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Molecular Characterization of Insulin Gene in Diabetic Foot Ulcer Patients: A Pilot Study from Bengal Bay Coastal Origin

Ahmad Fazilat[1], Swarupa R. Didla[2], M.S. Chakravarty[3], Saima Wajid[1]*

[1]Department of Biotechnology, Faculty of Science, Hamdard University (Jamia Hamdard), New Delhi - 110062, India; swajid@jamiahamdard.ac.in
[2]Department of Human Genetics, Faculty of Science, Andhra University, Vishakhapatnam – 530003, Andhra Pradesh, India
[3]Department of Biotechnology, Faculty of Science, Andhra University, Vishakhapatnam – 530003, Andhra Pradesh, India

Abstract

Objective: Many studies have showed that foot ulcer is the most frequent occurrence among diabetic patients in the south Indian origin. This study aimed to investigate on the hypothesis that in patients with diabetic foot ulcer in south Indian subjects there is a substitution within nucleotide sequence of coding region related to insulin gene and this may be signifying a link disequilibrium with the class III allele in the insulin gene locus, which can be considered due to its relevance with diabetes. We aim to examine this part of insulin gene to find out if there is any mutation within the insulin gene sequence that is relevant with diabetes mellitus. **Materials and Methods:** We used the PCR products of the amplified insulin gene from 40 patients diagnosed with foot ulcer seeking mutations, which might be in association with the class III allele and therefore indicates its relevance with the manifestation of diabetic foot ulcers. **Results:** We identified a β-chain mutant (i.e. insulin Chicago) using the restriction enzyme (MboII) in individuals afflicted with foot ulcer. A couple of patterns (α and β) were found indicating the presence of PstI polymorphism within the 3' un-translated part of the insulin gene. Besides, allele frequencies in subjects homozygous for α allele, homozygous for β allele and heterozygous were respectively 0.3 (12/40), 0.125 (5/40) and 0.575 (23/40). No significant variant regarding allelic frequency was characterized between the patients and normal controls, though a mutation in B-chain (insulin Chicago) was observed. **Conclusion:** Despite linkage disequilibrium between this polymorphism in the 3' un-translated portion of the insulin gene and the class III allele, it is unlikely to have advantage than the class III allele itself on prognosticating diabetes.

Keywords: Diabetes mellitus, Insulin Gene, Polymerase Chain Reaction

1. Introduction

This research study concentrates on differences in insulin gene among patients diagnosed with diabetes mellitus and reviews recent proceedings in molecular biology related to the insulin gene and diabetes mellitus in diabetic foot ulcer patients[1,2]. Foot ulcer is one of the most common clinical implications of diabetes with an approximate prevalence of 12-15% over diabetic patients. Foot ulcer (diabetic) is known as the major reason of hospitalizations among all complication of diabetes. Ulceration may have pernicious complications since they cause nearly 90% of lower severity amputations within patients[3]. Diabetic foot ulcers are the consequence of the complex impacts of diabetes related neuropathy and vascular disease[4-6]. Diabetic patients possess a higher prevalence

* *Author for correspondence*

of atherosclerosis, arteriolar hyalinosis, endothelial proliferation and the thickening of capillary basement membranes. Atherosclerosis may be developed in various sizes of arteries, including aortoiliac and femoropopliteal vessels, but atherosclerosis of the infrapopliteal segment is certainly prevalent. With the association of digital artery disease, ulcers are potentially able to lead to gangrene in the absence of an appropriate blood flow[6]. The strategy followed and implemented in this study was to amplify the sequence of insulin gene utilizing the polymerase chain reaction (PCR) technique[7] and then the fragments were digested by restriction enzymes, and eventually the resulting fragments were separated by performing Agarose gel electrophoresis. One of the B chain characterizing insulin Chicago 22 and/or adjacent to the A chain in the 3' un-translated region could be investigated among the possible intragenic polymorphisms[8,9].

2. Structure of the Coding Region, Flanking Regions and Intervening Sequences

The 3-dimentional arrangement of the insulin gene (in human) was first determined in 1980[10] with exploiting DNA sequencing and gene cloning techniques. Insulin gene contains all the required information for the generation of pre-proinsulin. This gene also contributes in many other traits with genetic materials of other eukaryotes. Different structural properties such as coding information, promoter within5' flanking sequence, couple of intervening sequences (introns) and 3' region (non-coding sequence). The promoter involves DNA sequences, which directs the binding of RNA polymerase[10], so the insulin gene is transcribed appropriately in the pancreatic β-cells. Also other less understood segment of DNA is likely to be involved in the insulin gene transcription in the pancreatic fJ-cells, which presumably hamper this process in other cells. Introns are portions of DNA molecule, which initiate production of messenger RNA as precursors of m-RNA is generated by transcription of introns, and then removed by splicing during the m-RNA maturation[11]. The human insulin gene contains two introns, and the second intron divides the coding region to two discrete portions[12,13]. The first coding division carries the B-chain codons, the pre-peptide codons and the first eight codons of the C-peptide. The second block (division)bears the remainder of the A-chain andC-peptide as well as the TAG codon, which is known as the terminator agent of the coding region. There are also other signals included in the 3' noncoding DNA mostly known as agents that appoint the addition of a polyadenylic acid tail to the pre-proinsulin mRNA, and other sequences that winds up gene transcription.

3. Materials and Methods

Total of forty diabetic patients diagnosed with foot ulcer were recruited in this case-control study and blood sample was collected. Also same number of 40 individuals with equal age and gender were considered as controls.

3.1 Samples and DNA Isolation

Samples were collected from South Indian subjects who were histopathologically diagnosed with diabetic foot ulcer (ulcers have been observed on the lower limbs, heels or feet). 2ml of patients' blood were obtained and stored at -20°C. Thereafter we isolated the DNA of patients sample using a simple, rapid and non-enzymatic method[14].

3.2 Amplification

In order to amplify the desired DNA sequences, a polymerase chain reaction (PCR) was done through denaturing the DNA, which is followed by replication using single free nucleotides, primers and a DNA polymerase specifically designed to aid the DNA bear the high temperatures during PCR. Since one nucleotide sequence of a DNA fragment is required to be known in order to run PCR, we exploited allele-specific primers. The nucleotide sequence of A-chain for the forward primer is 5'-GCTGGTTCAAGGGCTTTATTC-3' and its reverse primer is 5'-TGGGGCAGGTGGAGCTGGGCG-3'. Conversely, the B-chain forward primer was 5'-ATCACTGTCCTTCTGCCATGG-3' and its reverse primer was 5'-CCTGCA GGTCCTCTGCCTCCC-3'. PCR conditions for A-chain are denaturing for one minute at 99°C, annealing for 18 seconds at 55°Cand extension for 2.5 minutes at 72°C and for B-chain are denaturing for one minute at 99°C, annealing for three minutes at 68°C, and 2.5 minutes at 72°C for forty cycles[1].

3.3 Electrophoresis and Analysis

We applied agarose gel electrophoresis to run the DNA

samples through 2% agarose gel (100V, 30 minutes). Then, the migrated bands were analyzed under ultraviolet light after ethidium bromide staining. We compared size of bands with the molecular weight band markers (100-1000 bp) for further confirmation.

3.4 Restriction Enzyme Digestion

The A chain related PCR products of 40 subjects was digested with 50U of PstI and 50U PvuII. The PCR products related to B-chain were digested using MboII and HaeIII. The digested PCR products were separated based on their size on Agarose gel[2]. Size of restriction fragments were diminished in compared to 1kb DNA ladder.

4. Results

With use of MboII (to find out insulin Chicago 21) and HaeIII (general screen for polymorphism) four DNA fragments (with sizes of 20, 39, 94 and 51 base pair) were resolved and indicated on each lane of agarose gel after electrophoresis. Among all 10 south Indian NIDDM (noninsulin-dependent diabetes mellitus) subjects of those who have the class 3 allele, no polymorphism was found. Hence, mutations at these restriction sites are ruled out as a common cause of NIDDM within the subjects of this research.

For A chain PCR products, no polymorphisms were detected with PvuII and polymorphisms were observed with PstI with fragments size of 150, 85, 65 and 41 base pairs (figure2). In subjects homozygous for α allele, allele frequency 0.3(12/40), allele frequency of homozygous for β allele 0.125(5/40) and in heterozygotes allele frequency was 0.575 (23/40) (2x3x2=62, p<0.0001, Cramer's V= 0.52) (Table 1).

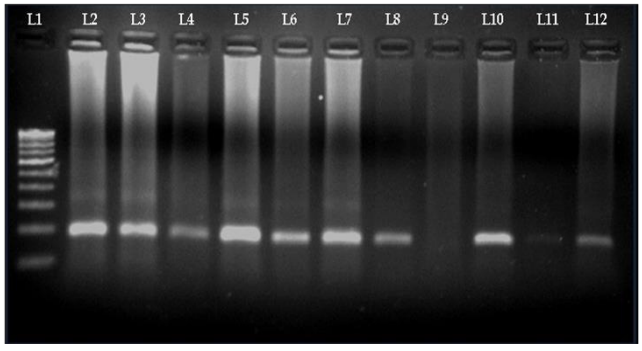

Figure 1. Shows L1-100 bp DNA ladder, L2-L12 amplified insulin gene products.

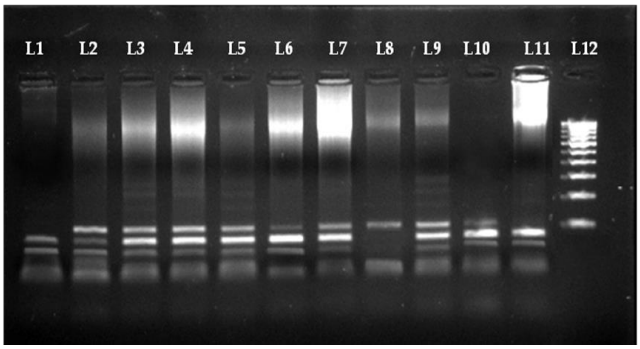

Figure 2. Shows PstI restriction map L1 &L11 shows homozygous for β, L2-L7, L-9 and L-10 shows heterozygous for α β, L-8 shows homozygous for α and L12-100 bp DNA ladder.

Table 1. Allele frequencies in α and β and heterozygous alleles compared between patients and controls

Allele frequency				
S. No	South Indian Population	Number	α-chain	β-chain
01	Control group	40	0.63	0.37
02	Diabetic foot ulcers Patients	40	0.60	0.40

5. Conclusion

The study results indicate primarily that mutant insulin (insulin Chicago) is not essentially a common cause of diabetic foot ulcers in the south Indian population and secondly, in all the south Indian people, there is a linkage disequilibrium between the mutation point in the 3' un-translated segment of insulin gene (the two loci being physically separated by approximately 2kb) and the class 3 allele in 5' flanking area of the insulin gene[8]. The Pstl intragenic insulin gene polymorphism was described first by Bell et al and Ull rich et al. in the two sequenced alleles, a mutation point in the 3' un-translated region at position 1628 involving C to A transversion accounted for the presence of gHI12-523/4 allele (or absence) gHI 14123/Ca allele of the PstI cutting site[8,9]. Similarly, it was studied by Southern blot analysis[15,16] in 50 alleles each from Caucasoids, Pima Indians and blacks; they found it to be non-polymorphic and therefore, concluded that the β type allele represented an isolated mutation[17]. The point mutation that makes the extra PstI site in the 3' un-translated (3UT) segment is known as a mutation[18,19], which appoints a tiny part of the genetic aptitude to

NIDDM patients among south Indian population.

6. Discussion

The function of the 3UT is unknown, though it forms part of the primary mRNA transcript; some researchers have postulated that this part would be important for RNA stability. So a feasible hypothesis is that this polymorphism may lead to decline mRNA translation and thereby occurrence of hypo-insulinaemia, which would account as a cause of diabetic condition in south Indian subjects. This would be in accordance with recent thoughts on the etiology of this disorder[20,21]. Alternatively, both the insulin gene and the described RFLP results of PstI could be in linkage disequilibrium with sequences involved in insulin regulation within the 5' flanking region of the insulin gene.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

7. Acknowledgement

The authors declare that they have no conflict of interest, and all authors have approved the publication of this manuscript. This article is not submitted to any other journal and all the authors are familiar with the contents of this manuscript.

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