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ASSOCIATION OF IFNΓ GENE POLYMORPHISMS IN TUBERCULOSIS PATIENTS

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ABSTRACT

Tuberculosis has caused the death of more people than any other single infectious disease, making it the most successful pathogen known to mankind. The aim of this work was to check the INF- γ gene polymorphism at +874 A/T position. Active Pulmonary Tuberculosis (APTB) (n=5) their Household Contacts (HHC) (n=50) who attended the PPM DOTS clinic. APTB was confirmed by sputum, culture and chest X-ray. Mantoux test was performed with 5 TU-tuberculin-Purified Protein Derivative (PPD), in APTB and HHC. Healthy Controls (HC) (n=50) were also included in the study.

Among the three genotypes, the AT genotype was more frequent in APTB, HHC &HC, whereas AA genotype was found to be significant in APTB ($p<0.04$ OR- 8.708; CI-1.031-73.55) compared to HC. This polymorphism showed.

KEYWORDS: Tuberculosis, IFN-γ Polymorphism, Mycobacteria, Denaturation, Agarose

INTRODUCTION

Tuberculosis remains a major health problem worldwide till today in spite of huge development in medical sciences and advanced medical technologies. It is the second leading cause of death from an infectious disease, the first place belonging to the human immunodeficiency virus (HIV), (Kranzer K et al, 2010). Infection with Mycobacterium tuberculosis is present in one third of the world's population and is responsible for approximately 2 million deaths per year (Giacomini E et al, 2001). Control over the Mycobacterium tuberculosis infection by the human body is a process involving pathogen recognition and activation, of both the innate immune system and the adaptive one (Fischer et al, 1990). TB also called phthisis pulmonalis, or consumption, is widespread, and in many cases critical, infectious disease caused by different strains of mycobacteria, generally Mycobacterium tuberculosis (Kawamuraet al. 1994). TB characteristically attacks the lungs, but is able to affect other parts of the body. It spreads through the air when people having active TB infection cough, sneeze, or otherwise transmit respiratory fluids through the air (Kawamura et al, 1994).

The common symptoms of active TB infection are a chronic cough with blood-tinged sputum, fever, night sweats, and weight loss (Durbin JE et al, 1996). Infection of other organs causes a wide range of symptoms. Diagnosis of active TB relies on radiology, microscopic examination and microbiological culture of body fluids (Anderson P et al, 1983). Diagnosis of latent TB relies on the tuberculin skin test (TST) and blood tests. Treatment is difficult and requires administration of multiple antibiotics over a long period of time (Pfizenmaier K et al, 1988) .

One-third of the world's population is thought to have been infected with M. tuberculosis, (Jung V et al, 1987) and new infections occur in about 1% of the population each year (Durbin JE et al, 1996). In 2013, 9 million cases of TB

occurred (WHO, 2014) and there were between 1.3 and 1.5 million associated deaths, most of the deaths were in developing countries (WHO, 2011). The rate of tuberculosis varies across the globe, about 80% of the population in many Asian and African countries tests positive in tuberculin tests, while only 5–10% of the United States population tests positive (Canetti G et al, 1993). More people in the developing world contract tuberculosis because of a poor immune system, largely due to high rates of HIV infection and the corresponding **development** of AIDS (Fennel C.W et al, 2001).

Transmission

When people with active pulmonary TB cough, sneeze, speak, sing, or spit, they expel infectious aerosol droplets 0.5 to 5.0 μm in diameter (Cole EC et al, 1998). Each droplet may transmit the disease, since the infectious dose of tuberculosis is very small; the inhalation of fewer than 10 bacteria may cause an infection (Golden MP et al, 2005).

People with prolonged, frequent, or close contact with people with TB are at particularly high risk of becoming infected, with an estimated 22% rate of infection (Kranzer K et al, 2010). A person with active but untreated tuberculosis may infect 10–15 or more other people per year (Jung V et al 1987). Transmission would occur from only people with active TB; those with latent infection are not contagious (Golden MP et al, 2005). The probability of transmission from one person to another depends upon several factors, including the number of infectious droplets expelled by the carrier, the effectiveness of ventilation, the duration of exposure, the virulence of the M. tuberculosis strain, the level of immunity in the uninfected person, and others (Kranzer K et al, 2010).

Interferon-Gamma (IFNγ)

IFNγ is the principal activator of macrophages and plays a role in both innate and adaptive cell-mediated immunity (Aguet M et al, 1987). In innate immunity, natural killer (NK) cells secrete IFNγ, stimulated by IL-12, in response to recognition of unknown organisms (Fischer T et al, 1990). In adaptive immunity, T cells secrete IFNγ in response to antigen stimulation, as well as IL-12 and IL-18 secretion (Kaplan MH et al, 1996).

The functions of IFNγ are especially important in adaptive cell-mediated immunity :

- IFNγ is a macrophage-activating cytokine, enhancing the microbiocidal activities of macrophages. It stimulates the synthesis of reactive oxygen intermediates and nitric oxide. These active molecules are produced in lysosomes and destroy organisms within the phagolysosome (Murray PJet al, 2011).
- IFNγ stimulates expression of class I and II MHC molecules and costimulators on APCs. It also stimulates the expression of many antigen-processing proteins. In this way it enhances MHC-associated antigen presentation and amplifies the recognition phase of immune responses by increasing expression of the ligands that T cells recognize (Fischer T et al, 1990).
- IFNγ activates vascular endothelial cells and potentiates some of the action of TNFα. These actions promote lymphocyte adhesion and extravasation to the site of infection (Shiloh MU et al,1990).
- The Th1-derived cytokine IFN-gamma inhibits the proliferation of Th2 lymphocytes, but the mechanism of inhibition is not known. Under certain disease conditions, an established Th2-mediated immune response is undesirable and a Th1-mediated response is beneficia (Oriss et al, 2005).
- In B cells, IFNγ promotes the switching of certain IgG subclasses (Darrah PA et al,2000).

- IFNγ activates neutrophils and stimulates cytolytic activity of NK cells (Canetti G et al, 1993).
- Although IFN production is induced by IL-12, IFNγ in turn initiates or augments IL-12 secretion (Kaplan MHet al, 1996).
- IFNγ also enhances the number of IL-12 binding sites expressed on individual macrophages (Kaplan MHet al, 1996).

IFN-γ Polymorphism

Interferon-Gamma (IFN-γ) is a key-helper type 1 cytokine produced by natural killer cells and T cells, with a Pivotal role in macrophage activation for controlling mycobacterial infection (Kawamura M et al, 1994). IFN-γ and its receptors Form a functional complex, and it is conceivable that, the biological effects of IFN-γ are influenced by a combination of genetic defects in the IFN-γ and IFN-γ receptor genes (Durbin JE et al, 1996). Patients with inherited complete or partial IFN-γ receptor deficiency are highly susceptible to infection by non-tuberculous mycobacteria and M. tuberculosis (Aguet M et al, 1987). Recently, IFN-γ and IFN-γ receptor polymorphisms have been reported to be associated with pulmonary TB (Golden MP et al, 2005). For example, the IFN-γ (+874 T/A) polymorphism was significantly associated with pulmonary TB in Sicilian, Spanish, South African, and Hong Kong Chinese populations (Trehanpati N et al, 2013).In addition, IFN-γ receptor 1 (IFN-γR1) polymorphisms have been associated with pulmonary TB in Croatia (Gray PW et al, 1989).However, the association of IFN-γ and IFN-γR1 gene polymorphisms with pulmonary TB is controversial (Kranzer K et al 2010). No association between IFN-γ polymorphisms and predisposition to pulmonary TB was found in Malawi or Colombian populations (Delgado JC et al, 2002). In addition, no association between IFN-γR1 polymorphisms and susceptibility to pulmonary TB has been observed in Iranian patients (Iranian Ministry of Health, 2011). Both the two coding polymorphisms studied within IFNGR1 have low heterozygosity in the populations studied (minor allele frequency, 3% for each), resulting in poor power to detect any effect on tuberculosis (Wu F et al, 2008). There was no evidence of disease association with these variants (overall p =0.53 and p=0.32, respectively). However, a small effect is seen with the -56 promoter variant. The -56CC genotype is significantly associated with protection from disease (unadjusted OR is 0.75; 95% CI, 0.59–0.97; p= 0.02; adjusted for age, sex, ethnicity, and HIV status, OR (odds ratio) is 0.75; 95% CI, 0.57–0.99; p =0.041). Haplotype analysis did not find evidence for disease association in the small number of Gambian trios studied. Given the rarity of the two no synonymous polymorphisms, the -56C/T polymorphism was the most informative, discriminating 94% of all haplotypes seen. Consistent with the case-control data, the -56C allele was not significantly under transmitted to affected offspring (180 observed vs. 189 expected, p = 0.14),(Littman AJ et al, 2005).

METHODOLOGY

Subjects

Active Pulmonary Tuberculosis (APTB) (n=35) their Household Contacts (HHC) (n=35) who attended the PPM DOTS clinic at Mahavir Hospital & Research Centre were studied.

APTB was confirmed by sputum, culture and chest X-ray. Mantoux test was performed with 5 TU-tuberculin-Purified Protein Derivative (PPD) in APTB and HHC. Healthy Controls (HC) (n=20) were also included in the study.

Sampling

2ml venous blood was collected in EDTA tubes for DNA isolationand to perform PCR.

METHODS

- DNA Isolation QIAGEN Kit method
- SNP's of IFN-γ (-874 A/T) by ARMS-PCR.

DNA Isolation

Flexi Gene DNA Kits provide a rapid and simple method for purification of DNA from human whole blood, buffy coat, and cultured cells. The procedure can be scaled up or down, allowing purification from variable amounts of starting material. Flexi Gene DNA Kits provide good yields of high-purity DNA that is free from contaminants or inhibitors. The purified DNA performs well in a range of downstream applications including PCR-based techniques, restriction digestion, blotting, and sequencing, or can be safely stored at 2–8°C or –20°C.

Principle

Lysis buffer is added to the sample. Cell nuclei and mitochondria are pelleted by centrifugation. The pellet is miixed and incubated in denaturation buffer, which contains a chaotropic salt, and QIAGEN Protease. This step efficiently removes contaminants such as proteins.

DNA is precipitated by addition of isopropanol, recovered by centrifugation, washed in 70% ethanol, dried, and resuspended in hydration buffer (10 Mm TrisHCl, pH 8.5).

Materials and Reagents

Buffer FG1-750µl, Buffer FG2/QIAGEN Protease-150µl, 100% isopropanol-150µl, 70% ethanol-150µl, Buffer FG3- 200µl, Pipettes and sterile, DNase-free pipette tips with aerosol barrier, For blood volumes <0.6 ml: 1.5 ml or 2 ml micro centrifuge tubes that tolerate 10,000 x g and a micro centrifuge capable of attaining 10,000 x g, equipped with a fixed-angle rotor, For blood volumes ≥0.6 ml: 15 ml and 20 ml conical centrifuge tubes that tolerate 2000 x g and a centrifuge capable of attaining 2000 x g, equipped with a swing-out rotor, Heating block or water bath, Vortex mixer.

Procedures

- Pipetted 750µl Buffer FG1 into a 1.5ml centrifuge tube. Added 300µl whole blood and mix, by inverting the tube 5 times.
- Centrifuged for 20s at 10,000 x g = 13500 RPM, in a fixed-angle rotor.
- Discarded the supernatant and leave the tube inverted on a clean sheet of absorbent paper for 2 minutes, taking care that the pellet remains in the tube. NOTE: In rare cases the pellet may be loose, so pour slowly. Inverting the tube onto absorbent paper minimizes back flow of supernatant from the rim and sides of the tube onto the pellet.
- Added 150 µl Buffer FG2/QIAGEN Protease and vortex immediately until the pellet is completely homogenized. Inspect the tube to check that homogenization is complete. NOTE: When processing multiple samples,

vortex each immediately after addition of Buffer FG2/QIAGEN Protease. Do not wait until buffer has been added to the samples before vortexing. Usually 3-4 pulses of high speed vortexing for 5s each are sufficient to homogenize the pellet. However, traces of pellet with a jelly-like consistency may remain. Add a further 30 µl Buffer FG2 in that case and vortex again.

- Centrifuged the tube briefly (3-5s), place it in a heating block or water bath, and incubate at 65°c for 5 minutes. NOTE: The sample color changes from red to olive green, indicating protein digestion.
- Added 150µl isopropanol (100%) and mix thoroughly by inversion until the DNA precipitate becomes visible as threads or a clump. NOTE: Complete mixing with isopropanol is vital, to precipitate the DNA and must be checked by inspection. For samples with very low WBC counts, invert the tube at least 20 times.
- Centrifuged for 3 minutes at 10,000 x g. NOTE: If the resulting pellets are loose, centrifugation can be prolonged or a higher g-force can be used.
- Discarded the supernatant and briefly invert the tube onto a clean piece of absorbent paper, taking care that the resulting pellet remains in the tube.
- Added 150 µl 70% ethanol and vortex for 5 seconds.
- Centrifuged for 3 minutes at 10,000 x g. NOTE: If the resulting pellets are loose, centrifugation can be prolonged or a higher g-force can be used.
- Discarded the supernatant and leave the tube inverted on a clean piece of absorbent paper for at least 5 minutes, taking care that the pellet remains in the tube.
- Air-dry the DNA pellet until all the liquid has evaporated (at least 5 minutes). NOTE: Avoid over drying the DNA pellet, sometimes over-dried DNA is very difficult to dissolve.
- Added 200 µl Buffer FG3, vortex for 5s at low speed, and dissolve the DNA by incubating for 1 hour at 65ºc in a water bath. NOTE: If the DNA is not completely dissolved, incubate the solution overnight at room temperature. If a reduced volume of Buffer FG3 is used, the incubation may need to be prolonged.

Estimation of DNA

Nucleic acids, like many other substances, have the property of absorbing light at a specific wavelength. Since nitrogenous bases absorb UV light, the more concentrated the DNA solution, the more UV light it will absorb. DNA and RNA absorb light maximally at a wavelength of 260 nm. Because of this property, they can be quantified spectrophotometrically with UV light source. The concentration of pure double-stranded DNA with an A260 of 1.0 is 50µg/ml. In order to get a linear relationship between absorbance and DNA concentration, we can use the following formula to determine the DNA concentration of a solution:

Unknown µg/ml = 50 µg/ml x Measured A260 x dilution factor

The purity of a nucleic acid solution can be determined, by calculating the A260/A280 ratio. The nucleic acid absorbs maximally at 260 nm and protein (a principle contaminant) absorbs maximally at 280 nm.

- Pure DNA has an A260/A280 ratio of 1.8.

- Pure RNA has an A260/A280 ratio of 2.0.

MATERIALS

- NanoDrop Spectrophotometer
- Double distilled water
- DNA sample
- Tissue
- Micro pipettes
- Gloves

Procedures

- Turned on the NanoDrop Spectrophotometer and its software, Opened NanoDrop program and pressed on nucleic acid button.
- Cleaned the surface of NanoDrop spectrophotometer with d.d.H2O and add blank as: 1 µl DNA Hydration Solution (TE Buffer).
- Read the blank by pressing the Blank button.
- Cleaned the surface of NanoDrop spectrophotometer with d.d.H2O and add DNA sample as: 1 µl DNA Template.
- Read the concentration of DNA by pressing the Measure button.
- Cleaned the surface of NanoDrop spectrophotometer with double distilled water.
- Took the readings of DNA concentration.

Polymerase Chain Reaction (ARMS – PCR)

In the PCR, two primers (short-stranded DNAs) are used that are complementary to opposite strands of the DNA sequence to be amplified. After heat mediated denaturation of the template DNA, the primer binds to their respective sequences (annealing) on the template DNA and DNA polymerase synthesize a complimentary strand in the 5'to 3' Direction. Each round of denaturation, annealing, and extension is known as a cycle. Theoretically, with each cycle the amount of the template DNA sequence amplified Doubles. Therefore, after 10 cycles the target sequence within the template DNA is multiplied by a factor of one thousand and after 20 cycles by a factor of more than one million. However, an ―amplification plateau‖ eventually be reached when additional cycles will not lead to any further increase in amplified product. This amplification plateau results from the exhaustion of reagents such as the dNTPs and primers. The optimum temperature at which each of these steps (denaturation, annealing, extension) proceeds is different and therefore the reaction is best performed in the thermal cycler, which automatically makes the temperature changes required. This machine, a programmed one, incubates the reaction mixture at a series of temperatures required for the reactions to proceed without any interruption. The discovery of thermostable DNA polymerases has made the steps in PCR cycle much easier.

Materials

- DNA template: PCR can amplify as little as one molecule of starting template. The sample DNA that provides one or more the target sequences can be used as a template.
- Enzymes: Thermostable DNA polymerases like Taq DNA polymerase (from Thermusaquaticus), which have been isolated are used.
- Primers: Forward and Reverse primers in which each primer must be complementary to its template strand in order for hybridization to occur and the 3' ends of the hybridized primers should point towards one another.
- Nucleotides (dNTPs or deoxynucleotide triphosphates): Single units of the bases A, T, G, and C, which are essentially "building blocks" for new DNA strands hence four dNTPs are used namely ATP, GTP, TTP, CTP for DNA synthesis. About 20-200 μM of dNTPs are normally used.
- Thermocycler: The entire PCR cycle includes three essential steps: 1. Denaturation 2. Annealing and 3. Polymerization. Thermocycler sometimes called PCR machine takes care of the adjustment of the three temperatures. It is automated to adjust the temperature and time required for each step.

Table 1

10 X PCR buffer 1μl
10 Mm DNTPS 0.5μl
FARWARD PRIMER 0.5μl
REVERSE PRIMER 0.5μl
1U TAQ POLYMERASE 0.1μl
DNA TEMPLATE 1.0μl
H2O 6.4μl
TOTAL 10μl

Table 2: Arms-PCR Cycling Program

Stage Cycles Step Temperature Time
1 1 Denaturation95°C5:00 min
DenaturationAnnealing95°C0:30 sec
235Extension62°C - 72°C0:40 sec
3 1 Extension72°C 8 min

Procedures

- Added 9ml of master mix and 1ml of template to prepare reaction mix and set the program depending on the primers we use.
- Tubes are subjected to spin by spinning machine micro centrifuge
- 3ml of orange dye is added to each tube.
- PCR products were verified by running them on 2% agarose gel.
- Prepared gel with 2% of agarose in 100ml of 1X TBE buffer is heated,15ml of ethidium bromide is added mixed and poured into plate with combs for the wells and left undisturbed till the gel thickens and gets solidify.
- 10μl of 100bp DNA ladder was loaded in one of the wells and load the samples in the other wells.

Agarose Gel Electrophoresis

Agarose is a linear polysaccharide made up of basic repeat unit agarobiose, which consists of alternating units of galactose and 3, 6-anhydrogalactose. It is a compound of agar-agar isolated from certain seaweeds. Agarose gel electrophoresis is a technique to separate and sometime purify macromolecules, especially proteins and nucleic acid that differ in size change or conformation. Fragments of linear DNA migrate through agarose with a mobility that is inversely proportional to their molecular weight. By using gels with different concentration of agarose facilitate separation of small DNA fragments. While, low agarose concentration allow resolution of layer DNA's.

Factors

The factors that determine the rate of migration of DNA through agarose gels are:

- Molecular size of DNA
- Concentration of agarose
- Conformation of DNA
- Presence of ethidium bromide in the gel
- Applied voltage
- Electrophoretic buffer

Materials

- Electrophoresis chamber & 150V power supply
- Gel casting tray & comb
- Electrophoresis buffer – TBE buffer
- loading dye
- Ethidium Bromide (10mg/ml)
- Agarose
- 100bp DNA ladder
- Electronic weighing balance
- Microwave oven
- Gel documentation system

Preparation of Reagents

Explain the reagents 100 ml of TBE buffer at the table fellow

Table 3

Trisbase -10.3%	21.6g
Boricacid- 5.4%	10.8g
EDTA -(0.5M)	1.189g
H2O	100ml

Preparation of 1.5% Agarose

- Measured 2gm of agarose powder and add it to 100ml of 1X TBE buffer in a 200ml conical flask and heated in microwave.
- Added 25µl of ethidium bromide to the warm gel.
- Sealed the ends of the casting tray and place the combs in the gel casting tray and pour the agarose.
- Carefully pulled out the combs and remove the tape.
- Placed the gel in the electrophoresis chamber and add TBE Buffer.
- 3µl of the PCR product was mixed with 3µl of 6x loading dye and loaded.
- 10µl of 100bp DNA ladder was loaded in one of the wells.

Running Gel

- Placed the lid on the gel box, connecting the electrodes.
- Connected the electrode wires to the power supply, making sure the positive (red) and negative (black) are correctly connected and adjust to 150 volts for 30 minutes until the blue dye approaches the end of the gel.
- Using gloves carefully remove the tray and gel and observed under Gel Doc.
- Bands are observed and are the base pairs are known with the help of the ladder.

RESULTS

The IFN-γ gene polymorphism at +874 A/T position was studied in 35 APTB, 35 HHC & 20 HCs. Among the three genotypes the AT genotype was more frequent in APTB, HHC &HC, whereas AA genotype was found to be significant in APTB ($p<0.04$ OR- 8.708; CI-1.031-73.55) compared to HC and was showing a positive association, while AT & TT genotypes did not show any significant difference between the groups. The A allele was found to be more frequent in APTB and HHC while in HCs the T allele was found to be more frequent. When the alleles were considered no significant association was shown between the alleles.

Table 4: Genotype and Allele Frequencies of IFN-γ(+874A/T) in APTB Patients Vs HC

Genotype	HC	APTB				
	N=20	N=35	Frequency (%)	P value	Odds Ratio	95% C.I.
AA	1	11	31%	0.041	8.708	1.031-73.55
AT	15	17	50%	0.100	0.3148	0.0939-1.056
TT	4	7	19%	0.99	1	0.2532-3.949
Allele	**HC**	**APTB**				
	N=40	**N=70**	**Frequency (%)**	**p value**	**Odds Ratio**	**95%C.I.**
T	17	39	56%	0.25	0.587	0.2681-1.287
G	23	31	44%	0.25	1.702	0.7768-3.73

Table 5: Genotype and Allele Frequencies of IFN-γ (-874A/T) in HHC Vs HC

Genotype	HC	HHC				
	N=20	N=35	Frequency (%)	P value	Odds Ratio	95% C.I.
AA	1	8	23%	0.17	5.63	0.6492-48.81
AT	15	20	57%	0.30	0.44	0.1321-1.496
TT	4	7	20%	0.99	1.00	0.2532-3.949
Allele	**HC**	**HHC**				
	N=40	**N=70**	**Frequency (%)**	**p value**	**Odds Ratio**	**95%C.I.**
T	17	36	51%	0.48	0.698	0.3191-1.527
G	23	34	49%	0.48	1.433	0.6549-3.133

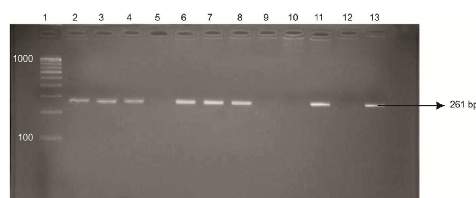

Figure: 1 IFN-γ (+874A/T) Gene polymorphism / Lane 1- 100bp Ladder; Lane 2-3-6-7-AT; 4-5-AA; 8-9-AA; 10-11-12-TT Genotype

DISCUSSIONS

The functioning of the IFN- γ pathway is mediated by its interaction with receptors located on the surface of the cells. The receptor for IFN- γ is composed of two structurally homologous polypeptides, called, IFN-γ-R1, and IFN-γ–R2. IFN-γ and IFN γ R form a vital complex in determining the outcome of the biological effects of IFN-γ. (Durbin JE et al., 1996) A previous study showed that defects in either of these two genes influence the availability of IFN-γ and therefore individuals were more prone to mycobacterial infections. Some polymorphisms have been detected along the IFN-γ sequence, especially in introns 1 and 3, and in the 30-UTR region (Kranzer K et al, 2010).One of the most studied polymorphisms in IFN-γ is located in the first intron (+874 T/A) and have been associated with TB susceptibility in several populations. A study that summarizes all the different studies is the meta-analysis performed on this particular SNP, which indicated a significant protection, with the T/T genotype, to tuberculosis in different ethnic population groups throughout the world. Therefore, it was shown that the +874 T/A SNP can be a major genetic marker for TB resistance (Darnell JE et al, 1994).

It has been reported that, the production of IFN-γ is genetically controlled and +874 T/A was found to be one of the well known polymorphism of this gene. Many studies have reported the resistance and susceptibility to TB at this position.

(Lopez-Maderuelo D et al, 2003), reported the least frequency of TT genotypes and highest frequency of AT genotypes in patient and control and also association of TT and AA genotypes in microscopic positive and negative form of the disease, which was also similar to our study, based on AA genotype association. Our study was similar to several studies showing association of AA genotype, with the disease (Delgado JC et al, 2002) has reported no significant difference in genotypes or alleles between patients and controls.

CONCLUSIONS

In conclusion the study demonstrates that, the gene interactions of different cytokine genes may help us to identify the high risk genotypes when analyzed in large samples. These high risk genotypes might be used as a marker, for identification of high risk individuals.

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