Abstract

Background: A variety of genetic mutations are thought to be responsible for acquisition of different infections such as tuberculosis (TB). An obvious example for these variations is the link between pulmonary TB and polymorphisms within interferon-gamma receptor 1 (IFN-γ R1) gene. This project is designed to identify the role of IFN-γR1 gene polymorphism in the development of pulmonary TB among Sudanese patients attending several hospitals in Khartoum State. Methods: One hundred (n = 100) patients with active TB and fifty (n = 50) matched healthy controls were investigated for the association of two genetic polymorphisms within IFN-γR1 gene and their risk of developing pulmonary tuberculosis. Polymerase chain reaction (PCR) assay and PCR-restriction fragment length polymorphism were performed. Results: Migrated IFN-γR1 DNA bands representing genotypes and polymorphic alleles were identified. Molecular findings revealed that two genetic variants, namely, −56C and +295C deletion within IFN-γR1 gene, were nonsignificantly linked with increased risk of development of pulmonary TB, \( P = 0.771 \) and 0.343, respectively. Two genetic variants within IFN-γR1 gene were examined for suggested role in inducing development of TB. Conclusion: The two genetic variants were found to have potential risk in association with active disease development among Sudanese patients. Further intensive research work involving use of large collection of samples should be conducted to verify these findings.

Keywords: Interferon-gamma receptor 1, Khartoum, polymerase chain reaction-restriction fragment length polymorphism, Sudan, tuberculosis, Ziehl–Neelsen stain

How to cite this article: Ali AH, Omer AA, Saeed NS, Mansour EE, Elhassan MM. Influence of interferon-gamma receptor 1 gene polymorphisms on the susceptibility to pulmonary tuberculosis among Sudanese Population. Int J Mycobacteriol 2018;7:26–31.
vulnerability to infections with mild mycobacteria, falls within this category. This genetic condition evolves due to genetic defects in IFN-γ receptors (IFN-γRs) or failure of expression on immune cell surfaces.[4,5] Similarly, a newborn with complete genetic disruption involving IFN-γR was prone to increased susceptibility and further dissemination of infection after inoculation with Bacillus Calmette–Guerin vaccine strain.[6,7]

As such, variations involving host genetics and immune response are vital in determining TB development and control.[8] Polymorphic host genotypes positioned at −56 and +874 within the IFN-γR1 gene were previously shown to confer risk of developing TB in different communities.[6,8,9] Yet, other studies revealed the reverse.[10] This issue is remained debatable requiring further investigation.

The current study is aimed to investigate two polymorphic genotypes, namely, +295 and −56 located within the promoter area of IFN-γR1 gene and their potential link in developing TB among Sudanese patients. The study involved polymerase chain reaction (PCR) amplification of these genotypes and enzymatic digestion using restriction fragment length polymorphism (RFLP) of DNA products to verify their identity through analysis of collected data.

**Methods**

**Type of study, sampling, and population**

One hundred (n = 100) TB participants showing typical signs of active pulmonary TB disease were recruited adopting a cross-sectional study. Patients were regularly attending several hospitals within Khartoum State. They belonged to different tribes and had variable socioeconomic status. Simultaneously, 50 unrelated healthy participants who tested negative for TB in the same environment were included as control subjects. Age and sex gender were matched in both groups. Seventy-five percent of participants were males while the remaining 25% were females. Ethical clearance and approval to carry out this project was obtained from the Federal Ministry of Health ahead with confidentially written consent from all candidates before starting the study. TB cases presented with typical clinical symptoms of TB were enrolled (sputum and blood samples were collected).

**Ziehl–Neelsen stain**

The presence of Mtb was provisionally detected by performing Ziehl–Neelsen for all sputum specimens.

**Culturing of pathological samples**

The samples were initially decontaminated and then processed for inoculation on appropriate Löwenstein–Jensen agar slope media to isolate and identify the pathogens following standard procedures.[12] Biochemical reactions include catalase, nitrate reduction, para-nitrobenzoic acid, and thiophene 2-carboxylic acid hydrazide. Susceptibility tests were further performed to support laboratory diagnosis.

**Amplification of interferon-gamma receptor 1 gene by polymerase chain reaction**

Extracted genomic DNA from human immune cells was employed as a template to amplify IFN-γR1 gene by PCR. Briefly, the PCR mixture was consisted of 1 µl DNA template, 1 µl (2.5 µM) for each of forward and reverse primer, 12.5 µl of GoTaq PCR Master Mix, and 14.5 µl PCR grade water (nuclease free). PCR amplification was performed on a thermal gene cycler (Biometra TProfessional Gene) utilizing specific primers according to Hamajima[13] as displayed below:

For amplification of IFN-γR1 – 56 T/C genotype, the primers were IFN-γR1 F: (5’-GGGCGTGGGCGGGGTCAA-3’) and IFN-γR R: (5’-CCTCCCTCCTCTCGTCC-3’).

At the same time and for IFN-γR1 295 deletion 12, IFN-γR1 295 confrontational pair (CP) we used: 5’-CTCTGCTCTTTTCACCGCTTT-3’; IFN-γR 295 del: 5’-AACCCTGGCTTTAACTCTGACC-3’; IFN-γR 295 del: 5’-CCATCAATTTCTCTAAMCCAGG-3’; and IFN-γR 295 del CP: (5’-CTAATAAAA GCAACATACAGAAGAC-3’).

Temperatures and cycling conditions for PCR amplification of polymorphic regions are shown in Table 1.

In the absence of polymorphism 295 del 12, the primer IFNγ-R1 295 del 12 will work to amplify with the primer IFNγ-R1 295 CP to give a PCR fragment of 232 bp, while in the presence of the 295 del 12, the primer IFNγ-R1 295 del 12 may work to amplify with the primers IFNγ-R1 295 del CP to give a PCR fragment of 160 bp. A third PCR product of 365 bp may be obtained as a result of the IFNγ-R1 295 CP and IFNγ-R1 295 deletion.

**Loading of polymerase chain reaction products and gel electrophoresis**

The amplified PCR products involving polymorphism located at −56 within the promoter region of IFNγ-R1 gene were enzymatically digested using PCR-RFLP before loading. The resultant DNA digest was mixed with 5 µl loading dye.

**Table 1: Appropriate gene cycling and temperature condition for amplification of polymorphic regions within IFN-γR1 gene by polymerase chain reaction**

| Polymorphism       | Starting temperature | Denaturation | Annealing | Extension | Final      | Number of cycles |
|--------------------|---------------------|--------------|-----------|-----------|------------|-----------------|
| IFNγ-R1-56         | 95°C/5 min (s)      | 94°C/1 min   | 66°C/1 min| 72°C/1 min| 72°C/5 min (s)| 35              |
| Thymine → Cytosine |                     |              |           |           |            |                 |
| IFNγ-R1 295 del 12 | 95°C/5 min (s)      | 94°C/1 min   | 62°C/1 min| 72°C/1 min| 72°C/5 min (s)| 35              |

International Journal of Mycobacteriology | Volume 7 | Issue 1 | January-March 2018
An agarose gel with 1.5% concentration was prepared and stained with 2 μl of ethidium bromide solution with 1 μg/ml concentration. The mixture was loaded into formed agarose gel wells and left to run in electrophoresis at a constant rate of 100 V for 1–2 h or until the front dye reached about two-thirds of the gel. IFN-γR1 DNA bands were visualized under ultraviolet (UV) light and gel was documented using DNA Gel Documentation System (GDS).

For CP, PCR amplicons were run on 2% agarose. In solution, staining was performed using 1 μg/ml ethidium bromide solution for 10–15 min and later visualized under UV light employing GDS. Genotype identification after amplification was designated according to the molecular length of obtained DNA fragments postenzymatic digestion and migration on agarose gel electrophoresis.

Statistical analysis of collected data
Collected data were statistically analyzed by using version 16 software of SPSS (SPSS Inc., Chicago, IL, USA). Chi-square test was applied to evaluate the association between different variables.

RESULTS

Demographic data analysis
Participants were randomly selected and found to originate from 26 tribes. Majority of participant patients belonged to seven main tribes including Galiyeen (19), Tama (11), Nuba (10), Zaghave (8), Haddaway (6), Fur (6), and Bargo (5). In addition, a few patients were assigned to other minority tribes. Seventy-five percent of TB participants were male, while the remaining 25% were females. The average age of cases was 34 years in comparison with 33 years for controls. When infected cases were grouped, the frequency of distribution was greater in the range from 20 to 30 years old accounting for 50%.

Genotyping analysis of polymerase chain reaction of −56 polymorphism
Following PCR amplification of polymorphic −56 within IFN-γR1 DNA and RFLP of the products, several DNA fragments were observed [Figure 1]. Two bright DNA fragments at molecular levels of 193 and 92 bp were seen and characterized as a homozygous 56CC genotype [Figure 1]. However, appearance of three bright DNA fragments at molecular sizes of 285, 193, and 92 bp implied a heterozygote 56T/C mutant [Figure 1].

Frequency of −56 polymorphic alleles
With reference to −56 polymorphism, −56CC alleles were carried by seven TB patients accounting for 7% frequency of distribution in comparison with only three healthy controls [Figure 2]. However, this variation in the frequency between the two target categories was statistically nonsignificant (P = 0.771). Polymorphic −56T/C alleles were harbored by 16 TB patients with 16% frequency of distribution [Figure 2]. By contrast, polymorphic −56T/C alleles were carried only by six healthy controls with 12% frequency of distribution [Figure 2]. When both allelic variants, i.e. −56T/C and −56CC, were added together, they had 23% frequency of distribution among infected patients compared to the healthy controls. Although the TT wild-type (WT) genotype was not shown in the above gel, its frequency of distribution was 77% in TB patients in comparison with 82% distribution in healthy controls.

Genotyping analysis of polymerase chain reaction of +295 polymorphism products
Genetic variation of homogenous +295C/C alleles showed frequency distribution of 6% among TB patients as opposed to 2% distribution for healthy controls. Accordingly, +295C alleles at deletion 12, six cases harbored such polymorphism in patients in comparison with their counterparts in the healthy controls [Figure 3]. This difference between groups was statistically nonsignificant (P = 0.343). Again, genotyping analysis of IFN-γR1 gene at position +295 deletion 12 revealed 90 TB cases exhibiting +295TT genotypes with 90% frequency of distribution in comparison with the same frequency of distribution in the healthy controls [Figure 4].

In relation to heterogeneous +295T/C allelic changes, they had 4% frequency of distribution across the gene in comparison...
with (8%) occurrence in healthy controls [Figure 4]. Similarly, frequency of distribution after addition of both +295C and +295T/C alleles was 10% in TB cases as similar as in healthy participants.

**Frequency of +295 polymorphic alleles**

With regard to genotyping of IFN-γR1 at position +295 deletion 12 within the gene, PCR with confronting two-pair primers (PCR-CTPP) assay amplified different bright bands of DNA after running the gel. DNA bright bands were observed at molecular sizes of 365 and 232 bp representing IFN-γR1 homozygotic WT type genotype +295TT [Figure 4]. On the other hand, appearance of two DNA bands at molecular position of 365 and 160 bp represented homozygote polymorphic type, +295CC [Figure 4]. Appearance of three DNA bands at molecular sizes of 365, 232 and 160 bp indicated IFN-γR1 heterozygote +295TC del. 12 [Figure 4].

**Statistical analysis**

Statistical analysis using SPSS and Chi-square test showed that genetic variations involving −56C and +295C deletion 12 located in the promoter region of IFN-γR1 gene were nonsignificantly (P = 0.771 and P = 0.343, respectively) associated with high chances of developing TB.

**Discussion**

With annual occurrence of nearly 2 million deaths and around 9 million fresh emergent cases, TB remains the major infectious bacterial disease that threatens humans’ public health.14 Patients with active disease and who show sputum smear-positive indicative of pulmonary TB constitute the main focus for spread of infection. It has been reported that only a minority proportion of people with waned immune system might get Mtb infection upon exposure and could present overt clinical signs of TB while a few people would never develop the disease.2 In several studies, TB has been documented as an infection related to host genetic susceptibility and familial inheritance.4 This belief evidenced by increased infection of homozygotic twins with Mtb than dizygotic twins and in close marriages than in distant relationships.2,4,15

Being a T-helper cytokine, IFN-γ gene and through its signaling pathway is responsible for activating macrophages to release bactericidal toxic products and combat progression of Mtb infection and other pathogens.11,16 Specific mutations in IFN-γR1 gene ligand binding chain were involved in Mendelian susceptibility to bacillary infection.17 Mice with genetic alterations in host IFN-γR1 gene failed to yield toxic reactive nitrogen intermediates and control wild pathogen after challenge.18 Similarly, children with inherited genetic disorder of partial or complete deficiency of IFN-γR1 were highly prone to infection with atypical mycobacteria.19 Moreover, a quite number of single-nucleotide polymorphisms (SNPs) in the IFN-γ such as +874 A/A genotype have been demonstrated in association with susceptibility of developing TB.3,5

In this study, pathological materials taken from diseased and healthy participants were examined microscopy and cultured to isolate and recover the causative agents. Genotyping analysis was performed on three potential polymorphic alleles located across the promoter region of IFN-γR1 gene at positions; −56 and +295 deletions 12. PCR assay combined with RFLP analysis by specific restriction enzyme or CTPP of genomic IFN-γR1 DNA isolated from immune cells of enrolled participants was used as tools to achieve the target. The purpose of the project is to evaluate whether genetic disruptions in the aforementioned SNPs within IFN-γR1 gene are associated with susceptibility and probability in conferring risk of developing pulmonary TB among the Sudanese patients. Identified SNPs and genotypes within the IFN-γR1 gene that might trigger pulmonary TB were further studied for their link with demographic characteristics including tribes from which diseased participants have originated.

Microscopic examination of stained sputum smears coupled with culturing of pathological materials as well as biochemical testing has confirmed that the enrolled participants who showed clinical symptoms were the main cause of TB.
active TB signs were infected with Mtb. Some of the genotypic findings of the two potential polymorphisms IFN-γR1 gene exploited in this project were consistent with those of others who have previously identified and explored them in different regions and populations worldwide.\cite{3,5,17} As such, we have attempted to investigate their genetic defects and subsequent effects in triggering active TB disease among Sudanese population.

The data from the present study demonstrated that PCR amplification of human genomic IFN-γR1 gene extracted from affected host immune cells followed by RFLP analysis has successfully helped in identification of DNA products resembling the three polymorphic alleles at −56 and +295 deletion 12 of the gene.

Distribution of −56CC genotypes was more in TB cases than those in healthy subjects. Hence, distribution of −56C >T was seen in TB cases while of −56C <T alleles was observed in the control subjects. However, this difference between the two groups was statistically insignificant ($P = 0.771$). Accordingly, −56C allele is probably associated with risk of developing TB. These findings were in concordance with similar study among West African citizens which revealed that the −56CC genotype was linked with risk of developing TB.\cite{22} Furthermore, Meyer et al. studied different variants within IFN-γR1 among Ghanaiian populations. Their results did not reflect any significant findings among susceptible population, despite a large number of screened samples and the different genes studied.\cite{20} Another study from Gambia yield similar findings.\cite{20} Moreover, similar findings were suggested among Chinese population by Chen et al.\cite{21}

Obtained statistical bias in the present project was probably related to the small size of collected samples. Conversely, these findings were not in agreement with others who reported that polymorphism within IFN-γR1 at −56C >T allele was significantly associated with twice fold reduction in the risk of susceptibility to TB in a similar population.\cite{19} On the other hand, and if we consider similar infections that are caused by mycobacterium other than tuberculosis, a significant relationship between mutation at position −56 and the susceptibility to this kind of infections was proved by Farnia et al. among Iranian community.\cite{23}

Distribution of +295C allele deletion 12 within IFN-γR1 was more frequent among infected patients ($n = 6$) compare to healthy controls (only one).Therefore, polymorphic +295C allele was more than T/C allele (+295C >T) suggesting nonsignificant ($P = 0.343$) indicating increased chances of developing TB. However, we could not find any evidence in literature to support these findings.

Evidence from earlier studies in vitro cell cultures which involved transformation of a construct harboring −56C allele into cells and expression of IFN-γR1 −56C resulted in low transcription and expression of the receptor on cell surfaces.\cite{5} Subsequently, this low immune response may be accompanied by less damage with reduced effects of immunopathology and providing more chances of protection against pulmonary tuberculosis.\cite{5} Undoubtedly, mutation in the promoter area of IFN-γR1 is greatly related to TB, but the host immune response and other genetic factors including production of IFN-γ may modify this pathway leading to increased susceptibility of TB or protection against it.\cite{5}

Collected demographic characteristics and genotyping data in this study showed that the Tama, Nuba, Zaghawa, and Fur tribes were in the top of lead in recording the highest number of positive cases linked with polymorphic alleles −56 T/C and −56CC at position −56 of IFN-γR1 gene probably triggering pulmonary TB cases. Yet, it was not significant when compared with healthy controls. At position +295 deletions 12, cases originated in Tama and Fur tribes had more mutations in association with positive TB cases. Increased prevalence of polymorphism in these tribes could be attributed to traditional habit of close marriage practiced by most families within the tribe. The act of close ancestral marriage was reported to trigger and precipitate IFN-γR1 genetic disorders in association with increased susceptibility to pulmonary TB.\cite{4}

To the best of our knowledge, this is the first (in home) project which implicated an investigation of two potential genetic polymorphisms within IFN-γR1 gene for their role in triggering development of TB among Sudanese populations. However, the current study has encountered several limitations. First, collected samples for further processing were quite small which has complicated statistical analysis and the findings. Second, most of the molecular epidemiological work was performed outside the country as facilities including advanced molecular techniques and expertise are not available. Finally, some molecular assays such as sequencing of IFN-γR1 DNA products could be of value in similar project.

With reference to demographic risk factors such as smoking and air pollution, both indoor and outdoor pose major threats to lung health as they reduce local defences and increase chances of TB disease.\cite{23,24}

**Conclusion**

This study was designed to determine the role of two polymorphisms located within the promoter region of IFN-γR1 gene in triggering development of TB among Sudanese patients. Collected data showed that the tested polymorphisms have potential link in increasing risk of developing TB among Sudanese patients. Demographic characteristics in relation to the identified polymorphisms were also sought out. Future research involving use of large number of samples in different regions of the country is warranted.

**Patient's agreement**

Written informed consent was obtained from all participants. A copy of this form was submitted to the research committee of medical laboratory sciences (approval No. MLT 143/2011).

**Financial support and sponsorship**

Nil.
Conflicts of interest

There are no conflicts of interest.

References

1. Albanese SP, da Costa AA, Pieri FM, Alves E, Dos Santos DT, Kerbauy G, et al. Prevalence and evolution of Mycobacterium tuberculosis infection in tuberculosis case contacts. Rev Soc Bras Med Trop 2015;48:307-13.

2. Abel L, El-Baghdadi I, Bousfiha AA, Casanova JL, Schurr E. Human genetics of tuberculosis: A long and winding road. Philos Trans R Soc Lond B Biol Sci 2014;369:20130428.

3. Tso HW, Ip WK, Chong WP, Tam CM, Chiang AK, Lau YL, et al. Association of interferon gamma and interleukin 10 genes with tuberculosis in Hong Kong Chinese. Genes Immun 2005;6:358-63.

4. Al-Muhsen S, Casanova JL. The genetic heterogeneity of Mendelian susceptibility to mycobacterial diseases. J Allergy Clin Immunol 2008;122:1043-51.

5. Cooke GS, Campbell SJ, Sillah J, Gustafson P, Bah B, Sirugo G, et al. Polymorphism within the interferon-gamma/receptor complex is associated with pulmonary tuberculosis. Am J Respir Crit Care Med 2006;174:339-43.

6. Jouanguy E, Altare F, Lamhamedi S, Revy P, Emile JF, Newport M, et al. Interferon-gamma-receptor deficiency in an infant with fatal Bacille Calmette-Guérin infection. N Engl J Med 1996;335:1956-61.

7. Casanova JL, Abel L. The human model: A genetic dissection of immunity to infection in natural conditions. Nat Rev Immunol 2004;4:55-66.

8. Berrington WR, Hawn TR. Mycobacterium tuberculosis, macrophages, and the innate immune response: Does common variation matter? Immunol Rev 2007;219:167-86.

9. Lio D, Marino V, Serauto A, Gioia V, Scola L, Crivello A, et al. Genotype frequencies of the +874T—>A single nucleotide polymorphism in the first intron of the interferon-gamma gene in a sample of Sicilian patients affected by tuberculosis. Eur J Immunogenet 2002;29:371-4.

10. Rossouw M, Nel HJ, Cooke GS, van Helden PD, Hoal HG. Association between tuberculosis and a polymorphic NFKappaB binding site in the interferon gamma gene. Lancet 2003;361:1871-2.

11. Attalla MA, Mogahid ME, Nagla GM, Miskelyem AE, Adil M. The role of interferon-γ receptor-1 gene (-56 T>C) polymorphism in development of susceptibility to pulmonary tuberculosis in central Sudan. Egypt Acad J Biol Sci 2011;3:31-5.

12. Morcillo N, Imperiale B, Palomino JC. New simple decontamination method improves microscopic detection and culture of mycobacteria in clinical practice. Infect Drug Resist 2008;1:21-6.

13. Hamajima N, PCR-CTPP: A new genotyping technique in the era of genetic epidemiology. Expert Rev Mol Diagn 2001;1:119-23.

14. World Health Organization. Global Strategy and Targets for Tuberculosis Prevention, Care and Control Beyond 2015. World Health Organization; 2013.

15. Allmann FJ, Reissner D. Twin studies on the significance of genetic factors in tuberculosis. Am Rev Tuberc 1943;47:549-74.

16. Collins HL, Kaufmann SH. The many faces of host responses to tuberculosis. Immunology 2001;103:1-9.

17. Awomoyi AA, Richardson N, Hull S, Koch AJ, Podinovskaia O, Todd M, et al. No association between interferon-c receptor-1 gene polymorphism and pulmonary tuberculosis in a Gambian population sample. Thorax 2004;59:291-4.

18. Cooper AM, Dalton DK, Stewart TA, Griffin JP, Russell DG, Orme IM, et al. Disseminated tuberculosis in interferon gamma gene-disrupted mice. J Exp Med 1993;178:2243-7.

19. Meyer CG, Intemann CD, Förster B, Owusu-Dabo E, Franke A, Horstmann RD, et al. No significant impact of IFN-γ pathway gene variants on tuberculosis susceptibility in a West African population. Eur J Hum Genet 2016;24:748-55.

20. Newport MJ, Awomoyi AA, Blackwell JM. Polymorphism in the interferon-gamma-receptor-1 gene and susceptibility to pulmonary tuberculosis in the Gambia. Scand J Immunol 2003;58:383-5.

21. Chen C, Chen L, Chen C, Chen Q, Zhao Q, Dong Y, et al. The distribution frequency of interferon-gamma receptor 1 gene polymorphisms in interferon-γ release assay-positive patients. Dis Markers 2017;2017:4031671.

22. Farnia P, Ghanavi J, Tabarsi P, Saif S, Velayati AA. The importance of single nucleotide polymorphisms in interferon gamma receptor-1 gene in pulmonary patients infected with rapid grower mycobacteria. Int J Mycobacteriol 2016;5 Suppl 1:S210-1.

23. Bates MN, Khalakdina A, Pai M, Chang L, Lessa F, Smith KR, et al. Risk of tuberculosis from exposure to tobacco smoke: A systematic review and meta-analysis. Arch Intern Med 2007;167:335-42.

24. Trinh QM, Nguyen HL, Nguyen VN, Nguyen TV, Sinzhenko V, Marais BJ, et al. Tuberculosis and HIV co-infection-focus on the Asia-Pacific region. Int J Infect Dis 2015;32:170-8.