A study on Insulin receptor gene mutations in diabetics (Type I & Type II) and healthy individuals from North Karnataka region, India.

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Abstract

Background: There is variation in requirement of insulin in cases of Type I and Type II DM patients with same blood sugar levels. The aims of the study were to identify, compare and correlate any mutations in INSR gene among Type I & Type II diabetics and normal healthy individuals.

Material and Method: Peripheral venous blood was collected from 150 Type II DM cases and 150 healthy subjects as controls. The patients diagnosed as per American association of diabetes criteria were included. Patients and controls with any other disease likely to have genetic etiology were excluded. Type I DM 37 cases were included. The DNA was extracted by Qiagen kit. PCR products were developed for Exon 2(INSR2), 14 (TK14) and 17(TK17) of INSR gene. The PCR products were subjected to 10 % PAGE vertical electrophoresis. The products showing variation in banding pattern were subjected for Sanger sequencing.

Results: Mutations in INSR gene were not seen in Type I DM cases. In case of Type II DM cases as well as controls clinically insignificant polymorphism was seen.

Conclusion: Mutations in INSR gene was not seen in cases of Type I DM cases studied. Clinically insignificant mutations were noted in Type II DM cases as well as controls indicating polymorphism.

Keywords : INSR, Diabetes mellitus, Mutations, Insulin resistance

Introduction:

Diabetes mellitus is today a prime concern in society for morbidity, disability and premature death. It is caused by combination of genetic and environmental risk-factors. Human insulin receptor gene (INSR) is responsible for coding of INSR protein receptor. Polymorphism in INSR gene may result in variation of insulin utilization, which may lead to insulin resistance in type-2 and increase in insulin dosage in type 1 diabetic patient. Susumu et al stated that human insulin receptor is present on all surface cells as integral part of membrane protein. The human INSR gene is located on the distal short arm of the human chromosome 19. The gene is composed of 22 exons and 21 introns. Bahram Kazemi et al in his study on Iranian population found 26% patients of type-2 diabetes mellitus having INSR gene mutations which were not reported earlier. Significant difference between black and white populations regarding frequency of DNA polymorphism in exon 17-21 has also been observed. Jasmine Sokhi et al observed that study that there are variations in polymorphism of INSR gene within ethnic Indian population. This study was undertaken to find mutations in INSR gene in our population which might help in early detection, screening and treatment of disease[1-3].

Materials and methods:

Patients and sampling criteria:

The subjects already diagnosed according to American Diabetic Association (ADA) and World Health Organization (WHO) criteria for Type-I - Insulin Dependent Diabetes Mellitus (IDDM) and Type-II – Non Insulin Dependent Diabetes Mellitus (NIDDM) diabetes mellitus from out patients department of Medicine, Belagavi Institute of Medical Sciences, Belagavi Hospital were selected based on following Inclusion and exclusion criteria. Ethical clearance was taken from the Institutional ethical committee (IEC) and Consent for voluntary participation of participant

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was obtained in prescribed consent format.

Inclusion criteria: Type I Diabetes mellitus patients- Abrupt onset of hypercatabolic, ketosis prone, dependent on daily insulin therapy.

Inclusion criteria: Type II Diabetes mellitus patients- 40 years old or older at the onset of disease. No history of diabetic ketoacidosis.

Exclusion criteria: All the patients with Type I & Type II Diabetes Mellitus with any other diseases having genetic etiology were excluded from the study.

Control Group: Normal healthy age and gender matched unrelated individuals, without any diabetic 1° relative.

Sampling criteria: 5 cc of venous blood in EDTA containing vacutainers was taken from identified cases and control group with all aseptic precautions. Total 337 samples were collected from Type I (37), Type II (150) and Normal healthy individual (150). Sample size was calculated upto 5% absolute error with the help of the prevalence rate of the disease.

DNA extraction from blood:

DNA was extracted from blood sample by QIAGEN kit. First 20 µl protease was taken in eppendorf tube then 200 µl blood sample was added, 200 µl of AL buffer was added. The above solution was vortexed for 15 seconds and was incubated at 56 oC for 10 minutes in dry water bath. The solution was briefly spinned to remove the drops from inside of the lid. 200 µl ethanol was added to sample and was mixed for 15 seconds by pulse voxtexing and was spinned briefly to remove the drops from inside of the lid. Carefully the mixture was added to spin column without wetting the rim. Cap was closed and centrifuged at 8000 rpm for 1 minute. Then spin column was placed in 2ml collection tube and tube containing the filtrate was discarded. Spin column was opened carefully and 500 µl AW1 buffer was added to it without wetting the rim. The cap was closed and centrifuged at 8000 rpm for 1 minute. Again the spin column kept in a clean 2mL collection tube and tube containing the filtrate was discarded. Spin column was opened carefully and 500 µl AW2 buffer was added to it without wetting the rim. The cap was closed and centrifuged at 14000 rpm for 3 minute. Again the spin column kept in a clean 2mL collection tube and tube containing the filtrate was discarded. Spin column was placed in eppendorf tube and 200 µl buffer AE was added to it. It was incubated at room temperature for 1 minute and centrifuged at 8000 rpm for 1 minute. Then spin column containing DNA extract was labeled and stored at -20°C. Concentration and the purity of DNA samples were determined by spectrophotometer using quartz cuvette for the greatest accuracy, reading between 0.1 and 1 was taken[3].

PCR amplification:

For each exon (INSR2, TK14, TK17) a pair of primers was designed as per table shown below. PCR reaction mixture was prepared in a PCR tube by adding master mix (MM), reverse and forward primers, H_2o and DNA extracted. The mixture was kept in thermal cycler with initial denaturation at 94°C for 5 minutes, followed by 30 cycles including denaturation at 94°C for 30 seconds, annealing at exon primer specific temperature for 60 seconds and extension at 70°C for 30 seconds. The final incubation was included at 72°C for 5 min.

Table 1: Showing the details of Primers.

Exon	Primer Sequence	Size of PCR product (bp)
2-INSR2	F 5` - TGT GTC CCG GCA TGG ATA TC – 3`	653
	R 5` - CCC CTA CCT AAT GAC CAT TT – 3`	
14-TK14	F 5`- CTC CTT CTC CTCCTC TCT TC –3`	210
	R 5`- CTG AGG CTG CCA TGG AGA C –3`	
17-TK17	F 5`- GCA TGG GTC CTG GAT CAC AG –3`	480
	R 5`- TAG GAG GAT ACA CCC TGT GTC –3`	

PCR product electrophoresis:

The PCR products was electrophoresed in horizontal 1.5% agarose gel for confirmation of quality and quantity of PCR product. The gel was stained with ethidium bromide, and the bands were visualized under UV transillumination and Photographed.

Scanning of PCR products by vertical gel electrophoresis:

The PCR product obtained was run on vertical gel electrophoresis made up of 10% polyacrylamide gel. 10% PAGE resolving gel was prepared with acrylamide bis acrylamide solution, TAE buffer, 1.5% APS, Temed and distilled water. 1-mm-thick gel prepared with 10% polyacrylamide made from 40% stock of acrylamide containing 99:1 acrylamide to bisacrylamide. The stacking gel was 5% polyacrylamide gel. The gel was setup by prerunning empty for 30 min. The PCR products after loading were electrophoresed for 60 min. at 90 V. After electrophoresis, one glass plate was removed and the gel on the second glass plate was stained with 1mg/ml ethidium bromide. The relevant section of the gel was cut, transferred with a piece of blotting paper to a transilluminator and released from the paper by wetting it with water. The gel was then Photographed[3].

Sequencing the suspected mutations:

PCR products of the samples with a suspected mutation, double band of PCR product detected by 10% vertical polyacrylamide gel electrophoresis were sent for purification and sanger sequencing analysis . Some random samples from controls were also sent for analysis.

Results:

Table 2: Showing variation in banding pattern of PCR products in Type II DM cases.

Exon No	Size of PCR product (bp)	No. of samples showing band variation on 10% PAGE
INSR2	653	4
TK14	210	5
TK17	480	8

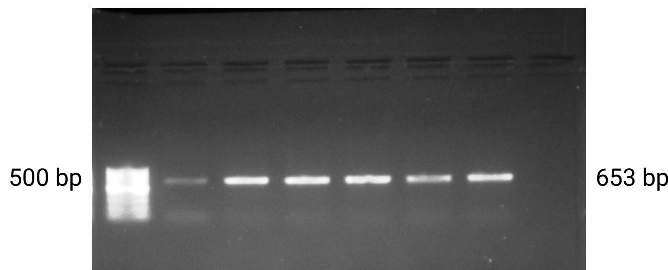

Figure 1: Shows image of horizontal gel electrophoresis of PCR product of INSR2 exon at 653 bp

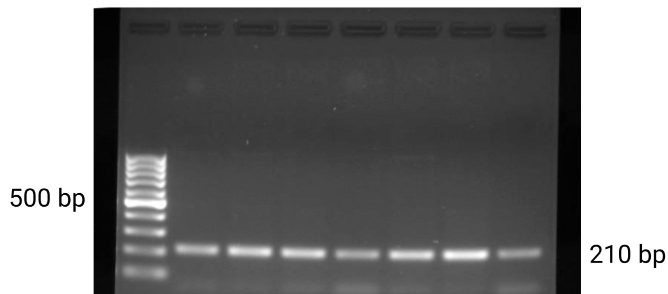

Figure 2: Shows image of horizontal gel electrophoresis of PCR product of TK14 exon at 210 bp

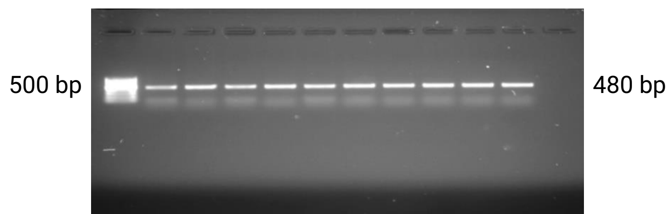

Figure 3: Shows image of horizontal gel electrophoresis of PCR product of tk17 exon at 480 bp

Photograph No. 1-3 show images of horizontal gel electrophoresis of PCR product of INSR2 exon at 653 bp, TK14 exon at 210bp and TK17 exon at 480bp respectively. From the image it is clear that the products were well formed in quantity and quality by the methodolgy mentioned in this study.

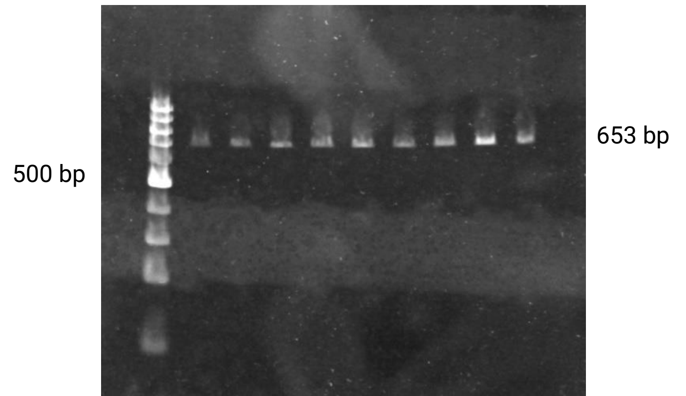

Figure 4: Shows image of vertical 10% PAGE electrophoresis of PCR product of INSR2 exon at 653 bp

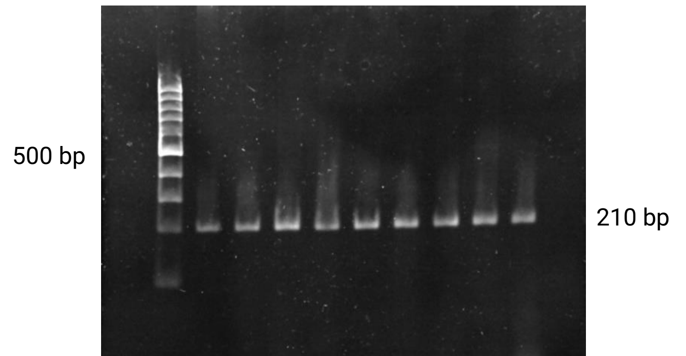

Figure 5: Shows image of vertical 10% PAGE electrophoresis of PCR product of TK14 exon at 210 bp

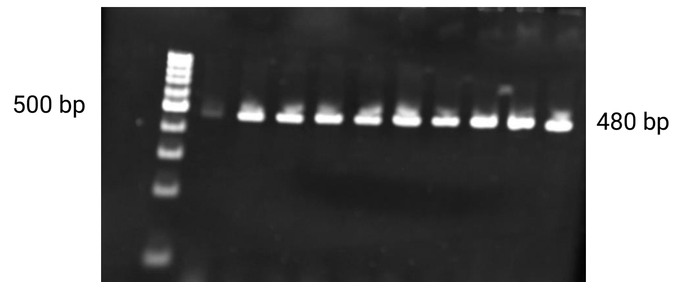

Figure 6: Shows image of vertical 10% PAGE electrophoresis of PCR product of TK17 exon at 480 bp

Photograph No. 4-6 show images of vertical 10% PAGE electrophoresis of PCR product of INSR2 exon at 653 bp TK14 exon at 210bp and TK17 exon at 480bp respectively. The image shows cases and controls in which normal banding pattern was seen when PCR products were subjected to 10 % PAGE electrophoresis.

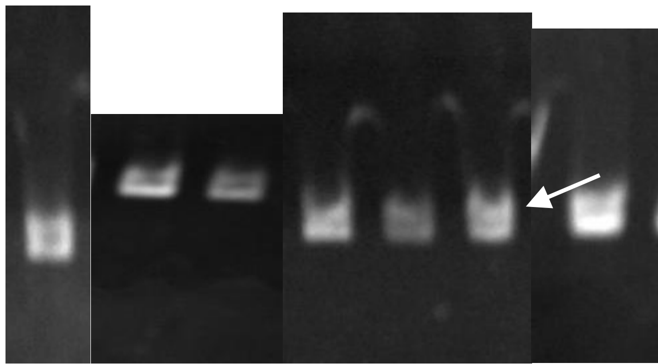

Figure 7: Shows image of vertical 10% PAGE electrophoresis of PCR products showing double bands.

Photograph No. 7 shows representative images of vertical 10% PAGE electrophoresis of PCR products showing double bands. Variations in banding pattern of PCR products was not seen in Type I DM cases and all controls but was seen in cases of Type II DM patients. Table No. 2 shows the distribution of these cases with respect to the different exons studied.

Table No. 3. Showing sanger sequencing analysis of PCR products in Type II DM cases & controls

Exon No	Nucleotide position	Nucleotide variation	Mutations seen In cases	Mutations seen In controls
INSR2	7267252 7267264	A → T A → C	2 out of 4 2 out of 4	4 out of 5 2 out of 5
TK14	Nil	Nil	Nil	Nil
TK17	7125185 7125285	T → A G → A	8 out of 8 3 out of 8	3 out of 4 2 out of 4

Table No.3 shows the results of sanger sequencing analysis of the suspected samples of cases as well as randomly selected controls for comparison. It is clear from the table that the mutations are present in cases as well as controls indicating clinically insignificant polymorphism in case of INSR gene in the studied population. The reference sequence used is genebank accession No. NC_0019.10. The analysis was also done with genebank accession No. M10051.1 as used in previous cases in which no mutations were observed in neither cases nor controls for the exons studied.

Discussion:

Susumu Seino et al in their article stated that the human INSR (hINSR) is an integral membrane protein present on the surface of all cells which varies widely from as few as 40 on circulating erythrocytes to >200,000 on adipocytes and hepatocytes. The mature hINSR is a heterotetramer of two α-subunits of 719 or 731 amino acids and two 620-amino acid β-subunits. The hINSR gene is located on the distal short arm of human chromosome 19. The gene is composed of 22 exons and 21 introns. The exons range in size from 36 bp (exon 11) to >2500 bp, whereas the 21 introns that separate the exons vary in size from -500 to >25,000 bp. All of the introns interrupt protein-coding regions of the gene. Exons 1-14 encode the extracellular region of the hINSR, whereas exons 16-22 encode the region that is localized within the cell. Hence in this study we choose one from either ends i.e. exon 2 and 17 and one from middle i.e. exon 14[1].

The insulin receptor membrane protein is composed of two extracellular α subunits that bind insulin and two β subunits which span the plasma membrane and have an intracellular tyrosine kinase domain. Insulin binding to the α-subunits causes a conformational change that results in the activation of the kinase activity of the β-subunits with subsequent autophosphorylation and activation of kinase activity toward intracellular substrates. A single gene codes for both subunits. The resulting preprotein is post-translationally cleaved into mature alpha and beta subunits that assemble together as a heterotetramer to generate the mature insulin receptor[1,2].

The majority of the mutations (64%, or 85 of 132) are missense mutations, 13% (17 of 132) are nonsense mutation, 4.7% are splice site mutations, 8.3% are deletions (11/132), 2.3% are insertions (3/132), 1.5% are insertions and deletions (indel, 2/132), 5.3% are gross deletions or complex gene rearrangements (7/132). Most of the mutations located in the first 11 exons affecting alpha subunit result in Leprechaunism most severe form of IR while the mutations in the later exons affecting beta subunit are found more frequently in patients with Rabson–Mendenhall syndrome a less severe form. Although there is no straightforward genotype–phenotype correlation markedly elevated insulin levels is a constant feature. Leprechaunism or Donohue syndrome disorder is a complete autosomal recessive insulin receptor binding defect resulting in a loss of insulin action while Rabson-Mendenhall syndrome constitutes an incomplete insulin receptor binding defect. Type A insulin resistance syndrome is characterized by decreased insulin receptor binding and signaling[4].

BahramKazemi et al. in their study on Type II DM found genetic mutations in 26% patients. These mutations include exon 2 (His171Asn, Ile172Ser, Cys196Ser and Ser210Arg), exon 3 (Gly227Asp and Gly232Ser), exon 8 (Thr543Ser), exon 9 (a heterozygote was observed with no change in phenylalanine at position 669), exon 13 (two heterozygotes: Arg890Pro with Asn865 remaining unchanged), exon 14 (Ala906Gly and Pro918Trp with Arg902 unchanged), exon 17 (Val1086Glu) and exon 19 (His1157Gln with Thr1172 unchanged)[2].

O. Ardon et al in their study found three of the four mutations identified caused premature insertion of a stop codon. The first patient, had a homozygous G to T variation at nucleotide 451 converting Glu 151 to a premature stop codon (c.451G >T,p.Glu151term). The second patient, showed a heterozygous C to T nucleotide change at position 1195 coding for a premature stop codon at amino acid position 399 (c.1195C >T, p.Arg399term). The third patient, had a heterozygous C to T nucleotide change at position 2734 resulting in a change of arginine 924 to a premature stop codon (c.2770 C >T, p.Arg924term). The fourth patient, showed a novel G to T missense mutation was identified at nucleotide position 425 resulting in a change of glycine 142 to a valine (c.425 G >T,p.Gly142Val)[4].

Vanessa R. Panz et al in the studied the frequency of DNA polymorphism in tyrosine kinase domain (Exons 17-21) of the insulin receptor gene in 30 black and 30 white south Africans, using single stranded conformation polymorphism and direct sequencing analysis. A comparison of frequencies of the normal versus the combined polymorphic alleles found only in exon17, showed a significant difference between black and white[5].

Steven C in their research on exon 17 detected a Met-for-Val substitution at position 985 in 1/18 pedigrees. In the study done by MasaharuKan, Fumihiko Kanai three patients with heterozygous missense mutation Thr831→Ala831 in exon 13 and one patient with heterozygous missense mutation Tyr1334→Cys1334 in exon 22 of the βsubunits were identified. AbolhasanRezaei study results showed that except for the first 21 and the last 12 nucleotides at the end of sequences, other regions of exon 17 had high sequence homology between them[6-8].

Ashish Sethi et al in their study analysed families by targeted next generation sequencing of the known hyperinsulinism genes. In First family a novel heterozygous INSR variant p.(Met1180Lys) (c.3539T>A) in the proband, her sister, mother and maternal grandfather was identified. In second family novel heterozygous INSR variant p (Arg1119Gln) (c.3356G>A) in the proband and her mother was seen. In third family novel heterozygous INSR variant p.(Arg1191Gln) (c.3572G>A) in the proband and her father was detected. No further disease-causing variants were identified in all families[9].

In the case control study done by Jasmine Sokhi et al in three ethnic groups for INS (rs689) , INSR (rs1799816) and PP1G.G (rs1799999) in context to type Type II DM it was revealed that INS (rs689) polymorphism conferred risk towards Type II DM susceptibility in all the three ethnic groups, whereas INSR (rs1799816 polymorphism conferred risk towards Type II DM in Brahmins only and P1G.G (rs1799999) polymorphism indicated Type II DM risk in Jat Sikhs only[3].

Dhanasekaran Bodhini et al in their study found only one polymorphism, His1085His [rs1799817, (C→T)], in exon 17. The frequency of the “T” allele of the His1085His polymorphism was significantly lower in the T2D subjects (31%) compared with the non-glucose tolerant subjects (35%) and showed significant protection against diabetes. Iqra Hameed, Dil-Afroze, studied a total of 468 ethnic Kashmiri subjects (198 type 2 diabetes mellitus patients and 270 non diabetic controls). They analyzed a nonsynonymous transition Leu233Pro (T↔C), and two synonymous substitutions Asp234 (C↔T) and Gln276 (G↔A) in the exon 3 of INSR gene in patients with type 2 diabetes mellitus for polymorphic alleles[10,11].

Insulin resistance (IR) leads to insulinemia >1000 IU/dl for normal glucose levels; its prevalence in childhood ranges from 3.0 to 8.4%. The underlying etiopathogenesis may be primary (gene mutation) or secondary (obesity and/or lipodystrophy). Hereditary IR may be classified as polygenic IR of moderate severity, which is characteristic of type 2 or monogenic diabetes; IR associated with syndromes characterized by a phenotype with centripetal obesity or lipodystrophy; or IR associated with isolated hereditary receptor disorders. The disorders are majority autosomal recessive but there are also autosomal dominant varieties of inheritance. A multiplex ligation dependent probe amplification (MLPA) assay could be used to detect single exon deletions in the INSR gene. Insulin sensitizers are used as first choice treatment to lower insulinemia but Insulin is sometimes required. Recombinant IGF1 improves survival in neonatal presentations of the disease. Heterozygous alteration in exon 20 (p.Asp1177Glu, C.3531C >G)y treated with metformin, with good glycemic control (HbA1C 6.5%[12].

Insulin resistance has been related to cancer development because of the similarity between insulin receptors and growth factor receptors. Increased IGF1 and IGF2 receptors induce changes in receptor dimerization (iso-receptors A and B) and a cascade of highly potent activating signals that increase endometrial and breast proliferation through the activation of sex hormones, while decreasing the metabolic response and apoptosis. Variable phenotype among the family members, ranging from neonatal hypoglycaemia to adult-onset diabetes mellitus has been observed which highlights the importance of genetic analysis and long-term follow up of these patients[13].

Conclusion:

Mutations in INSR gene was not seen in cases of Type I DM cases studied. Clinically insignificant mutations were seen in Type II DM cases as well as controls indicating polymorphism. Susceptibility to insulin resistance is less in studied population of DM. Limitations to this study may be because of Sanger sequencing technique limitations and study of only three exons of INSR gene.

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Conflict of interest: Nil

Source of funding: Advance research wing, RGUHS, Bengaluru, Karnataka

Date received: Mar 15, 2023

Date accepted: Oct 20, 2023