Investigation of IL12R\(\beta1\) Gene Polymorphisms in Leprosy Patients from Tamil Nadu

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Abstract: Leprosy as a social disease has been a major public health problem because of the social stigma and ignorance attached to it. This has made it difficult for our health care delivery system in their pursuit for early diagnosis and prompt treatment. This study was done with the objective of assessing the present knowledge, attitude and practice of leprosy affected persons and their family members in a rural setting. The patients and family members had adequate average level of knowledge about leprosy, but their attitude toward the disease and their practices were not adequately favorable. Leprosy is still a major health problem in India which has the highest number of cases. Multiple loci variable number of tandem repeat analysis (MLVA) and single nucleotide polymorphism (SNP) have been proposed as tools of strain typing for tracking the transmission of leprosy. Mutations in IFNGR1, IFNGR2, IL12RB1, IL12B, STAT1 and NEMO result in a common clinical phenotype known as Mendelian Susceptibility to Mycobacterial Diseases (MSMD). Interleukin-12 receptor \(\beta1\) (IL-12R\(\beta1\)) deficiency is the most common genetic etiology for MSMD.

Keywords: Leprosy, present knowledge, attitude and practice, (SNP), (MSMD), (IL-12R\(\beta1\)).

1. Introduction

Leprosy is a chronic granulomatous infectious disease of human whose etiological agent, Mycobacterium leprae, was identified by in 1873. It was the first bacterium to be identified as a disease-causing agent in man, yet it remains as one of the disease that is least understood. It is prevalent in warm, wet areas in the tropics and sub tropics. Leprosy is also caused by Mycobacterium lepromatosis. It affects the peripheral nervous system and skin leading to disability and disfigurement. Leprosy is not contagious and 95% of people have a natural immunity of it. Initially infections are without symptoms and typically remain this way for 5 to as long as 20 years. Symptoms that develop include granulomas of the nerves, respiratory tract, skin and eyes. This may result in a lack of ability to feel pain and this loss of parts of extremities due to repeated injuries. Weakness and poor eyesight may also be present. The main symptom of leprosy is disfiguring, skin sores, lumps or bumps that do not go away after several weeks or months. The skin sores are pale colored. Nerve damage can leads to loss of feeling in the arms and legs and muscle weakness. It usually takes about 3-4 years for symptoms to appear after coming into contact with the leprosy causing bacteria. (Hansen 1873).

With more than 100 affected patients worldwide, IL-12R\(\beta1\) deficiency is the most common genetic etiology for Mendelian susceptibility to Mycobacterial Disease (MSMD). Mutations on the IL12RB1 gene are associated with impaired response to IL-12 and IL-23. With only one exception, all IL-12R\(\beta1\)-deficient patients described displayed no detectable IL-12R\(\beta1\) on their cell surface due to mutations that either interrupt the open reading frame (nonsense and frame shift mutations) or disrupt folding of the protein (missense mutations). Unexpectedly, mutation 1623-1624delinsTT was found in 5 out of 6 Argentinean IL-12R\(\beta1\)-deficient patients (3 homozygous and 2 heterozygous) from 6 unrelated families. Altara (1998).

Leprosy is a chronic granulomatous disease which usually presents with characteristic skin lesions, thickening of peripheral nerves with variable sensory and or motor loss. Leprosy has a worldwide distribution. According to the WHOIndia contributes about 60% of new case detected cases in the world. Out of 127326 new cases detected in India, 11,389 were children. Mohanty (2016).

2. Materials and Methods

Sample Collection

Blood from 30 Leprosy patients was collected form sacred heart leprosy hospital Kumbakonam, Thanjavur district, Tamil Nadu. 5ml blood was collected from the each experimental subject using EDTA tube.

Genotyping

Whole Blood DNA Extraction

Whole genomic DNA was collected by following kit protocol Gill et al., (1987).

Protocol for DNA Isolation From Blood Samples

a) Four volumes of reagent A was added to 10ml of blood sample and centrifuged at 3600rpm for 10min.

b) The supernatant was discarded and the pellet was disturbed. Then 5ml of reagent B and 1.25 ml of reagent C were added and shaken thoroughly. Then 3ml of Tris Phenol and 3ml Chloroform-isoamyl alcohol (24:1) were added, mixed gently and centrifuged at 3000rpm for 10min.

c) The two layers were formed. The bottom layer was phenol, middle or the portion containing the precipitated part and the upper layer was aqueous. The aqueous phase was taken in another tube using blunt tips.
Garose Gel Electrophoresis

Principle
Agarose gel electrophoresis is a procedure used to separate DNA fragments based on their molecular weight and is an intrinsic part of almost all routine experiments carried out in molecular biology. Preparation of 1% Agarose Gel:
- 1X TAE was prepared by diluting appropriate amount of 50 X TAE buffer stock solutions. (0.4 mL of 50X TAE was made up to 200 mL with distilled/dematerialized water).
- 0.5 g of agarose weighed and added to 50 mL of 1X TAE. This gave 1% agarose gel.
- The solution was boiled till agarose dissolved completely.
- Meanwhile the combs were placed in electrophoresis set such that it is approximately 2 cm away from the cathode.
- Ethidium bromide (EtBr) was added to the molten agarose at a final concentration of 0.5 µg/mL (from a stock of 10mg/mL in water) and mix gently.
- The agarose solution was poured in the central part of the tank when the temperature reaches approximately 60°C. Air bubbles were avoided. The thickness of the gel was around 0.5 to 0.9 cm. The gel kept undisturbed at room temperature for the agarose to solidify.

Electrophoresis
- 1X TAE buffer was poured into the gel tank till the buffer level stood at 0.5 to 0.8 cm above the gel surface.
- The combs lifted gently, ensuring that wells remained intact.
- The power cord was connected to the electrophoretic power supply according to the convention red: anode, black: cathode.
- DNA samples were loaded in the wells in the desired order and order recorded with tracking dye (6X Tri-track loading dye, Bangalore genei).
- The voltage set at 50 V and the power supply switched on.
- The power was switched off when the tracking dye from the well reached 3/4th of the gel.
- After electrophoresis, DNA samples were visualized under UV transilluminator, (they appear fluorescent. No destaining was required in this case).

Quantification of DNA
Standard DNA was prepared using salmons sperm DNA at various concentrations (10, 25, 50, 75 µg/mL). The control DNA was serially diluted in distilled water.50 mL of the isolated genomic DNA was diluted in 1 mL distilled water and OD was measured at 260nm using spectrophotometer. An OD of 1 corresponds to 50 µg/mL for double stranded DNA. Using standard curve obtained from the standard DNAs OD, the concentration of the template DNA was quantified. Similarly, OD was measured at 280nm. The ratio of reading at OD 260 and 280nm provides an estimate of purity of DNA.

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<th>Table 1: Patients’ Preoperative Characteristics</th>
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<th>Table 2: Quantification of DNA</th>
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Polymerase Chain Reaction (PCR) Amplification
The primers of FMR1 gene (Bioserve, India) used for amplifying genomic DNA were chosen in accordance with (Hixon and Vernier 1991). They were as follows:
Forward primer: 5’- TGTTGTAACATATGTCACAGATCTTCTCTG-3’
Reverse primer: 5’- TGTTGTACTATATGTCAACAGATCTTCTG-3’

The reaction volume used was
Template DNA (200 ng) ----------------------- 4.0 µL
Forward and Reverse primers (1µM) ---1.0 µL each
PCR Master Mix (2X) ------------------------12.5 µL
MilliQ water-----------------------------8.5 µL
Total volume --------------------------25.0 µL
The PCR conditions for amplification were as follows:
An initial denaturing step at 95°C (10 min) followed by 30 cycles of
95 °C (30 s) - Denaturation step
60 °C (30 s) - Annealing step
72 °C (1 min) - Extension step
And a final elongation step of 72 °C (10 min).
The PCR products were electrophoreses on 2% agarose gels containing EtBr and viewed under ultraviolet light.
Sample preparation

- Genomic DNA isolated were amplified by PCR.
- One microliter of the PCR product was combined with 9µl of 95% formamide loading dye (containing 10mM NaOH, 0.05% of bromophenol blue and 0.05% of xylene cyanol) was boiled for 5 minutes and immediately chilled on ice.
- Five micro liters of the denatured DNA was applied onto 10 to 12% polyacrylamide gel depending on its length.

Electrophoresis:

- Vertical slab gel units with gels of 160 mm × 160 mm × 1 mm were used. The cooled denatured mixture was loaded onto 10% polyacrylamide gel.
- Run the gel in 0.5 x TBE buffer (45mM TrisHcl, pH 8.0, 45m borate, and 1mM EDTA ) that has been pre-cooled to 10°C. Place the gel tank in a tray filled with ice. Set the voltage to 100 V and run the gel for about 5 h. The temperature at the run is about 17 ºC.
- Then electrophoresed at 4°C and at 20°C in 0.5x TBE buffer (45mM TrisHcl, pH 8.0, 45m borate, and 1mM EDTA )
- After electrophoresis, the gel was silver-stained according to the following conditions: gel was fixed in solution of 10% (v/v) ethanol and 10% (v/v) glacial acetic acid for 10 min, stained in 0.1% (w/v) silver nitrate solution for 40 min developed in 3% (w/v) sodium hydroxide solution containing 0.15% (v/v) formalin, and finally stopped with distilled water.

3. Result and Discussion

We found numerous SNPs of IL12Rβ1 gene associated with various of inflammatory diseases by using bioinformatics analysis tool (www.snp database).

The blast result of our target sequence also showed its presence in exon 4 region of IL12Rβ1 gene of 250bp. Further the sequence was analyzed using NEB cutter tool for the presence of AvaII restriction site in the whole 250bp. Our analysis result showed the presence of AvaII restriction site in our target sequence. AvaII restriction site was found at nucleotide 137 which results from two DNA fragment of 129bp and 121bp. While in mutated case the AvaII site is not recognized by the enzyme that results in the same 250bp after digestion with restriction enzyme.
Figure 5. a, b.: Showing the 1.5% of Ethidium bromide stained Agarose Gel Electrophoresis checking for Restriction Fragment Length Polymorphism in after digestion of AvaII enzyme. The enzyme cutting specific recognition site, show the lane 1-30, Controls of 250 bp PCR-Amplification product with AvaII digestion product of homozygous normal type of both TT alleles.
Eukaryotic synthetic construct chromosome 19
Sequence ID: CP034522.1, Length: 64242768, Number of Matches: 1
Range 1: 18120291 to 18120540

Alignment statistics for match #1

- Score: 462 bits (250)
- Expect: 2e-126
- Identities: 250/250 (100%)
- Gaps: 0/250 (0%)
- Strand: Plus/Minus

Query 1: CTCCCCTCTCCTTCCAGAACCAGTGGCTCTGAATATCAGCGTCGGAACCAACGGGACCAC
Sbjct 18120540: CTCCCCTCTCCTTCCAGAACCAGTGGCTCTGAATATCAGCGTCGGAACCAACGGGACCAC

Query 61: CATGTATTGGCCAGCCCGGGCTCAGAGCATGACGTATTGCATTGAATGGCAGCCTGTGGG
Sbjct 18120480: CATGTATTGGCCAGCCCGGGCTCAGAGCATGACGTATTGCATTGAATGGCAGCCTGTGGG

Figure 5: Showing the blast search in IL12Rb1-rs121434493 gene DNA sequence find the correct position in chromosome 19
Blast matching of IL12Rβ1-rs121434493 gene mutation site:

Figure 6: Showing the blast search to compared IL12Rβ1-rs121434493 gene Normal and Mutant DNA sequence, finds the mutation allele region of correct position.

4. Conclusion

IL12Rβ1-250T was also found to be associated with several infectious diseases. SNPs variation plays a main role in leprosy disease. This study identified in IL12Rβ1 a gene -250 C/T single nucleotide polymorphism in leprosy. Our results find TT Homozygous was observed using Sequence Specific Primer method. Hence our present study provides consistent evidence for the involvement of IL12Rβ1 gene in susceptibility to leprosy.

5. Acknowledgement

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References

[5] Penna GO, Pinheiro AM, Nogueira LSC, De Carvalho LR, De Oliveira MBB,Carreiro VP: Clinical and epidemiological study of leprosy cases in the University

[6] Prado-Montes de Oca E, Velarde-Félix JS, Ríos-Tostado JJ, Picos-Cárdenas VJ, Figuera LE. 2009. SNP defensin 1 (DEFB1) and is associated with lepromatous leprosy. Infect 668C (~44) alters a NF-kappaB1 putative binding site in non-coding strand of human beta-


