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A Novel Variant Of Regenerating Iα Gene (REG) In Type II Diabetics Among Pakistani Targeted Population

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Abstract

Objective: Regeneration of pancreatic β-cells, is an essential step towards diabetes management. The regenerating (REG) Iα gene is secreted from damaged β-cell for the synthesis of β-cell. This study aimed to identify REG Iα gene polymorphisms and their association with Type II diabetes (T2DM).

Methods: Patients (110) with T2DM and age-related controls were selected from PNS Shifa Hospital, Karachi. DNA was extracted PCR was performed and amplified products were sequenced to identify polymorphisms. For six exons of the REG 1a gene, 6 sets of primers were designed. The selected (51) samples were amplified and sequenced for 306 (51x6) times. Odds ratios were calculated through logistic regression analysis. The correlation was used to find an association between REG Iα and diseases. $p< 0.05$ was considered significant.

Results: Blood samples were drawn from 90 finalized patients, including 70 diabetics and 20 controls with an M: F ratio of 12:8. Twenty patients opted to withdraw. The REG Iα and disease duration in type II diabetics showed a negative correlation ($r= -0.355$, $p=0.005$). The single nucleotide polymorphisms (SNPs) of eight sites were detected: g.-385T>C, g.-243T>G, g.-145G>A, g.+142A, g.+209G>T, g.+226A>G, g.+2199G>A, g.+2360A>G. The novel SNP g.-145G>A was found in all patients (controls, T2DM). Among all SNPs, only g.+209G>T showed a positive association (OR= 2.4, $p=0.01$) with T2DM. Whereas, g.-243T>G showed a positive association (OR=8.06, $p=0.0003$) with smoking.

Conclusion: A novel variant g.-145G>A REG Iα gene was found among all participants. The SNPs g.+209G>T had a significant positive association with T2DM and SNP g.-243T>G showed an increased risk of the disease among smokers.

Keywords: REG Iα gene, Type II diabetes, β-cells regeneration, Polymorphisms.

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1. Introduction

Diabetes and its management is becoming a challenge for healthcare providers. Worldwide efforts are being made to identify novel molecular markers that have the potential to improve the long-term survival and functional performance of cells.[1,2] It has been hypothesized that insulin-secreting cells multiply and encourage the production of a variety of progenitor stem cells. Numerous genes and associated transcription factors, including the proteins that can be derived from Reg islets such as, "NeuroD1, Sox 9, Netrin 1, and Neurogenin-3", [3] participate in insulin production. Members of the REG gene family found in humans, have been linked to the neogenesis and regeneration of cells. [4] When islets form or cells are damaged, the REG gene is expressed.[5,6] It improves experimentally caused diabetes and regenerates the population of beta cells.[7,8] DNA replication takes place either in an autocrine or paracrine manner in -cells. It was discovered that the signals for -cell growth and regeneration are transmitted by a supposedly Reg protein receptor.[9]

REG I is made by exocrine pancreatic acinar cells and is found in insulin-secreting granules within beta cells.[5] An approximately 2.7 kb gene on chromosome 2p12 that codes for REG I has six exons and five intervening regions.[10] Early stages of pancreatic development and islet cell growth have been associated with REG I gene expression.[11] Recently, a study claimed that insulin insufficiency in chronic pancreatitis is associated with the destruction of acinar cells. Based on a review of the literature, it is hypothesized that the exocrine pancreas's acinar cells, which secrete regenerative proteins, lose their ability to protect against injury to the -cells that secrete insulin cells in chronic pancreatitis.[12] Elevated serum Reg I levels have been linked to numerous kinds of diabetes[13,14] as a sign of renal dysfunction and apoptosis in cells.[15,16] Following gastric bypass

surgery, reg protein overexpression in those with type II diabetes may aid in the disease's remission.[17]
Furthermore, it is important to investigate the relationships between risk factors for diabetes, such as BMI, age, gender, family history of the disease, smoking, hypertension, and the genetic variations of REG I. We sought to investigate REG I gene variants in Pakistani individuals and their potential relationship with type II diabetes and related risk factors to carefully investigate the potential REG I gene association with type II diabetes.

2. Materials & Methods

Blood samples were drawn from 90 finalized patients, including 70 diabetics and 20 controls with an M: F ratio of 12:8. Patients (110) T2DM and age-related controls were selected from PNS Shifa Hospital, Karachi. Twenty patients opted to withdraw from the study. A total of 90 participants were finalized, out of which 70 were diabetic patients and 20 were healthy controls. The certificate from the ethics review committee (Letter No. 02/CREAM-A/Sadaf Saleem) was sought before the collection of samples.
Patients selected with diabetes diagnosed under the American Diabetes Association (ADA). The participants' verbal or written consent was taken before the sampling. An aseptic environment was used for blood sample collection and then DNA extraction was done according to the manufacturer's protocol with some modifications (Sambrook and Russell 2001). BLAST software was used for primer designing of the targeted sites of exons and intronic regions. The Forward (F) and Reverse (R) primers used are as follows:

Exon 1; F: 5'TCCCAAAACTCACCGCTTGC3', R: 5' CTGAGACACCCACACCTTC3',

Exon 2; F:5'AGGTAATAGGTGCTTTGCTCTCC, R: 5'TCCCCAAATCCACCATCACG3',

Exon 3; F:5'CCTTTTCCTTACCCTGAGAGCC3', R: 5'CATTGCAGCCACTGAACACA3',

Exon 4; F: 5'TTTTCTGACCCGTCCTCTTGG3', R 5'GAGACCAGAACTTGAACCTCCT3',

Exon 5; F:5'GGCCCAGTGATTCCATGTAT3', R: 5'GGAGACCCGAAAGAGTATGACC3',

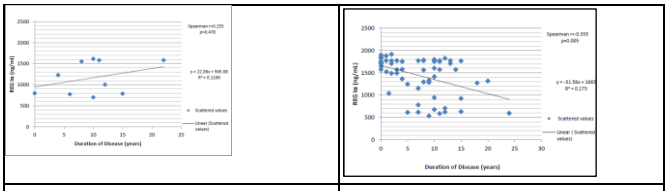
Exon 6; F: 5'TCAAGCACAGGTGAGAGGCA3', R: 5'GTTGAGTTGGAGAGATGGTCCG3', All samples were amplified using the above primer sets, the optimization was done on Bioer's XP PCR Thermal cycler "Pre denaturation at 94^0C for 4 minutes, denaturation at 94^0C for 30 seconds, annealing at 55^0C (Exon 1, 5 and 6) and 53^0C (Exon 2, 3 and 4) for 40 seconds, extension at 72^0C 1kb/min for 35 cycles, post extension 72^0C for 10 minutes, hold 4^0C". the amplified PCR products were run on 2%agarose gel and visualized on Gel Doc imaging system(Herolab EasyWin 32 program). For sequencing 51 samples were selected which were sequenced 6 times 51x6= 306 sequences were obtained for study. Before sequencing, PCR product purification was done by kit method "PCR cleanup kit using Spin Column Method". The Sanger dideoxy method was used to sequence DNA of targeted sites by using Big Dye Terminator v 3.1 cycle sequencing kit on Genetic Analyzer.
Software programs "Clustal X" and "Sequence Scanner 2" were used, respectively, to analyze the alignment of the sequences and to align the REG I gene sequence of the entire study population with the known sequence. The SNPs were found in both the type II diabetes group and the control group. The newly discovered SNPs were compared to known polymorphisms for the target gene from the Gene Card database and dbSNP Nucleotide BLAST.
Statistical package of social sciences (SPSS) software was used to analyze the clinicodemographic and anthropometric data. Shapiro Wilk test was used to determine whether the data were normal. Through the use of logistic regression analysis, odds ratios, a 95% confidence interval, and matching p values were determined. The correlation was used to find an association between RegI and diseases $<p$ 0.05 were regarded as significant.

3. Results

A total of 90 individuals, both control and diabetes were finalized for the study. The controls (n=20) included males (12) and females (8). The type I diabetes (n=10), Male: Female ratio was 7:3 whereas, in the type II diabetics (n=60), Male: Female ratio was 44:16. The mean age (mean $\pm$ SD) of controls was 50 ± 3, and type II diabetics was 55 ± 9 and type I was 34 ± 16. A negative correlation between type II diabetes and

duration of disease was found and the results were statistically significant Figure 1(A, B).

The correlation was studied between the duration of disease and serum REG Iα levels in type I (r=0.255, p=0.476) and type II diabetics (r= -0.355, p=0.005).
Further n=51 patients were selected for polymorphism identification of Reg Iα. There was a total of 6 females and 9 males in controls (n=15) while the group of type II diabetics (n=36) included 11 females and 25 males. The mean age of type II diabetics was 52 ± 9 and the control was 50 ± 3. The mean duration of disease among type II diabetic patients was 6 ± 5, whereas the age at the onset of the diseasewas 46.2 ± 9.



A B

Figure 1(A, B): Correlation between serum levels of REG Iα and disease duration in type I and type II diabetics

In our study population, single nucleotide polymorphisms (SNPs) identified were eight in the REG Iα gene located on chromosome 2p12 (Gene ID: 5967)(Figure 2).

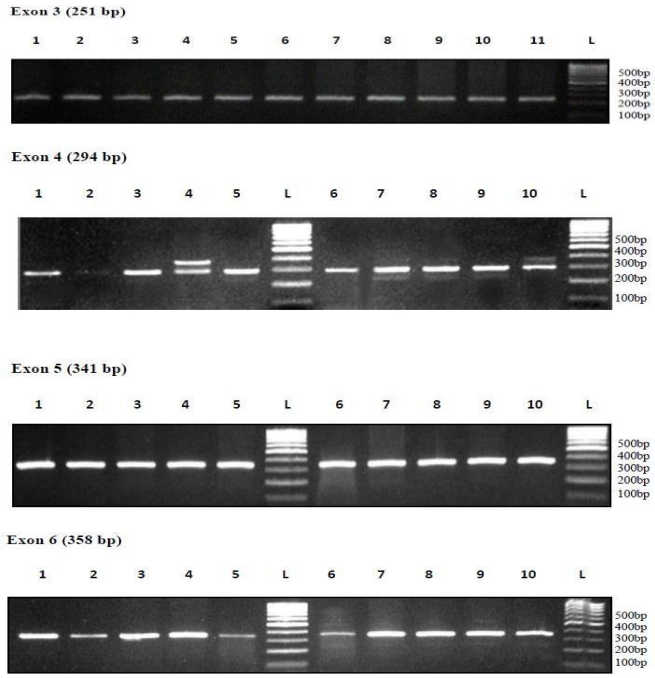


Figure 2: Amplification bands of REG 1alpha exonic region (1-6) (bp: base pair)

When compared with the diabetic patients seven were reported previously and their reference sequence sites (rs) are as follows: (g.2360A>G [rs 12228], g.2199G>A [rs 3739142], g.226A>G [rs 192054590], g.209G>T [rs2070707], g.142A missing [rs11339710], g.-243T>G [rs 283890], and g.-385T>C [rs10165462]). A novel SNP, g.-145G>A, was found in all the targeted populations including the controls and type II diabetics (Figure 3).

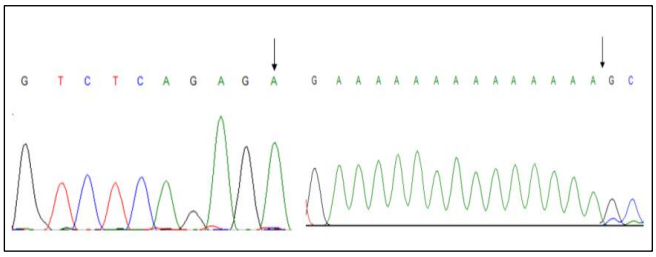


Figure 3: Left (SNP2: -145 G>A) The electro-chromatogram showing substitution of G>A, allele A present in all study populations (indicated by arrow). Right (SNP: +142 A) The electro-chromatogram showing deletion of A, allele A missing in all study subjects). (indicated by arrow)

The genotype of four single nucleotide polymorphisms (SNPs) was calculated in Table 1. For SNP1 (g.-385T>C), the distribution of the genotypes CC, TC, and TT by the clinical presentation in type II diabetics was found to be statistically significant($p<0.05$). The genotype CC was found higher in controls (86%) than diabetics (72%) and lower in smokers (33%). For SNP2 (g.-243T>G), the genotypes GG, TG, and TT were found but the TT genotype was present in the mainstream: 63% in hypertensive, 64% in upraised BMI, 64% in age < 46 years old subjects, 60% in patients with diabetes family history, 62% and 70% in males and females respectively and 22% were smokers. The distribution of the genotypes among diabetics and controls was also found significantly different (p=0.0000)(Table 1). Significant differences (p=0.00) were detected for SNP3 (g.+209G>T) among three detected genotypes (GG, GT, and TT). The genotype GG was the most common among all the subjects: hypertensive (77%), diabetic (81%), and smokers (78%) (Table 1). For SNP4 (g.+2199G>A), the most frequent genotype was AA. It was present in 59% of hypertensive patients, 70% in subjects with a diabetes family history, 65% in both sexes, and 33% in smokers (Table 1).

Table 1: Genotypic distribution of SNP g.-385T>C, -243T>G, +209G>T, +2199G>A

Characteristics	Genotype Frequency															
	-385T>C				-243T>G				+209G>T				+2199G>A			
	TTn (%)	TCn (%)	CC n (%)	*p*-value	TTn (%)	TGn (%)	GGn(%)	*p*-value	GGn(%)	GTn(%)	TTn(%)	*p*-value	GGn(%)	GA,n (%)	AA,n(%)	*p*-value
Diabetic	9 (25)	1(2.8)	26(72.2)	0	22(61)	5 (14)	9 (25)	0	28(78)	3(8)	5(14)	0	12(33)	1(03)	23(64)	0
Control	2(13.3)	0	13(86.7)	0.0001	11(73.4)	2(13.3)	2(13.3)	0.02	15(100)	0	0	0	5(33)	0	10(67)	0.0001
Male	7(20.6)	1(2.9)	26(76.5)	0	21(62)	6 (18)	7 (20)	0.0007	30(88)	1(3)	3(9)	0	11(32)	1(3)	22(65)	0
Female	4(23.5)	0	13(76.5)	0	12(70.5)	1 (6)	4(23.5)	0.0004	13(76)	2(12)	2(12)	0.01	6(35)	0	11(65)	0
Age <46	2(14.3)	0	12(85.7)	0.0001	9(64.3)	3(21.4)	2(14.3)	0.10Ω	9(64.3)	3(21.4)	2(14.3)	0.10Ω	3(21)	0	11(79)	0.0001
Age >46	7(31.9)	1(4.5)	14(63.6)	0	13(59)	2 (9)	7 (32)	0.0001	19(86)	0	3 (14)	0	9(41)	1(4.5)	12(54.5)	0
BMI <23	3(21.4)	0	11(78.6)	0.0001	9(64.3)	2(14.3)	3(21.4)	0.01	13(93)	0	1(7)	0.0001	4(29)	0	10(71)	0.0001
BMI>23	8(21.6)	1(2.7)	28(75.7)	0	24(64.8)	5(13.5)	8(21.6)	0	30(81)	3(11)	4(8)	0	13(35)	1 (3)	23(62)	0
Hypertensive	5(22.7)	0	17(77.3)	0	14(63.6)	3(13.6)	5(22.7)	0.001	17(77.3)	1(4.5)	4(18.2)	0	9(41)	0	13(59)	0
Non-hypertensive	6(20.7)	1(3.4)	22(75.9)	0	19(65.5)	4(13.8)	6(20.7)	0.0004	26(90)	2(7)	1(3)	0.01	8(28)	1 (3)	20(69)	0
Smoker	6(66.7)	0	3(33.3)	0.002	2 (22)	1 (11)	6 (67)	0.03	7(78)	1(11)	1(11)	0.07	6(67)	0	3 (33)	0.002
Non smoker	5(11.9)	1(2.4)	36(85.7)	0	31(74)	6 (14)	5 (12)	0.0005	36(85.7)	2(4.8)	4(9.5)	0	11(26.2)	1(2.4)	30(71.4)	0
FH (Yes)	7(23.3)	1(3.3)	22(73.3)	0	18(60)	5 (17)	7 (23)	0.0007	24(80)	3(10)	3(10)	0.0008	8(27)	1(3)	21(70)	0
FH (No)	4(19)	0	17(81)	0	15(71)	2 (10)	4 (19)	0.0007	19(90.5)	0	2(9.5)	0	9 (43)	0	12(57)	0

In Table 2, the odd ratio (OR= 2.33) calculated to determine the relevance of the frequency of allele T and allele C in control and diabetics for SNP1 - 385T>C showed an insignificant difference (p>0.05) among all variables except smokers and non-smokers showed significant association (p=0.0002), thus, allele C had 0.11 % more protective effect as compared to allele T in smokers. For SNP2 -243T>G, the OR showed significant results among smokers and non-smokers (OR=8.06, p=0.0003) and showed an 8-fold greater risk in smokers with allele G. Compared with the controls, type II diabetics have a significant association with allele T in SNP3 g.+209G>T, ($\gamma 2 = 6.20$, $p = 0.01$) whereas other variables were found insignificant**.** In SNP4 g.2199G>A again smokers versus non-smokers were found significant (p=0.008)

Table 2: Association among SNPs and different variables

Variables	Single Nucleotide Polymorphism											
	-385T>C			-243T>G			+209G>T			+2199G>A		
	OR	γ2	p-value	OR	γ2	p-value	OR	γ2	p-value	OR	γ2	p-value
Diabetes	2.33 (0.71-7.55)	2.06	0.15	0.53 (0.19-1.5)	1.48	0.22	2.4	6.20	0.01	0.94 (0.38-2.31)	0.01	0.89
Control												
Male	1.06 (0.34-3.30)	0.01	0.91	1.37 (0.45-4.2)	0.31	0.57	2.31 (0.67-7.89)	1.81	0.17	1.10 (0.38-3.16)	0.03	0.84
Female												
Age <46	0.32 (0.09-1.10)	3.45	0.06	0.58 (0.20-1.6)	1.01	0.31	0.47 (0.14-1.59)	1.49	0.22	2.78 (0.94-8.22)	3.57	0.05
≥46												
BMI<23	0.50 (0.15-1.65)	1.30	0.25	0.51 (0.16-1.6)	1.31	0.25	1.71 (0.33-8.66)	0.42	0.51	1.16 (0.36-3.70)	0.07	0.79
>23												
Hypertensive	1.37 (0.47-3.94)	0.34	0.55	0.89 (0.32-2.4)	0 4	0.83	1.76 (0.49-6.40)	0.77	0.37	0.69 (0.25-1.89)	0.50	0.47
Non-Hypertensive												
Smoker	0.11 (0.03-0.39)	13.8	0	8.06 (2.3-27.7)	12.8	0	1.06 (0.25-4.43)	0	0.93	0.21 (0.06-0.70)	7.0	0.008
Non-smokers												
FH(yes)	0.89 (0.29-2.75)	0.03	0.85	0.90 (0.31-2.6)	0.03	0.85	1.14 (0.31-4.21)	0.04	0.81	2.69 (0.96-7.48)	3.70	0.05
FH(No)												

4. Discussion

In this study, eight SNPs in the REG Ia gene were identified, among variants g.- 145G>A is being reported for the first time in any population, and it might be the Pakistani population representative. A negative correlation between diabetes and genes was found in this study which was contrary to a study that found no correlation.[18,19] In Korean population studies looking for genetic variations in REG I, certain polymorphisms with weak correlations with type II diabetes with early onset were discovered.[20] The variant g.+209G>T might be the link with diabetes susceptibility. A Korean study on type II diabetes patients found no association of REG Iα gene polymorphisms with the risk of diabetes. The risk, however, of early onset of the disease was found low in variants g.-385C and g.2199A and high with variant 1385G (OR, 1.398 [1.055 to 1.854]).[20] In the current study, we recognized that the REG Iα variant, g.+209G>T might be linked with the overall vulnerability to diabetes. The polymorphism g.209G>T might be significantly associated with the general predisposition to the disease in our population. To our interest, the controlled populace in this study had just allele G (homozygous for GG) while patients of type II diabetes had both T and G alleles (TT, GT or GG). As the T allele exists only in individuals with diabetes, this suggests that we ought to anticipate that the polymorphism g.209T can be a risk factor in type II diabetes. Other studies have also identified the variant g.209G>T such as Koo et al in individuals with type II diabetes, whereas, Mahurkar et al in patients with trophic calcific pancreatitis, but they did not find any significant association with the overall susceptibility to the disease.[20,21] As far as we searched, our study possibly is the first ever study to report the relationship of the SNPs in the gene of REG Iα with the risk of diabetes type II. In this study g.-243T>G, g.-385T>C, and g.2199G>A variants were found having an association with the smokers. The variant g.-243T>G, increased the risk of diabetes by eight-fold in smokers, on the other hand, the variants g.-385T>C and g.2199G>A had protective effects in smokers. At this point, we can conclude that while grouping the diabetic individual according to the illness the genetic variants g.- 385C (rs 10165462) and g.2199G>A (rs 3739142) were found to diminish the risk of disease in smokers while the variant g.- 243G (rs 283890) extends the disease risk in smokers up to eight folds. Earlier studies on the REG Iα gene and protein were done mainly investigating its relationship

with cancer. Studies on patients with fibrocalculous pancreatic diabetes also having type I diabetes, investigation, did not find any significant link between disease and variants.[20] Similarly, in trophic calcific pancreatitis patients, researchers made a wide-range scrutiny of the REG Iα gene and found a few SNPs in the promoter region but were unable to find any disease association.[21]

Oxidative stress caused by smoking is fully endorsed by many studies. It is well documented that oxidative stress which plays a pathogenic role in the body initially leads to inflammation which is the first line of defense. [22,23]

A study in the Slavonic population by Azorova et al found that smoking triggers an association between the human insulin-like growth factor 2 (IGF2BP2) gene, rs11927381 polymorphism, and the development of type II diabetes.[27] Age was also found related to disease risk. Patients less than 46 years old with hereditary variant g.-385C have less prevalence of diabetes. These results are by the Korean study which concluded that people with variant g.- 385C were found to have less risk of type II diabetes[20] Considering several inevitable limitations, enrolments of patients with type II diabetes, their family history, BMI, sex, hypertension, onset of disease, age; missing or uncollected data in controls, unavoidable selection bias and related polymorphisms to the association diabetes its development and risk factors require further research with bigger sample size better prospective studies.

The sample size was limited in this study, primarily due to the need to sequence six exons of a gene using six sets of primers for each of the 51 samples, resulting in a total sample size of 306. The budget constraints prevented the inclusion of additional samples.

5. Conclusion

The REG Iα gene novel polymorphism g.- 145G>A was discovered for the first time in Pakistani people. A positive association with significant results were seen between the SNP g. +209T and type II diabetes in any population. The association between smoking and the SNP -243G (rs 283890) was found significant making this SNP risk factor for diabetes in the smokers. Further research with a bigger sample size and a better prospective study on polymorphism in the REG Iα gene, Inflammation triggers cytokine production and activated immune cells cause insulin resistance and diabetes.[24] Smoking, however, does not affect every smoker and makes him diabetic. The genetics play a huge role and only individuals who are genetically predisposed to diabetes are affected. This suggests that smoking may be the genetic modifier of the disease. Many genetic variants have been identified by various studies showing an association with the risk of diabetes.[25,26] In this study, we found a link between SNP g.- 243G(rs 283890) and smoking which may be a cause of developing diabetes. It can be predicted that smoking may be the avoidable and achievable cause of diabetes.

diabetes type II is recommended to identify risk factors that may be avoidable.

CONFLICTS OF INTEREST- None

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Contributions:
S.S, S.B, S.F, M.S - Conception of study
S.S, S.B, S.F, M.S - Experimentation/Study Conduction
S.S, S.B, S.F, M.S - Analysis/Interpretation/Discussion
S.S, S.B, S.F, M.S - Manuscript Writing
S.S, S.B, S.F, M.S - Critical Review
S.S, S.B, S.F, M.S - Facilitation and Material analysis

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