ORIGINAL ARTICLE
Year : 2018 | Volume : 7 | Issue : 1 | Page : 26-31

Influence of interferon-gamma Receptor 1 gene polymorphisms on the susceptibility to pulmonary tuberculosis among sudanese population

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Date of Web Publication 7-Mar-2018

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Source of Support: None, Conflict of Interest: None

DOI: 10.4103/ijmy.ijmy_206_17

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 12 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

Introduction

Tuberculosis (TB) is a great infectious bacterial killer to humankind though its health impact is on decline. The disease has different forms, but the pulmonary form is of concern since the patient remains infectious and continues to shed infection among people in close contact.[1]Despite its serious public health risk and threat, only a small proportion (5%) of population manifests a clinical disease, while a similar percentage incubates a dormant bacillus infection that may reactivate later in life to active disease.[1] Yet, a minority may contract infection and suffer from a severe clinical phenotype disease. Arguably, a few individuals developed protective immunity against *Mycobacterium tuberculosis* (Mtb) infection and would never become sick.[2] However, it is uncertain why the bacillus behaves differently in various human hosts and populations.

Interferon-gamma (*IFN-γ*) is a cytokine produced by $CD4^+$ T-cells and crucial for macrophage activation and signaling pathway between immune cells, production of toxic products, and subsequent boosting of host immune response to destroy virulent pathogens such as Mtb.[3]However, genetic disorders within *IFN-γ* may be linked to predisposing host genetic susceptibility against bacillus infection.[3] An inherited condition termed Mendelian susceptibility to mycobacterial disease, which mediates high 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],[9],[10] Yet, other studies revealed the reverse.[11] 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'-CCTCCCTCCCTCTCGTCC-'3).

At the same time and for IFN-γR1 295 deletion 12, IFN-γR1 295 confrontational pair (CP) we used: 5'-CTCTGCTCTTTCTACCGCTTT-'3; *IFN-γR* 295 del12: 5'-AACCCTGGCTTTAACTCTGACC-'3; *IFN-γR* 295del12: (5'-CCATCAAATTCTCTTAAAGCCAGG-'3), and *IFN-γR* 295del CP: (5'-CTAATAAAA GCAAACATACAGAAGAC-'3).

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

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

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In the absence of polymorphism 295 del 12, the primer *IFNγ-R1* 295 del. 12 will work to amplify with the primer *IFN-γR1* 295 CP to give a PCR fragment of 232 bp, while in the presence of the 295 del. 12, the primer *IFN-γR1* 295 del. 12 may work to amplify with the primer *IFN-γR1* 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 IFN-γR1 295 CP and *IFN-γR1* 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 *IFNγR1* gene were enzymatically digested using PCR-RFLP before loading. The resultant DNA digest was mixed with 5 μl loading dye. 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), Zaghawa (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].

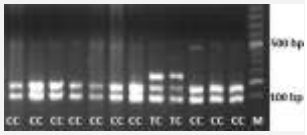

Figure 1: Amplification of allelic polymorphism at position −56 of interferon-gamma receptor 1 gene by polymerase chain reaction-restriction fragment length polymorphism. Lanes CC, homozygous mutant; lanes TC, heterozygous mutant; lane M, 100 bp DNA ladder

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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.

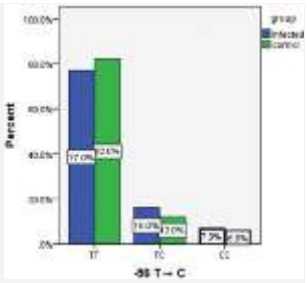

Figure 2: Frequency distribution of −56 polymorphic alleles within the promoter region of interferon gamma receptor 1 found among tuberculosis patients as compared to healthy controls

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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].

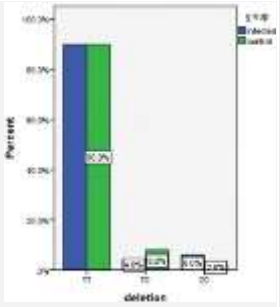

Figure 3: Frequency distribution of +295 deletion 12 within promoter region of interferon gamma receptor 1 in tuberculosis patients as compared to healthy controls

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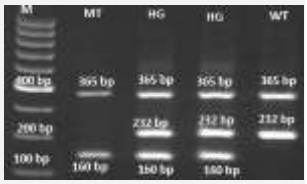

Figure 4: Polymerase chain reaction-CTPP amplification of allelic polymorphism at position +295 deletion 12 within interferon-gamma receptor 1. Lanes; MT, Homogenous mutant (+295CC); HG, heterogeneous mutant (+295TC); WT (wild type) represented by +295 TT genotype of healthy human controls; M, 100 bp DNA ladder

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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 +295T/C 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.[7] 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 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.[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 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.[5]Furthermore, Meyer *et al*. studied different variants within IFN-γR1 among Ghanaian populations. Their results did not reflect any significant findings among susceptible population, despite a large number of screened samples and the different genes studied.[19] Another study from Gambia yield similar findings.[20] Moreover, similar findings were suggested among Chinese population by Chen *et al.*[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-γ* R1 at −56C >T allele was significantly associated with twice fold reduction in the risk of susceptibility to TB in a similar population.[11] 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.[22]

Distribution of +295C allele deletion 12 within *IFN-γ* R1 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.[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.[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.[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-γ* R1 genetic disorders in association with increased susceptibility to pulmonary TB.[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-γ* R1 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.[23],[24]

Conclusion

This study was designed to determine the role of tow 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).

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