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The Role of IFN-ɣ Receptor-1 gene Polymorphism in Development of Pulmonary Tuberculosis among Sudanese Patients

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Abstract

Background: Tuberculosis (TB) is a killing infectious bacterial disease with socioeconomic and grave public health implications. Approximately two thirds of global population is infected with *M. tuberculosis* (Mtb) and only 10% of individuals develop clinical disease. Sudan is one of the few countries which suffer from high burden of disease accounting for 209 cases/100,000 population. A number of host genetic factors including gamma interferon influence disease susceptibility. The cytokine mediates immunity for control of progressive infection. Thus, mutations within gamma interferon receptor 1 (*IFN-γ*R1) result in increased susceptibility to pulmonary TB (PTB).

Objective: To determine the role of IFN-ɣ receptor-1 gene polymorphism in **d**evelopment of pulmonary tuberculosis among Sudanese patients.

Materials and methods: A total of 100 participants with active TB and 50 matched healthy controls were investigated for association of three genetic polymorphisms within IFN-γR1 gene and their risk of developing PTB. Polymerase chain reaction (PCR) assay and restriction fragment length polymorphism (PCR-RFLP) followed by gel electrophoresis were performed. Migrated IFN-γR1 DNA bands representing genotypes and polymorphic alleles were identified. Supportive immunoassay using ELISA was also carried out. Demographic factors which have probable role in triggering chances of the disease were also investigated.

Results: Molecular findings revealed that two genetic variants namely; -56C and +295C deletion 12 positioned in the promoter region of IFN-γR1 gene were non-significantly (p=0.771) and (p =0.343) respectively associated with increased risk of novel development of pulmonary tuberculosis (PTB). However, polymorphism at position +95C in the promoter region of IFN-γR1 gene was significantly (*P*-value =0.343) associated as a risk factor in developing TB.

Conclusion: The three genetic variants had a potential risk in association with active disease development among Sudanese patients.

Key words: IFN-ɣ receptor-1 gene polymorphism, Pulmonary tuberculosis, Sudanese patients

Introduction

Tuberculosis (TB) is a chronic infectious bacterial disease that threatens public health and responsible for killing nearly 2 million infected individuals a year together with a further figure of about 9 million fresh emerged active cases worldwide as estimated by the World Health Organization. It is caused by *Mycobacterium tuberculosis* (Mtb) which belongs to *Mycobacterium tuberculosis* complex (MTBC)[1].
Other members of the complex include; *M. bovis*, *M. africanum*; *M. microti, M. pinnipedii, M. caprae*, and *M. canetti.* These members have distinguished phenotypic properties and wide host range; though they are genetically and closely related species. Genomic sequence analysis of Mtb in comparison with *M. bovis* has shown minor differences mounting only to <0.05% . Although the latter species is primarily an animal pathogen, it also infects humans[2].

Transmission of TB occurs by inhalation of infected droplet aerosols released by an infected person through coughing or sneezing to a susceptible one in close contact. Following infection with Mtb, only a small proportion of the population gets the disease. While the remaining majority incubates infection in a latent granulomatous stage which reactivates under appropriate conditions. TB is mainly pulmonary affecting the lungs, and accounts for >85%. But, an extra-pulmonary form exists as exemplified by lymphadenopathy (inflammatory disease of lymph nodes) or TB meningitis. This extra-pulmonary form occurs due to weakening of immune response to clear infection or failure of the host to suppress bacterial dissemination to different parts of the body. Active TB is clinically exhibited by symptoms of fever, persistent cough with or without blood, weight loss and night sweating[3].

Pulmonary tuberculosis is an infectious chronic bacterial disease that cripples humans' health causing increased morbidity and mortality across the globe. Sudan is among the top 22 countries with high TB burden. Several risk factors including familial genetic make-up predispose susceptibility to the disease. IFN-γR1 protein release has a central key role in potentiating host immune responses against TB. A number of single-gene mutations within IFN- γR1 signaling pathway or changes in the promoter region have been identified and reported to associate with severity of mycobacterial disease. Yet, relevance of genetic polymorphism spanning such genes to the common phenotypic clinical characteristics of TB has remained uncertain.

In Sudan and with the exception of one scientific publication in which the authors linked a single *IFN-γR1* mutation to increased resistance of TB[4], there isn't any research work to date that associates any genetic defects in the development of pulmonary TB. The undergoing study was designed to investigate the effect of IFN-γR1 gene polymorphism or mutation in predisposing active pulmonary TB among Sudanese patients attending hospitals within Khartoum State. Additionally, other laboratory tests were carried out to permit support or early detection of *IFN-γR1* genetic defects in order to prevent further development of any complications.

Materials and methods

This was a qualitative, prospective, hospital-based, analytical study, descriptive, cross-sectional study. It was carried out at various hospitals in Khartoum State (Sudan). The study population was 100 randomly selected patients exhibiting clinical features of active pulmonary TB. 50 healthy individuals with respiratory infections other than TB were enrolled as controls. Inclusion criteria were patients with clinical signs of active pulmonary disease; and patients with positive ZN-stained sputum smears. Exclusion criteria were TB patients presenting with clinical signs of active pulmonary disease but with negative ZN-stained sputum smears. Study variables were demographic data (age, gender, tribe, past history of BCG vaccination), molecular sensitivity pattern, molecular genotypes, and sequencing.

A confidential written consent was obtained from all participants. Approval to conduct this research project was sought from the Ethical Committee of Federal Ministry of Health, Sudan. Collected data was analyzed by the statistical package of social sciences (SPSS) program. Chi-square (χ^2) test was used to analyze and evaluate these data.

Sampling was a non- probability purposive, convenience type where participants were chosen on the basis of accessibility. The sample frame was tuberculous patients. Sample size was 100 patients exhibiting clinical features of active pulmonary TB. Demographic data collected were age, gender, tribe, past history of BCG vaccination, and TB risk factors (smoking, alcoholism, other diseases, socioeconomic status). Such data was collected by a structural questionnaire. 100 sputum and 100 whole blood samples were collected before starting chemotherapy from patients enrolled in the study. Also 50 sputum and 50 blood samples were simultaneously collected from healthy controls. All reagents and primers were pre-tested using control strains; and equipment was calibrated.

Each patient was provided with a clean, disposable, screw capped plastic sputum container and instructed to expectorate sputum specimen by deep coughing in a well ventilated area. The specimens were immediately transferred to the National Tuberculosis Reference Laboratory (Khartoum) for further investigation.

ZN staining: Provisionally, ZN-stained sputum smears were prepared. Direct sputum smear was made on slide, fixed by gentle heating and stained by the standard ZN method.

Culture: Positive sputum smear samples were decontaminated for further processing. This was performed by adding 50 ml of sputum sample in a disposable plastic tube to an equal amount of 4% of sodium hydroxide, vortexed and allowed to stand at room temperature for 20 minutes. The specimen was neutralized with 1N HCl containing 0.1% phenol red as an indicator. The prepared samples were inoculated on three LJ slope media: two of them containing glycerol and the third one was supplemented with pyruvate. All cultural procedures were carried under biological safety cabinet and chemical agents were prepared following the standard methods.

Decontaminated sputum samples were further processed for inoculation on appropriate Lowenstein-Jensen (LJ) agar slope medium to isolate Mtb and identify the causative agent following standard procedures. Cultures were also inoculated and incubated in slope position for 3 days in tubes with loosen caps to evaporate the excess fluid. Bottle caps were loosely closed,

left on upright position and lately examined for contamination.
Re-examination of cultures was performed in first week for rapid growers and then every week until the eighth week for detection of slow growers. The result was recorded as negative if no growth appeared. Number of colonies was recorded as:
(+) If the number of colonies is less than 20
(++) If the number of colonies is 20-100
(+++) If the number of colonies is 100-200.
Positive cultures were maintained through alternative sub-culturing on LJ medium. Pure cultures were stored in 20% glycerol in distilled water at -20^{o}C for future processing.
Identification: Bacterial isolates were firstly identified depending on growth rate and colonial morphology. Identification was performed by pigment production, catalase test, nitrate reduction test, para-nitrobenzoic acid (PNB) susceptibility test, and thiophen-2-carboxylic acid hydrazide (TCH) susceptibility test.
Drug susceptibility testing (DST): Mc Farland turbidity solution was prepared by adding 1% anhydrous barium chloride to 1% sulphuric acid. Bacillary suspension was prepared by adding subculture colonies to 1 ml of distilled water containing glass beads, homogenized, vortexed, and opacity of the suspension was adjusted by addition of distilled water to match Mc Farland tube standard No.1turbidity.
Quality control for drug susceptibility testing was prepared by inoculating *M. tuberculosis* H37Rv sensitive strain to LJ slopes when performing drug susceptibility testing.
Drug-containing media for susceptibility testing were prepared by adding:
* 5 ml of isoniazid INH solution to 500 ml LJ medium to provide 0.2 ug/ml drug concentration.
* 2.5 ml of rifampicin RIF solution to 500 ml of LJ medium to provide 40ug/ml drug concentration.
* 5 ml of streptomycin SM solution to 500 ml of LJ medium to provide 8ug/ml drug concentration.
* 5 ml of ethambutol EMB solution to 500 ml LJ medium to provide 2ug/ml drug concentration.
100 µl of 10^{-2} and 10^{-4} bacillary suspension were inoculated separately onto two LJ slope media and left as controls. Suspension of mycobacterial isolate cultures containing 10^{-2} bacilli were also inoculated onto two LJ slope media containing separately INH, RIF, SM, and EMB. Cultures were incubated and examined after six weeks. If the percentage of colonies on drug-containing media were less than 1% compared with growth on control, the isolates were considered resistant. But if the proportion of growth was equal to or greater than 1% compared with growth on control, the isolates were considered sensitive.
Molecular investigation: Nine hundred µl Red Blood Cells (RBCs) Lysis Solution was pipetted into 1.5 ml microtube following instructions in the Kit (Jena Bioscience GmbH, Jena, Germany). 300 µl of the whole blood was added and mixed by inverting the tube 10 times. Contents in the tubes were incubated at room temperature with occasional inversion for 3 minutes (min.). The tubes were then centrifuged at 15,000 g revolution for 30 seconds (sec.). The supernatants were removed with pipettes leaving behind visible cell pellet representing host white immune cells.

Then, each tube was vigorously vortexed for 10 sec. to resuspend the white immune cells in the residual liquid. The immune cells pellet in each tube was completely resuspended. 300 µl cell lysis Solution were added to the re-suspended cells to extract genomic IFN-γR DNA.
Then 300 µl isopropanol were pipetted into a 1.5 ml microtube and the supernatant containing extracted DNA was added. The sample was gently mixed by inverting the tube for 1 min. Then, contents were centrifuged at 15,000 g for 1 min. The supernatant was discarded and tube containing the precipitated DNA was briefly drained on a clean absorbent paper. To wash the DNA, 500 µl washing buffer were added and the tube was inverted several times, and washing buffer discarded.
100 µl protein precipitation solution were added to the prepared cell lysate. Then, contents were thoroughly and vigorously vortexed for 20 sec. The tube was centrifuged as before for 1 min. The supernatant containing extracted IFN-γR1 DNA was pipetted and collected in a clean tube; while the precipitated sediment discarded.
A 170 bp DNA fragment was PCR-amplified using 100 ng of genomic DNA in a total reaction volume of 30 µl using the following primer pair:
IFNγR1 E1F (5`-CGGGGTTGGAGCCAGCGAC-`3)
IFNγR1 E1R (5`-CCTCCCTCCCTCTCGTCC-`3)
The PCR conditions were programmed as initial temperature 95ºC for 5 minutes, followed by 35 cycles of 94ºC as melting temperature for one minute, annealing temperature at 60ºC for one minute and 72ºC as an extension temperature, and a final prolongation step at 72ºC for 5 min. Polymerase chain reaction of *IFN-γR1* DNA products were analyzed on an agarose gel with a concentration of 1.5%. The agarose dissolution was left to become warm during which 2 µl of (10 mg/ml) ethidium bromide was added. Following complete agarose solidification, the gel was placed into an electrophoresis tank filled with 1X TAE buffer. Specific amounts of tested IFN-γR1 DNA samples were gently mixed with certain amounts of loading dye and contents were loaded into the formed wells. IFN-γR1 DNA bands were visualized under ultraviolet (UV) trans-illuminator and gel was documented using DNA gel documentation system. Agarose gel electrophoresis showed absolute absence of digestion of 170bp amplicon at position +95 promoter of IFN-γR1 gene extracted from immune cells of infected patients and healthy controls and amplified by PCR restriction fragment length polymorphism (PCR-RFLP) method (Fig. 1).
50 µl DNA Hydration Solution were added to the DNA pellet in the tube. Contents in the tube were vortexed and gently mixed at a medium speed for 5 sec. Samples containing the DNA were incubated at 65°C for 30 min. to accelerate rehydration. Purity of isolated genomic *IFN-γR1* DNA was checked by agarose gel electrophoresis and its concentration was determined by spectrophotometer.

Results

75% of TB participants were males while the remainder 25% were females. The average age of cases was 34 years old in comparison with 33 years old for controls. When infected cases were

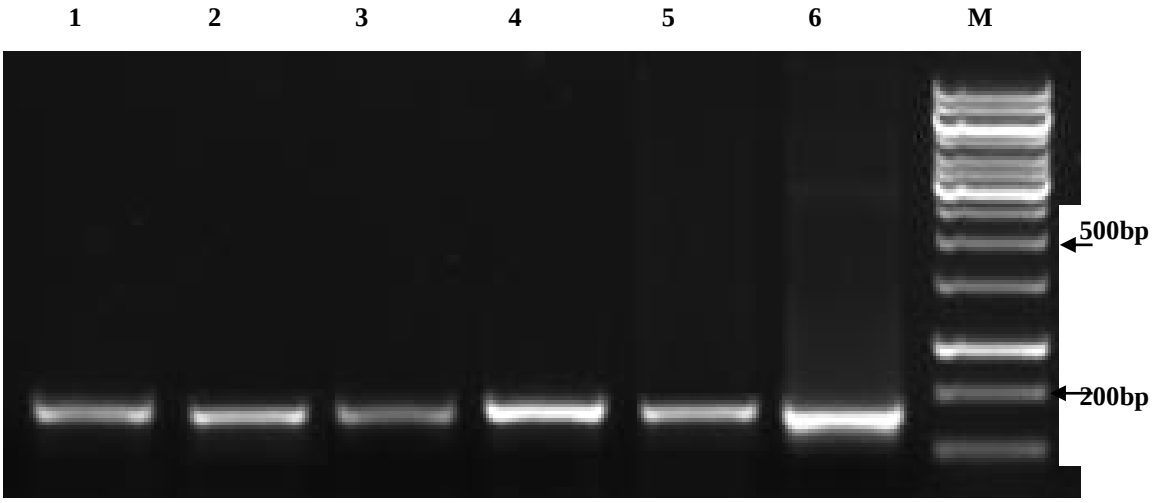

Fig. (1): Agarose gel electrophoresis bands of IFN-γR1 gene extracted from infected patients and healthy controls

grouped, the frequency of distribution was greater in the range from 20-30 years old accounting for 50%. In relation to frequency of BCG-vaccination against TB, were more in males than females. But, the correlation between vaccination and gender was statistically non-significant (*P*= 0.580). Non-significant correlation was also seen with associated disease and alcoholism; while a significant association (*P*=0.033) was observed in smoking.

Collected demographic characteristics and genotyping data in this study showed that the Tama, Nuba, Zagawah 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.

ZN stained positive sputum and culture smears showed rod-shaped acid fast bacilli. Following incubation, non-pigmented, dried and rough colonies were seen. Suspected organisms reacted positively to catalase enzyme. TB cultures on inoculation in nitrate broth reduced nitrate to nitrite. Para-Nitrobenzoic acid showed inability of tubercle bacillus to grow in its presence. When TB isolates were able to grow in the presence of thiophen 2-carboxylic acid hydrazide. Growth of TB isolates in media containing the drugs EMB and SM appeared morphologically similar to that of controls lacking the drugs. However, scarce growth of TB organisms were observed on media containing either RIF or INH indicating drug resistance.

Regarding genotyping, agarose gel electrophoresis showed absolute absence of digestion of 170 bp amplicon at position +95 promoter of IFN-γR1 gene extracted from immune cells of infected patients and healthy controls and amplified by PCR restriction fragment length polymorphism method. Significant difference (*P*=0.033) was shown between wild type +95TT and 95T/C polymorphism and the groups.

However, incomplete digestion of genomic *IFN-γR1* DNA at position -56 yielded three DNA fragments at molecular sizes of 285bp, 193bp and 92bp characteristics of -56T/C heterozygote. The latter polymorphic -56T/C alleles were harboured by 16 TB patients with 16% frequency of distribution. By contrast, polymorphic -56T/C alleles were carried by 6 individuals of healthy controls with 12% frequency of distribution.

With reference to polymorphic -56C alleles, they were carried by 7 TB patients with 7% frequency of distribution in comparison with only 3 individuals of healthy control group bearing the same alleles, and having 6% frequency of distribution. Therefore, frequency distribution of -56C alleles was more in TB cases than those in healthy controls. But, the difference in occurrence between the two groups was statistically non-significant (*P*-value =0.771).

The PCR-amplified products showed different DNA patterns after running electrophoresis. The pattern showed two DNA bands at molecular sizes of 365bp and 232bp representing WT type *IFN-γR1* homozygotic +295TT genotype at position of +295 del. 12. On the other hand, formation of two DNA bands at molecular levels of 365bp and 160bp represented homozygote polymorphic type +295CC alleles . Appearance of three DNA bands at molecular sizes of 365bp, 232bp and 160bp represented heterozygote +295T/C alleles at position 295 del. 12.

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. Similarly, frequency of distribution after addition of both +295C and +295T/C alleles was 10% in TB cases as well in healthy participants.

Although there were some differences in absorbance between test samples and controls, these differences were statistically insignificant. Thus, immunological findings did not link identified genetic alterations in *IFN-γR1* gene with development of pulmonary TB in this study (fig. 2).

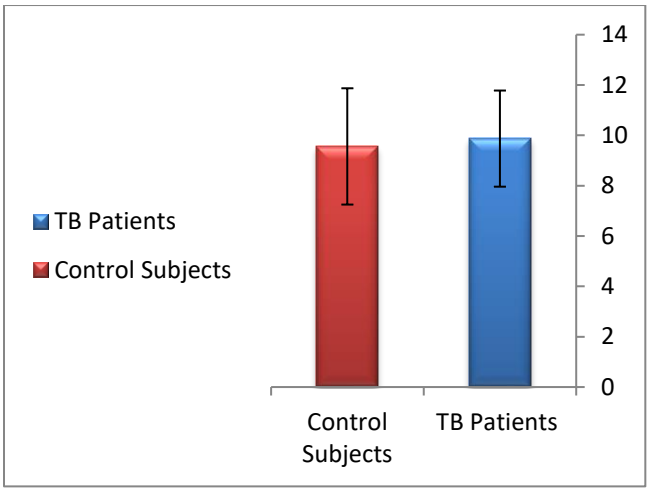

Fig. (2): Gamma interferon (IFN-γ) levels in sera of patients with pulmonary tuberculosis

Discussion

In this study, some of genotypic findings of the three 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[5]. As such, we have attempted to investigate their genetic defects and subsequent effects in triggering active TB disease among the Sudanese populations. The data from the present study showed that PCR-amplification of human genomic IFN-γR1 gene extracted from affected host immune cells followed by RFLP analysis has successfully identified DNA products resembling the three polymorphic alleles at positions; +95, -56, and +295 deletion 12 of the gene.

Also in this study, the lack of polymorphic *IFN-γ*R1 +95C/C and T/C alleles was surprising and difficult to explain. Frequency of +95TT genotype found in the promoter region of *IFN-γ*R1 was overrepresented in both TB patients and controls with 100% frequency of distribution, and significantly (*P*=0.033) indicative of susceptibility to the disease. In addition, +95CC genotype; and +95C/C and +95T/C variants have previously been demonstrated in association with PTB in several African populations[5].

Furthermore, distribution of -56C>T was seen in TB cases while -56C<T alleles was observed in the control subjects. However, this difference between the two groups was statistically insignificant (*P*-value =0.771). Accordingly, -56C allele is probably associated with risk of developing TB. These findings were in concordance with Cooke and his colleagues (2006)[5] who showed that the -56CC genotype was linked with risk of developing TB. The obtained statistical bias in the present study could probably be 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[6].

In the present context, distribution of +295C allele deletion 12 within *IFN-γ*R1 was found to be more frequent (6 cases) in TB patients than in healthy controls (only one case). Therefore, polymorphic +295C allele was more than T/C allele (+295C>T) suggesting non-significant (*P*-value=0.343) difference and indicating increased chances of developing TB. However, we could not find any evidence in literature to support these findings.

Collected demographic characteristics and genotyping data in this study showed that the Tama, Nuba, Zagawah 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 deletion 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 ancestral marriage practiced by most families within the tribe. The act of this ancestral marriage was reported to trigger and precipitate *IFN-γ*R1 genetic disorders in association with increased susceptibility to pulmonary TB[7].

The current study had encountered several limitations. Firstly, sample size was quite small and this had complicated the statistical analysis and the findings. Secondly, advanced molecular

facilities techniques and expertise personnel are unavailable in Sudan. Due to this, researchers had to travel abroad and pay extra costs. Thirdly, a couple of important laboratory tests were not performed as it was impossible to investigate fresh samples collected from participants enrolled in the study. Fourthly, some crucial molecular assays such as sequencing of *IFN-γ*R1 DNA products, which can verify our results could not be carried out. Lastly, the biased study findings were not unexpected since the current work relied wholly on the PCR-RFLP analysis which was probably an insensitive method to apply.

From this study, it may be recommended that more research work need to be carried out in this field using advanced molecular tools.

Conclusion: Polymorphisms have a potential link in increasing risk of developing TB among Sudanese patients.

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