5]. *REG Ia* is encoded by an approximately 2.7 kb gene, comprising six exons and five intervening sequences located on chromosome 2p12. Expression of the *REG I* gene has been observed early in pancreas development and islet cell expansion. Reduced levels of Reg I protein and insulin were observed in the case of aging and pancreatitis-induced diabetes[10]. Lately, a study suggested that damage to acinar cells is related to the insufficiency of insulin in chronic pancreatitis. Based on the review of studies, it is proposed that lost defense from acinar cells of exocrine pancreas secreting regenerating proteins results in damage of insulin-secreting β-cells in chronic pancreatitis[11].

Reg proteins upregulation in individuals with type 2 diabetes after gastric bypass surgery may contribute to its remission[12]. Furthermore, Reg I overexpressed after virus infection induced diabetes in *REG I* knockout mice specifies their crucial role in beta cell regeneration[13]. Raised levels of Reg Ia and Iβ demonstrated a better survival rate in pancreatic cancer[14]. Studies are being done to identify miRNAs targeting human Reg family members that may provide new insights into Reg protein biology[15].

However, genetic variants of *REG Ia* and their clinical associations are overlooked areas that need to be explored. A study carried out to screen the comprehensive *REG Ia* gene showed no association between *REG I* gene abnormalities and diabetes. Korean population research for genetic variants in *REG Ia* found some of the polymorphisms showed modest associations with type 2 diabetes of early onset[16]. Moreover, risk factors associated with diabetes like BMI, family history of the disease, age, sex, smoking and hypertension and their association with the genetic variants of *REG Ia* need to be explored. To meticulously explore the possible *REG Ia* gene link with type 2 diabetes, the study aimed to identify polymorphisms of *REG Ia* gene exon 1 variant in Pakistani targeted people and their possible link with type 2 diabetes and associated risk factors.

METHODS

A total of n=51 participants were included in this case-control study. The type 2 diabetes n=36 patients (aged, 52±9 years; males 25, females 11) were recruited from PNS Shifa Hospital, Karachi medical OPD, and n=15 healthy controls (aged, 50±3 years; males 9, female 6). The study was approved by the Ethical Review Committee of the Centre for Research in Experimental and Applied Medicine (CREAM), Army Medical College, (Letter No. 02/CREAM-A/Sadaf Saleem). The patients with type 2 diabetes diagnosed under the American Diabetes Association (ADA) criteria were selected. The participants were well-versed in the study purpose and written informed consent was taken before preceding sampling.

Blood samples were drawn under an aseptic technique for biochemical measurements and DNA extraction. Whole blood was collected in EDTA tubes and DNA extraction was done using a modified Sambrook and Russell 2001 methodology. Primers were designed for exon 1 and their corresponding intronic regions using Primer-BLAST software as follows: FORWARD PRIMER: TCCCAAAACTCACCGCTTGC, and REVERSE PRIMER: CTCTGAGACACCCACACCTTC. The primers amplified the region of 322 bp.

All samples were amplified using primer set for exon 1 after its optimization on Bioer's XP PCR Thermal cycler (Pre denaturation at 94°C for 4 minutes, denaturation at 94°C for 30 seconds, annealing at 55°C for 40 seconds, extension at 72°C 1kb/min for 35 cycles, post extension 72°C for 10 minutes, hold 4°C. PCR products amplified were run on agarose gel (2%) and visualized on GelDoc imaging system using Herolab E.A.S.Y Win 32 program. PCR products were purified through a PCR cleanup kit using Spin Column Method. PCR product quantification was done by Spectrophotometer using 260 nm UV absorbance. Sanger dideoxy method was used to sequence DNA of interest using "BigDye Terminator v 3.1 cycle sequencing kit" on Genetic Analyzer.

Sequence Scanner 2 and Clustal X software were used to analyze the chromatograms and alignment between sequences, respectively. Alignment between *REG Ia* gene sequence of all the study population and known sequence from the National

Center for Biotechnology Information (NCBI) was done using Nucleotide-BLAST and SNPs were identified in both the type 2 diabetes group and controls. Known polymorphisms for the gene of interest from the Gene Card database and dbSNP NCBI Nucleotide BLAST were used for comparison with the newly identified SNPs.

To analyze all the data SPSS statistical software was used. Descriptive statistics were calculated for clinico-demographic and anthropometric data. The normality of the data was checked by applying the Shapiro-Wilk test. Mean ± SD and Median (IQR) for non-parametric data were calculated. An Independent sample t-test was applied and $p < 0.05$ was considered significant and $p < 0.001$ highly significant. Chi-square (χ^2) test was used to determine whether each polymorphism obeys Hardy-Weinberg equilibri-um (HWE). Odds ratios, 95% confidence interval along with corresponding p values were calculated through logistic regression analysis.

RESULTS
A total of 36 individuals with diabetes (group II) and 15 controls (group I) were included in the present study. There were 9(60%) men and 6(40%) women as controls while individuals with type 2 diabetes included 25(69%) men and 11(31%) women. The mean age of Group I was 50±3 and group II was 52±9 (p=0.479). The Body mass index (BMI) of controls was 24.6±4, which was less than the diabetic group (26±3) of both groups. Whereas the Fasting blood glucose was also found high 8 (6.1-10.9) in dietetics than controls at 4.1 (3.7-5.6) 9, p <0.001). Table 1 shows the clinicodemographic and anthropometric data of the groups under study.

Table 1: Clinicodemographic and anthropometric characters of the control and diseased groups.

Characteristics	Group I (Control) (n=15)	Group II (Diabetes) (n=36)	*p*-Value
Age (years)	50±3	52±9	0.479
Age with the onset of disease	-	46±9	-
Disease/ time duration	-	6±5	-
Body weight in kg	68±8.6	71±11.5	0.315
Height in inches	66±3.3	65±3.5	0.390
Body mass index (kg/m^2)	24.6±4	26±3	0.248
Systolic Blood Pressure (mmHg)	120 (110 - 120)	130 (120 -140)	0.001*
Diastolic Blood Pressure (mmHg)	80 (70 - 80)	80 (80 - 90)	0.007*
Fasting blood glucose (mmol/l)	4.1 (3.7 - 5.6)	8 (6.1 - 10.9)	<0.001**

*Data is stated as Means± SD (Median (IQR) for non-parametric data). p <0.001** and p <0.05* were considered significant and highly significant respectively.*

The 322 bp of exon 1 was amplified and checked on agarose gel electrophoresis (Figure 1). The Sanger sequencing results showed many SNPs but two were selected' g.-243T>G [rs 283890], and g.-385T>C [rs10165462]) for analysis and association with different parameters (Figure 2).
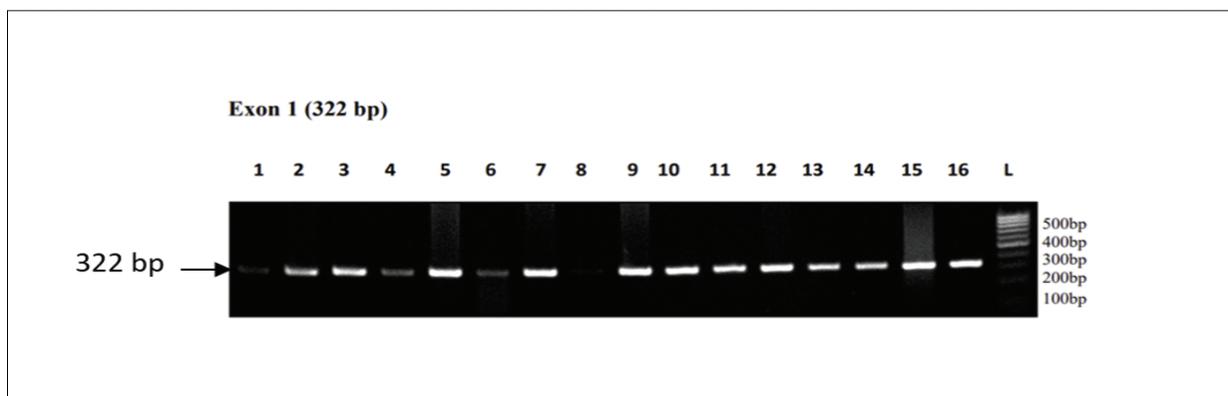
Figure 1: The 322 bp of exon 1 was amplified and checked on agarose gel electrophoresis.
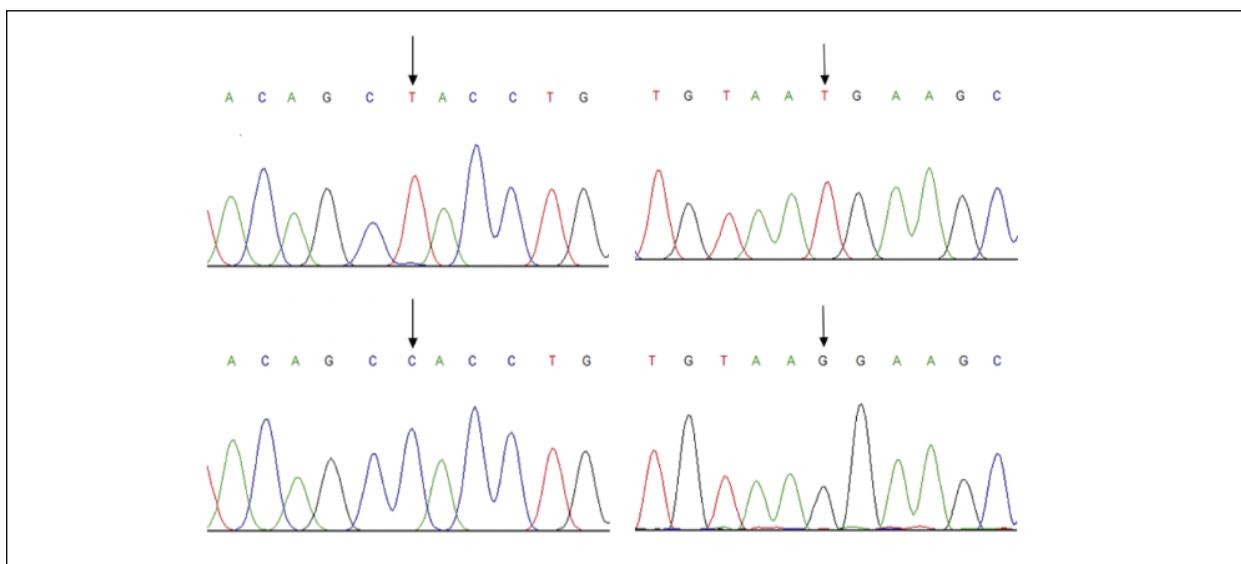

Figure 2: Left (SNP -385T>C): The electro-chromatogram of a study showing substitution of T>C (as indicated by an arrow). Right (SNP -243T>G): the electro-chromatogram of a study showing substitution of T>G (as indicated by an arrow).

Hardy Weinberg Equilibrium (HWE) was applied to determine the genotype and allelic frequencies in the SNPs -243T>G region. The selected population deviated from the HWE. For SNP2 (g.-243T>G), the OR calculated to test the relevance of frequencies of allele-T and allele-G in both controls and diabe-tes individuals was 0.51 with p = 0.22. However, the OR for diabetes individuals when further classified into smokers and non-smokers was 8.06 with a highly significant $p<0.001$ indicating an 8-fold increase in the risk of the disease in smokers (Table 2).

Table 2: Odd Ratio (OR) calculation of main variables with frequencies of alleles for SNP -243T>G.

-243T>G	Comparative Frequencies of Cases		Total	OR/ 95 % CI	$\gamma2$	p-Value
	Control Group	Group with Diabetes				
Allele-T	24	49	73	0.53 (0.19-1.5)	1.48	0.22
Allele-G	6	23	29			
	30	72	102			
-243T>G	Male	Female				
Allele-T	16	33	49	1.37 (0.45-4.2)	0.31	0.57
Allele-G	6	17	23			
	22	50	72			
-243T>G	FH (-)	FH (+)				
Allele-T	16	33	49	0.90 (0.31-2.6)	0.03	0.85
Allele-G	8	15	23			
	24	48	72			

-243T>G	Age at disease onset					
	≤ 45	**>45**				
Allele-T	21	28	49	0.58 (0.20-1.6)	1.01	0.31
Allele-G	7	16	23			
	28	44	72			
-243T>G	**BMI<23**	**BMI≥23**				
Allele-T	9	40	49	0.51 (0.16-1.6)	1.31	0.25
Allele-G	7	16	23			
	16	56	72			
-243T>G	**Smoker**	**Non-Smoker**				
Allele-T	5	44	49	8.06 (2.3-27.7)	12.8	**0.0003**
Allele-G	11	12	23			
	16	56	72			
-243T>G	**Hypertension (-)**	**Hypertension (+)**				
Allele-T	16	29	49	0.89 (0.32-2.4)	0.04	0.83
Allele-G	8	13	23			
	30	42	72			

For SNP1 (g.-385T>C), the OR calculated to test the relevance of frequencies of allele T and allele C in controls and diabetes individuals was OR=2.33 (0.71-7.55). The calculations were also made when diabetes individuals were further grouped. The OR calculations for smoker and non-smoker diabetes individuals was 0.11 with *p*<0.001 indicating that smokers with allele C have 11% less chance of the disease compared to non-smokers. For groups with age at disease onset ≤ 45 and > 45 OR=0.32 and *p* = 0.06 (Table 3).

Table 3: Odd Ratio (OR) calculation of main variables with frequencies of an allele for SNP -385T>C.

Alleles	Comparative Frequencies of Cases		Total	OR/ 95 % CI	γ2	*p*-Value
	Control Group	**Group with Diabetes**				
-385T>C						
Allele-T	4	19	23	2.33 (0.71-7.55)	2.06	0.15
Allele-C	26	53	79			
	30	72	102			
-385T>C	**Male**	**Female**				
Allele-T	13	6	19	1.06 (0.34-3.30)	0.01	0.91
Allele-C	37	16	53			
	50	22	72			
-385T>C	**FH (-)**	**FH (+)**				
Allele-T	6	13	19	0.89 (0.29-2.75)	0.03	0.85
Allele-C	18	35	53			
	24	48	72			
-385T>C	**Age at disease onset**					
	≤ 45	**>45**				
Allele-T	4	15	19	0.32 (0.09-1.10)	3.45	0.06
Allele-C	24	29	53			
	28	44	72			
-385T>C	**BMI<23**	**BMI≥23**				
Allele-T	6	13	19	0.50 (0.15-1.65)	1.30	0.25
Allele-C	10	43	53			
	16	56	72			
-385T>C	**Smokers**	**Non-Smoker**				
Allele-T	10	9	19	0.11 (0.03-0.39)	13.8	**0.0002**
Allele-C	6	47	53			
	16	56	72			
-385T>C	**Hypertension (-)**	**Hypertension (+)**				
Allele-T	9	10	19	1.37 (0.47-3.94)	0.34	0.55
Allele-C	21	32	53			
	30	42	72			

DISCUSSION

The study identified two SNPs in the exon 1 of the gene for *REG Ia* in the selected subjects and found that the variant, g.-243T>G was found to be associated with the increased risk of diabetes in smokers by eight-fold while the variants g.-385T>C was found to have a protective effect in smokers.

High values of serum *Reg Ia* have been reported across various types of diabetes[12-15]. As a marker of β-cell apoptosis and impaired kidney function[16,17]. Previous studies investigating the *REG Ia* gene in patients with fibrocalculous pancreatic diabetes and type I diabetes found no significant association between the disease and variants in the gene[18,19]. Wide-range scrutiny of the *REG Ia* gene in trophic calcific pancreatitis patients documented few polymorphisms including the one in the promoter region but did not show any disease association[20]. A study conducted on patients with type 2 diabetes of Korean origin found some *REG Ia* gene polymorphisms exhibiting associations with type 2 diabetes of early-onset nonetheless they did not find any link of these variants with general susceptibility to disease[21,22].

Studies have shown that smoking causes oxidative stress and increases inflammation in the body and may lead to insulin resistance and diabetes[23-25]. However, not every person who smokes acquires the disease. The effect of smoking is more marked in individuals who have a genetic predisposition to diabetes. This suggests that smoking may act as the genetic modifier of the disease. Studies have shown many genetic variants associated with the risk of diabetes in smokers[26,27]. The results suggest that smoking may be a link between the SNP g. -243G (rs 283890) of the *REG Ia* gene and the development of the disease. A recent study by Azarova et al. found an association between polymorphic variant rs11927381 of IGF2BP2 gene and increased risk of type 2 diabetes therefore smoking is a foremost avertible cause of the disease[28].

In addition, the hereditary variant g. -385C was found to reduce the disease risk in the age group less than 45 years. Our outcomes are in concurrence with the investigation led by the Korean populace, indicating that g.-385C brought down the risk of early-stage type 2 diabetes[27]. In the patients with type 2 diabetes after adjustment with numerous risk phenotypes, for example, sex, BMI, hypertension, age at disease onset, and family history, it was revealed that no polymorphism was associated significantly with the disease risk factors. Therefore, the wide-ranging quest for polymorphisms in the gene for *REG Ia* and the estimation of their association with type 2 diabetes are novel in Pakistani people.

CONCLUSION

A significant association between the SNP -243G (rs 283890) was found to increase the risk of disease in smokers. More studies are needed with a bigger and more diverse populace to elucidate the relationship between polymorphism in the *REG Ia* gene, diabetes type 2 and its related risk factors.

CONFLICT OF INTEREST
The authors declared no conflict of interest.

ETHICS APPROVAL
The study was approved by the Ethical Review Committee of the Centre for Research in Experimental and Applied Medicine (CREAM), Army Medical College, (Letter No. 02/CREAM-A/Sadaf Saleem).

PATIENT CONSENT
Informed consent was taken from patients.

AUTHOR'S CONTRIBUTION
SSU contributed to conception and design, drafting, acquisition of data, analysis and interpretation of data. The manuscript and revising it critically for important intellectual content. BC had made substantial contributions to the acquisition of data, analysis and interpretation of data. AKN had been involved in revising the manuscript and given final approval of the version to be published. All the authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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