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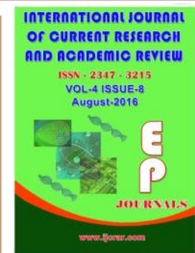

Association of IGF2BP2 Gene Polymorphism on Type 2diabetes: A Case Control Study on Chennai Population, India

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KEYWORDS

Type 2 Diabetes,
GWAS,
RFLP,
Gene
Polymorphism

A B S T R A C T

Type 2 Diabetes is the most common type of clinical disorder found worldwide. Recently, advanced technique like Genome Wide Association Studies (GWAS) has confirmed the effect of certain susceptible genes and their loci playing significant role in Type 2 Diabetes. To identify the mutant variants of IGF2BP2 gene prevalent in South Indian type 2 diabetic population against the non-diabetic controls using Restriction fragment length polymorphism (RFLP) analysis. The study focuses on association between Insulin-like growth factor 2 (IGF2) mRNA-binding protein-2 (IMP2/IGF2BP2) in South Indian diabetic population, which is involved in development of pancreas, growth and stimulation of insulin action. Three possible genotypes were screened and compared amongst both type 2 diabetic and non-diabetic controls. PCR analysis was done using DNA isolated from blood samples to amplify IGF2BP2 gene using the primers TD5 and TD6. Restriction fragment length polymorphism (RFLP) analysis was performed on PCR ampliconsusing*Msp1* enzyme that recognizes homozygous mutant. Agarose gel electrophoresis was performed on the digested amplicons to determine the genotype. Electrophoresis results post enzyme digestion indicated the following; in homozygous normal and mutant, 2 fragments and 1 single fragment were observed respectively, whereas in heterozygous mutants 3 fragments were observed upon digestion. Statistical analysis was done using SPSS v10.2 software. Overall, the frequency of G allele was higher in the diabetic patients when compared to the controls. A large scale study is further required to confirm the association of the IGF2BP2 gene polymorphism with Type 2 diabetes.

Introduction

Under normal physiological conditions, insulin is the key regulator of glucose and lipid metabolism. However, there are evidences that prove, Insulin-like Growth Factor (IGF)-I play an important influential role. Insulin and the Growth Hormone (GH)-IGF systemis known to coordinate the growth and energy uptake in many organisms, which is achieved by the regulation of IGF-I through IGF-binding proteins. Insulin seems to control the IGF system by modifying the IGFBPs (Ayman *et al.,* 2009; Bach *et al.,* 2005; Wheatcroft *et al.,* 2007). Insulin-like growth factor binding protein (IGFBP) play a major role in metabolism and growth. Presently, six mammalian IGFBPs (IGFBP-1–6) have been characterized. Prevalence ofIGFBP2 is found to be higher in blood and is highly expressed largely in liver followed by adipocytes, and central nervous system, and has been found to be involved in metabolic homeostasis and insulin resistance. Insulin-like growth factor binding protein IGFBP2 is known to be carrier for Insulin-like growth factor (IGF-1)which plays a key role in regulating growth and development, and also has a variety of functions related to cardiovascular disease, cancer, diabetes, liver cirrhosis and Laron syndrome (Kang *et al.,* 2015; Kang *et al.,* 2016).Insulin-like growth factor closely resembles insulin, but has distinct metabolic actions. IGF-I is able to coordinately link growth hormone and insulin actions and has direct effects on intermediary metabolism.IGF-1at supraphysiologic concentrations have an indirect effect in suppression of insulin secretion. IGF-I has been shown to be linked with lowering glucose and improving insulin sensitivity in both type 1 and type 2 diabetes (David R. Clemmons, 2012).Type 2 Diabetes often begins with resistance to Insulin, i.e. when the cells are unable to recognize Insulin hormone thus preventing metabolism of glucose to take place. Obesity, which evolves due to the adipose tissue expansion, is known to be the major contributing factor towards insulin resistance leading to Type 2 Diabetes, and other diseases. Studies have shown that obese humans have reduced IGFBP-2 levels in serum, which plays key role in IGF regulation (Maddux *et al.,* 2006; Wheatcroft *et al.,* 2007).

Materials and Methods

Blood samples of both diabetic and control patients were obtained from three places in Chennai, i.e. SS labs, Thiruvanmiyur, New Asian Hospital, Besant Nagar and Dr. Rai Memorial Medical Centre, Teynampet. The patients and volunteer controls were first asked to complete an interview based questionnaire, through which the following details were obtained; i) personal details, ii) age of onset of diabetes, iii) familial history, iv) medical history. The normal and abnormal levels of blood sugar was scrutinized in control and diabetic patients according to the Chart in Table 1 (www.medindia.net). Once the informed consent was obtained, 5mL of blood was collected from both the patients and controls.

Isolation of DNA

The genomic DNA was isolated using modified Miller’s protocol (1988).

Reagents required

RBC Lysis Buffer

7.462g Ammonium Chloride (NH_4Cl) dissolved in 900mL of double distilled water.

2.06g Tris dissolved in 100mL of distilled water.

Both the solutions are mixed and made up to 1L with double distilled water and autoclaved at 15psi for 15min and stored at room temperature.

1M Tris

12.114g of Tris is dissolved in double distilled water and pH was adjusted to 8.0 and then the solution was made up to 100mL and autoclaved at 15psi for 15min and stored at room temperature.

1M Sodium Chloride

5.844g was dissolved in double distilled water and made up to 100mL which was further autoclaved at 15psi for 15min and stored at room temperature.

0.5M Na_2EDTA

18.612g of Na_2EDTA was dissolved in double distilled water and made up to 100mL. This solution was autoclaved at 15psi for 15min and stored at room temperature.

WBC Lysis Buffer

1mL of 1M Tris, 4mL of 1M NaCl and 0.4mL of 0.5M Na_2EDTA was dissolved in double distilled water and made up to 100mL and stored at room temperature in autoclaved reagent bottle.

10% Sodium Dodecyl Sulphate (SDS)

2.5G of SDS was dissolved in 25mL of autoclaved double distilled water and stored at room temperature in autoclaved reagent bottle.

6M NaCl (55.44)

35.064g of NaCl was dissolved in autoclaved double distilled water and made up to 100mL and stored at room temperature in autoclaved reagent bottle.

Ethyl alcohol (absolute)

Commercially purchased from Tedia Co. and stored in refrigerator.

70% Ethonol

30mL of autoclaved double distilled water was added to 70mL of Absolute ethyl alcohol to make 100mL of the solution which was stored in autoclaved reagent bottle in refrigerator.

The purpose of the study was explained to the volunteers taking part and the consent forms were signed by control volunteers and the diabetic patients.Five milliliter of peripheral venous blood was collected at the elbow joint using disposable syringe (Dispovan). This was transferred to 15ml sterile centrifuge tubes which had a pinch of Na_2 EDTA. The tube was inverted for few times and stored at 4ºC for further processing. The blood samples were centrifuged at 3000rpm for 30min and the buffy coat was carefully transferred to a fresh sterile 15ml centrifuge tube using a Pasteur pipette. 10ml of RBC lysis buffer was added to the buffy coat and aspirated vigorously with Pasteur pipette and was incubated at 37ºC for 10min followed by centrifugation at 3000rpm for 20min. The supernatant was decanted. The RBC lysis step was repeated until a clear white pellet of WBC was obtained.

3ml of WBC lysis buffer was added to the pellet and aspirated vigorously, to which 200µL of 10% SDS was added and the tubes were gently inverted for 10min. The tubes were incubated overnight (16±2 hours) at 37ºC. 1mL of 6M NaCl was added and inverted several times for 20sec and were

centrifuged at 3000rpm for 20min. The supernatant was transferred to 15ml centrifuge tubes to which double the volume of ice-cold absolute ethanol was added and inverted until the DNA precipitates. This was distributed into 1.5ml micro centrifuge tubes and centrifuged at 10,000rpm for 10min and the supernatant was discarded. To the DNA pellet 1ml of 70% ethanol was added and inverted for a couple of times and centrifuged again at 10,000rpm for 10min. The supernatant was discarded and the pellet was air dried at room temperature. 100µl of TE buffer was added to the semidried pellet and left at room temperature overnight to dissolve. This was then stored at 4ºC.

Agarose Gel Electrophoresis of DNA Samples

The 0.7% Agarose gel is the appropriate concentration for electrophoresis of human genomic DNA.

Reagents

10X Tris-Borate EDTA (TBE buffer)

108g of Tris base, 5.5g of Boric acid and 40mL of EDTA was dissolved in double distilled water to made upto 1000mL. The pH was adjusted to 8.3 and autoclaved at 15psi/15min and stored at room temperature.

1X TBE (working solution)

10ml of 10X TBE was made up to 100ml with double distilled water.

6X loading buffer

25mg of Bromophenol blue and 3ml 60% Glycerol was dissolved dissolved in double distilled water to made up to 5ml and stored at 4ºC.

Ethidium Bromide (EtBr)

10mg of EtBr and 1ml of autoclaved double distilled water was dissolved and stored at 4ºC in 1.5 ml micro centrifuge tubes.

Electrophoresis tank was set up using the above reagents. 5µL of the DNA sample along with 2µL of loading dye was loaded into each well. Electrophoresis was carried out at 50 volts for 2hours, the gels were observed under an UV transilluminator, (Uvitec, lark inoovative Inc.) and photographs were taken.

Polymerase Chain Reaction (PCR)

PCR was carried out to amplify the IGFBP2 gene of human with rs7651090, to detect the polymorphismpresent.

Reagents required

A) Theprimers wereobtained in the lyophilized for from MWG Biotech Germany. Details of the primers are given in the Table-2.

The primers were diluted according to the vendor's description to a working concentration of 10pmol.

B) Working dNTP mix:Each dNTP was supplied in 10mM concentration. Equal volume of each dNTPs was mixed to get a final concentration of 2.5mM of each of the dNTPs. It was stored at -20 ºC

C) $MgCl_2$was diluted so as to get a final concentration of 2mM in the PCR reaction mixture.

D) Taq polymerase was obtained at a concentration of 1 U/µL and was stored at -20ºC.

The PCR reagents were mixed in sterile conditions on ice. Centrifugation was carried out at 4ºC. Total reaction volume was 20µL for each reaction. The PCR components were added to the PCR reaction tube in a stepwise manner. The various components and their respective volumes are represented below in the sequential order of addition in Table 4.

Initial denaturation and denaturation of DNA was carried out at 94ºC for 5min and 45sec respectively. Annealing temperature was standardized to 59.7ºC for 45sec. Extension was carried out at 72ºC for 45sec. The above steps of the PCR cycle were repeated 30times followed by final extension at 72ºC for 5min. The holding temperature is 4ºC.

After each PCR cycle the products (5µL) were electrophoresed on a 2% agarose gel dissolved in 1X TBE buffer along with 2µL loading dye to check for amplification.

RFLP Analysis

Genotyping was performed by RFLP (Restriction fragment length polymorphism) analysis. PCR amplicons were digested with *MspI* enzyme, which recognizes homozygous mutant and produce 2 fragments in normal cases (146bp+236bp). In mutant forms, *MspI* will not recognize any site, therefore only one fragment is formed. In heterozygous mutant type *MspI* is able to catalyze the gene into 3 fragments (146bp+236bp+382bp).

RFLP-reaction

Reagents: 0.9µL of double distilled water, 1.0mL of 10X Buffer and 0.1µL of *MspI*is dissolved and made up to 2.0µL.

The digested amplicons were analysed by electrophoresis. 8µL of the digested sample along with 2µL of loading dye is sufficient to confirm if the digestion has taken place. The digested DNA samples in the gel was viewed under UV transilluminator and photographed.

Results and Discussion

Sample collection

This study is a part of large study involved in the comparative screening of genes in Diabetes patients and controls. A total of 118 persons voluntarily enrolled for the study out of which 68 were type 2 Diabeticand remaining 50 were control. DNA was isolated from all the volunteers and checked qualitatively and quantitatively using 0.7% agarose gel. The agarose gel electrophoresis revealed a good yield of DNA with minimal shearing and contamination. This denotes that the DNA sample was suitable for further analysis using PCR followed by RFLP.

PCR Amplification

All the collected samples were processed for the presence of rs7651090 polymorphism in the IGFBP2. The samples were amplified using primers TD5 and TD6 and the annealing temperature was standardized to 59.7ºC. At this temperature, an intense specific band of the expected size of 382bp was observed.

RFLP Analysis

The restriction digestion using *MspI* enzyme was carried out for all the amplicons. The digestion products were analyzed on the agarose gel suitable for the analysis of digested products, which was standardized to 2%. The expected size of the digested product was observed. In homozygous normal, the enzyme recognized 5'- GGCC – 3' producing 2 fragments (146bp+236bp).

Whereas in homozygous mutant no sites are recognized, thus digestion does not take place. In heterozygous mutant type, 3 fragments are produced by *MspI* enzyme (146bp+236bp+382bp). The frequency of the different genotypes, GG, GA, AA has been compared between the diabetic and controls.

Statistical Analysis

Statistical analysis was done using SPSS (version 10.2) software. The data from the genotype and distribution of the alleles for the *IGFBP2* gene in diabetic and non-diabetic has been tabulated.

Table.1 Blood Sugar Chart

Category	**Fasting Value (mg/dl)**		**Post Prandial (mg/dl)**
	Minimum Value	**Maximum Value**	**Value 2 hours after food**
Normal	70	100	Less than 140
Early Diabetes	101	126	140 to 200
Established Diabetes	More than 126	-	More than 200

Table.2 Details of the designed primers.

S.No.	**Primer Name**	**Primer Sequence**	**Primer Length**	**GC%**	**Temperature**
1	Forward Primer	5' – CAC CTG GCA ATG GAC ACT CG – 3'	19 - mer	57.9	58.8 ºC
2	Reverse Primer	5' – CCA CCA CAC CCA GCT AA TTT – 3'	20 – mer	50	57.3 ºC

Table.3 Concentration of PCR Components.

Autoclaved double distilled water	11.6μL
10X PCR buffer with $MgCl_2$	2.0 μL
2.5mM dNTPs (each)	0.1 μL
Forward Primer	2.0 μL
Reverse Primer	2.0 μL
Taq polymerase (1U/μL)	0.3 μL
Template DNA	2.0 μL

Table.4 Genotype and allele frequencies of intron 3 rs7651090 G/A polymorphism of IGFBP2 gene: All subjects

Subjects	Genotype				Allele Frequency		χ^2
	N	**GG**	**GA**	**AA**	**G**	**A**	
Control	50	6	37	7	0.490	0.510	11.548
Diabetic	68	12	52	4	0.559	0.441	20.636

χ^2– Hardy-Weinberg Equilibrium.

Table.5 Percent and gene frequencies of intron 3 rs7651090 G/A polymorphism of IGFBP2 gene: All subjects

Genotypes	Control (%)	Case (%)	P value*
Homozygous reference (GG)	12	17.6	0.6990
Heterozygous (GA)	74	76.5	0.2852
Homozygous variant (AA)	14	5.9	0.2273
MAF#	**51**	**44.1**	**0.3603**

*- Corrected value

- Minor Allele Frequency

Fig.1 0.7% Agarose gel electrophoreto grams of human genomic DNA. 5μL of DNA sample + 3μL of loading dye,electrophoresis at 50V for 2hrs.

HUMAN GENOMIC DNA.

1 2 3 4 5 6 7 8

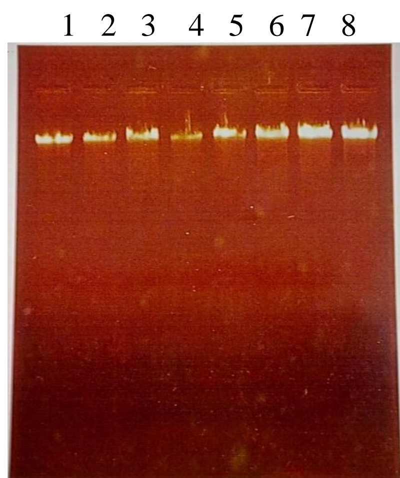


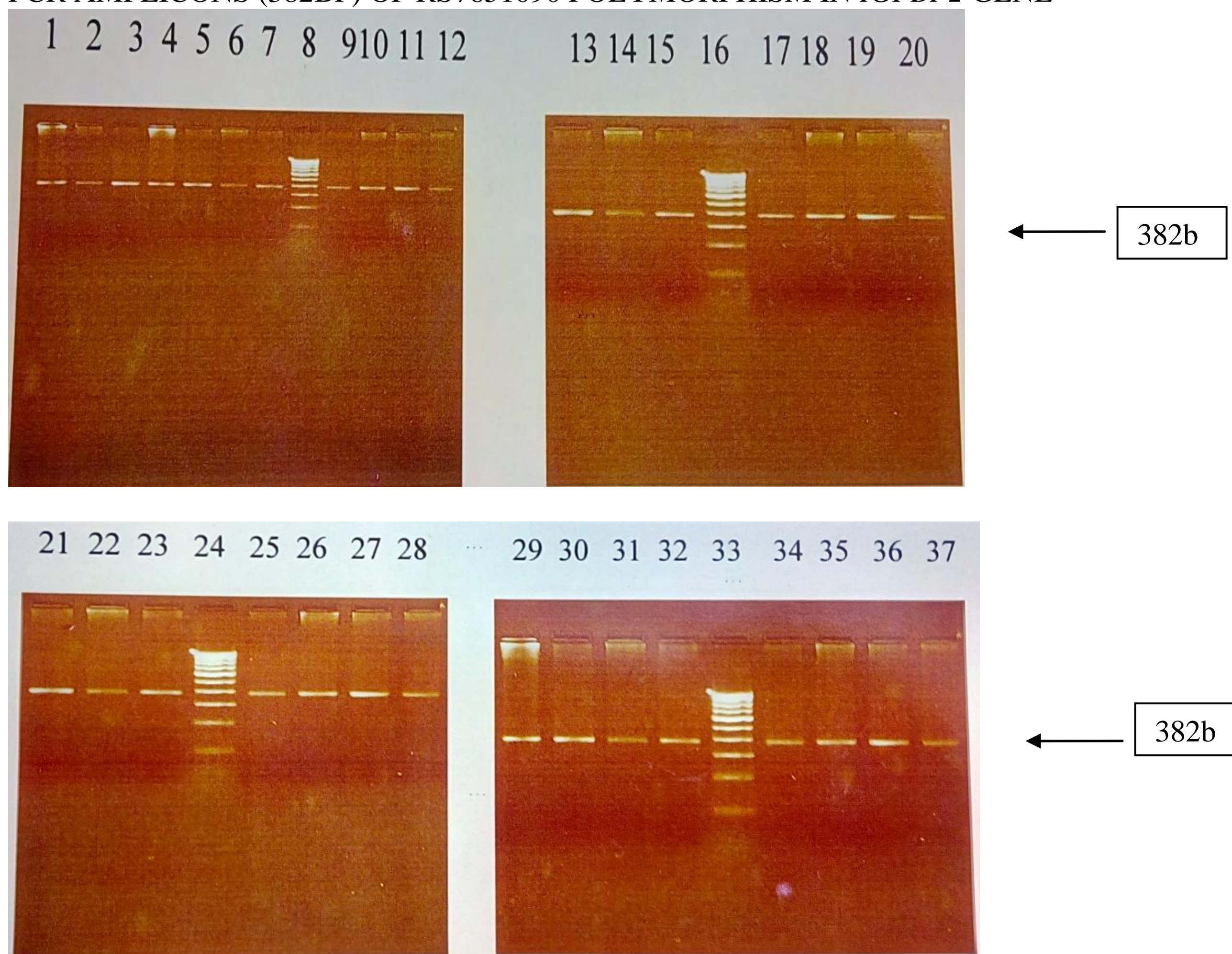
Table.6 Odds ratios of intron 3 rs7651090 G/A polymorphism of IGFBP2 gene: All subjects

Case vs. Control	ODDs Ratio	P value**	95% CI	
GA vs. AA	2.46	0.28516	0.671	9.014
AA vs. GG	0.29	0.22728	0.059	1.375
GA vs. GG	0.70	0.69896	0.242	2.042
AA + GA vs. GG	0.64	0.55922	0.221	1.830
AA vs. GA + GG	0.38	0.23866	0.106	1.392
A vs. G	1.82	**0.36030**	1.087	3.063
		** Unadjusted value		

Fig.2 2% Agarose gel electrophoretograms of PCR amplicons (382bp) of rs7651090 polymorphism in IGFBP2 gene (using primers TD5 and TD6).

Lanes 1-7, 21-23, 25-32, 34-40 - Amplified products from patients.
Lanes 8, 16,24, 33, 45, 51 - 100bp DNA ladder
Lanes 9-15, 17-20, 41-44, 46-50, 52,53 - Amplified products from controls

PCR AMPLICONS (382BP) OF RS7651090 POLYMORPHISM IN *IGFBP2* GENE



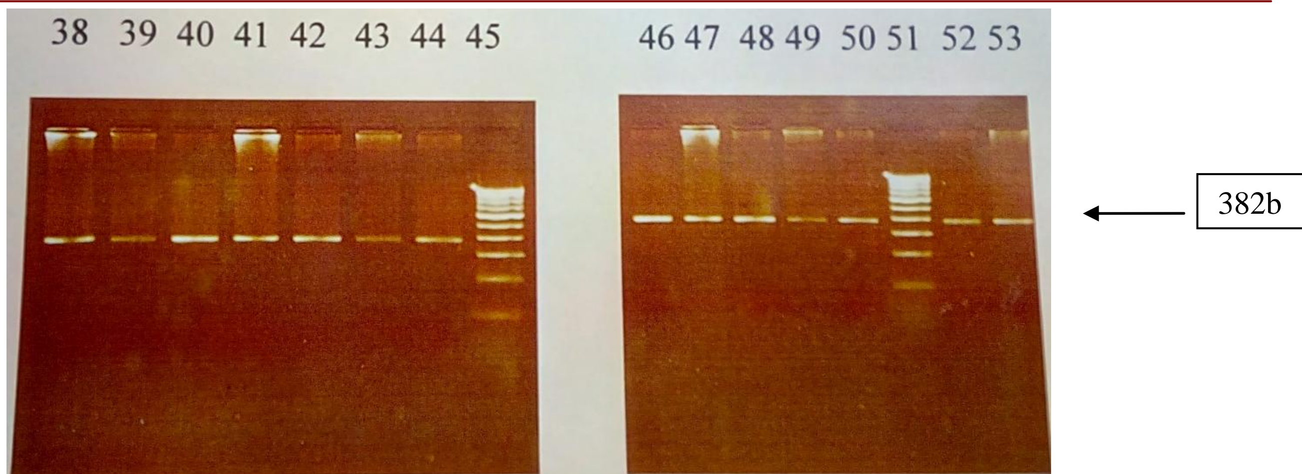


Fig.3 2% Agarose gel electrophoretograms showing the restriction digestion patterns of rs7651090 polymorphism in IGFBP2 gene (using primers TD5 and TD6) using the MspI enzyme.

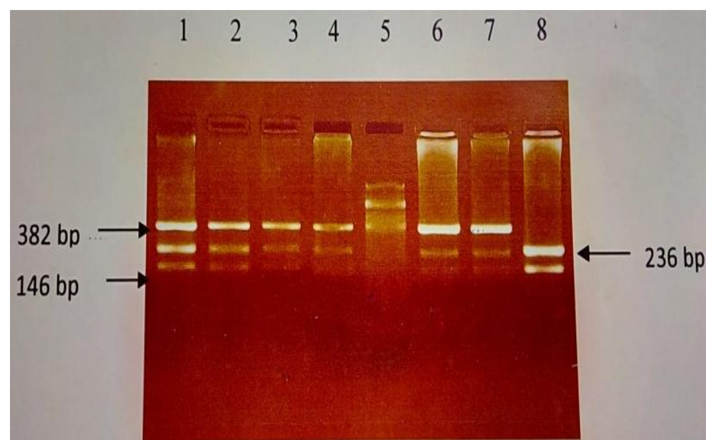
Lanes 1-4, 6,7, 9-11, 15,16, 18,20-22,24,25, 27,29-31, 33,37-40, 42,43, 46, 48,50-52,56 - Restricted homozygous products for the rs7651090 polymorphism showing 3 bands corresponding to 236bp and 146bp respectively for GA genotype.

Lanes 8,23,32,34-36,44,45,55 - Restricted homozygous products for the rs7651090 polymorphism showing 2 bands corresponding to 236bp and 146bp respectively for GG genotype.

Lanes 13,17,26,47,49,54 - Restricted homozygous products for the rs7651090 polymorphism showing single band corresponding to 382bp respectively for AA genotype.

Lanes 5,12,19,28,38,50 - 100bp DNA ladder.

Restriction digestion patterns of RS7651090 Polymorphism in *IGFBP2* gene



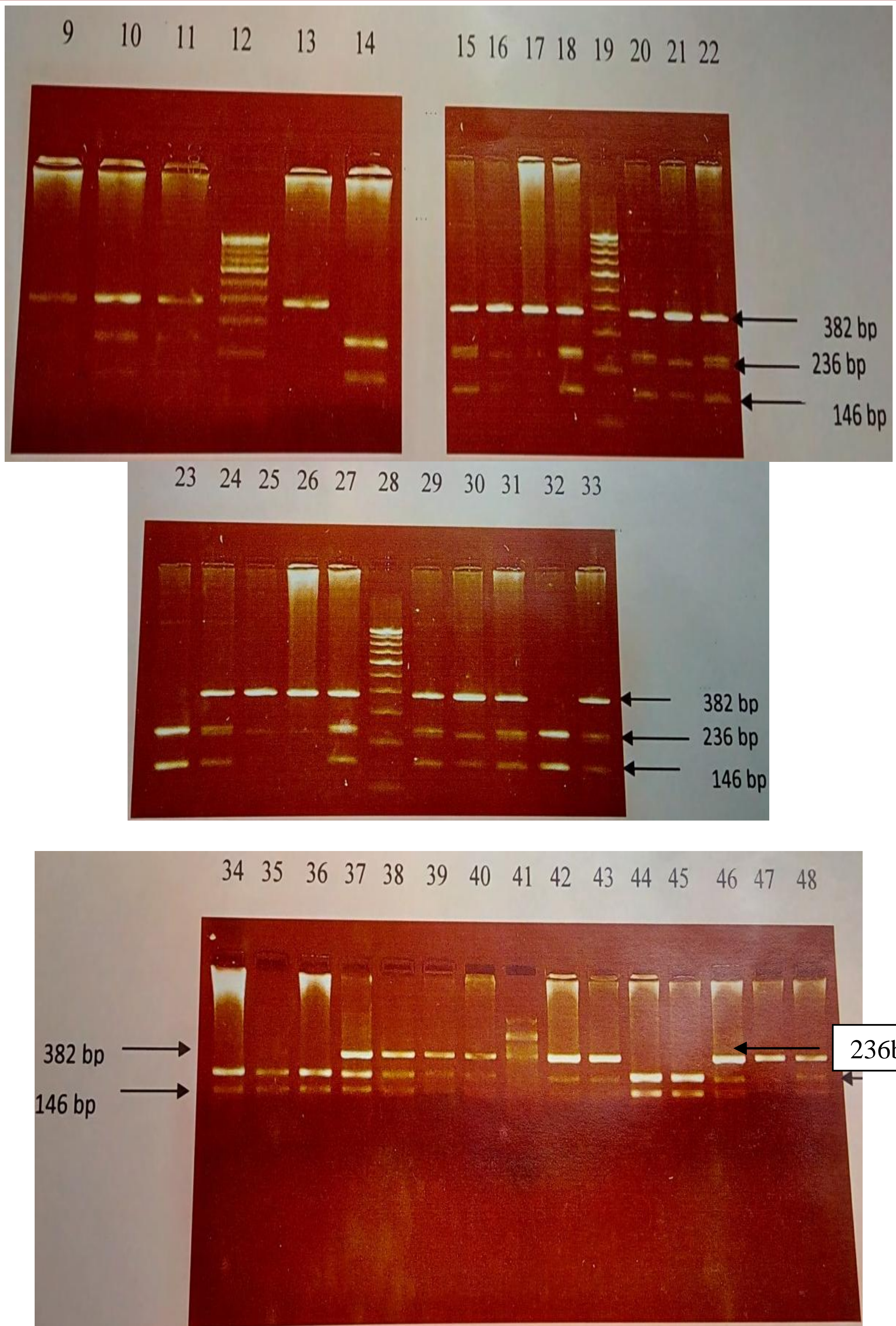

9
10
11
12
13
14
15
16
17
18
19
20
21
22
382 bp
236 bp
146 bp
23
24
25
26
27
28
29
30
31
32
33
382 bp
236 bp
146 bp
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
382 bp
146 bp
236b

Fig.4 IGFBP2 intron 3 (rs7651090) Genotype frequency between Type 2 Diabetic patients and Controls

Genotype frequency

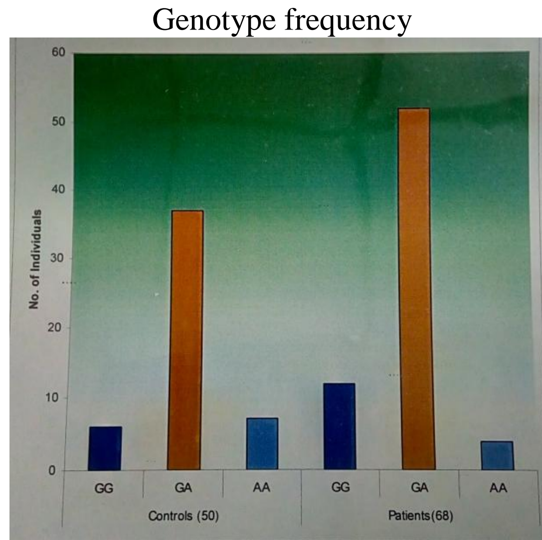
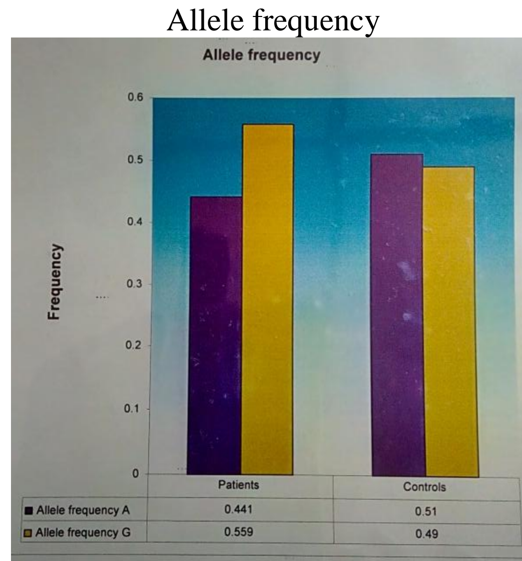


Fig.5 IGFBP2 intron 3 (rs7651090) Allele frequency between Type 2 Diabetic patients and Controls.

Allele frequency



It is a known fact that Diabetes Mellitus is a complex disease that occurs due to the malfunctioning of Insulin and β-cell of islets of Langerhans in Pancreas which enables glucose to be metabolized. Overall increase in diabetic patients is due to some internal and external factors such as, stress, obesity, food habits, lack of exercise, etc. Genetic mutations also play a major role in increasing the susceptibility to Diabetes, which has been proven by Genome Wide Association Studies (GWAS) based on single nucleotide polymorphism (SNP).

The gene *IGFBP2* has a major role in the late onset of diabetes. The gene is found to

be involved in the development of pancreas, growth and stimulation of insulin action (Hoek *et al.,* 2009). The *IGF* gene also plays a critical role in placental and fetal development. One of the SNP, rs7651090 of *IGFBP2* is found to play an essential role in the occurrence of T2D. A significant association of this rs7651090 with T2D was confirmed in Chinese population with the ORs ranging from 1.114 to 1.282 9 ($p<0.05$) (Hu *et al.,* 2009).

The gene *IGFBP2* has been found to play an important role in the development of breast tissue, and possibly in the progression of cancers. It plays an essential role in regulating proliferation, differentiation and apoptosis (Neuhausen *et al.,* 2009).

The genotype and allele frequencies of *IGF2* intron 3 G/A polymorphism is depicted in Table 4 and Figures 3 and 4. The frequencies of the GG genotype was higher in diabetic patients (17.6%) compared to the controls (12%). Also the GA genotype was differed between the cases and controls (Table 5 and Fig. 5).

The odd ratios were compared for the comparison of various genotypes and alleles. The polymorphism shows a contrasting result, that the ratio of A vs. G p = 0.36030, and that this polymorphism might not be associated with T2D. Our results demonstrates that there is no association of the A allele of *IGFBP2* gene polymorphism with T2D.

Apart from environmental factors, genes play an important role in type2 diabetes, which is found to be more common in people who are obese and have no physical activity. The gene IGFBP2 with the SNP rs 7651090 was analyzed in both patients and control samples. The statistical analysis showed that there is an association of A allele in IGFBP2 gene polymorphism with type 2 Diabetes. According to the evidences found, this study has established the link between IGFBP2 gene polymorphism and type 2 diabetes. A large cross- sectional study needs to be done in south Indian population to confirm the result obtained in this study.

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