A Correlation between Macrophage Migration Inhibitory Factor (MIF) Gene Promoter Polymorphism and Susceptibility to Active Pulmonary Tuberculosis in Yunnan Population, China

Aihua Liu

A Thesis Submitted in Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Microbiology
Prince of Songkla University
2018

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ABSTRACT

Tuberculosis (TB) is a infectious disease caused by the bacterium *Mycobacterium tuberculosis* (Mtb), which resulted in estimated 10.4 million new cases and 1.7 million deaths in 2016 and still is serious health problem in the word. Approximately 1/3 of the world's population carry the disease but don't have any symptoms, however approximately 10% of these people will likely develop active disease during their lifetime and able to transmit the bacterium, but the exact pathogenesis of TB is not clarified yet and is urgent to be studied. The MIF gene encoding macrophage migration inhibitory factor (MIF) has been proposed as candidate tuberculosis (TB) susceptibility gene and need to be deeply explored.

The aim of this study was to extensively investigate the potential association between the −173 G/C single-nucleotide polymorphisms and −794 CATT5–8 microsatellite polymorphism of the MIF gene and susceptibility to new cases and retreatment cases of TB, respectively, and to systematically evaluate the correlation between functional G/C SNP and CATT microsatellite polymorphisms in the MIF gene promoter regions and the susceptibility to new cases and retreatment cases of TB, which are one of the dominant drug-resistant TB pools.

In order to elucidate whether MIF gene variants are associated with susceptibility to retreatment cases of TB, and prevent drug-resistant TB prevalence, we conducted a study based on paired human population data. Genotypes of MIF

−173 G/C single nucleotide polymorphisms (SNP) and MIF −794 CATT5–8 microsatellite polymorphisms among 200 tuberculosis (TB) patients and 100 healthy controls were performed to study the relationships between G/C polymorphism at position −173 and TB or between CATT polymorphism at position −794 and TB in new patients and retreatment cases, respectively. Significant increases in MIF – 173 G/C and −794 CATT, genotype GC + CC (60.0% vs. 43.0%, OR = 0.503, 95% CI = 0.309–0.818, $p < 0.01$), genotypes 7/8 (6.0% vs. 1.0%, OR = 6.32, 95% CI = 0.81–49.31, $p < 0.05$) and allele CATT 8 (3.0% vs. 0.5%, OR = 6.15, 95% CI = 0.79–47.67, $p < 0.05$), were observed in TB patients compared with the controls. Moreover, significant differences in the genotypic frequencies of MIF −173 G/C (GG vs. GC+CC) were demonstrated upon comparing the total cases, the new cases of TB and the retreatment cases of TB with the controls (OR = 1.99, 95% CI = 1.22–3.24, $p < 0.01$, OR = 1.83, 95% CI = 1.05–3.21, $p < 0.05$, and OR = 2.16, 95% CI = 1.23–3.81, $p < 0.05$, respectively), and MIF −794 CATT (5/X + 6/X vs. 7/7 + 7/8) were also proved upon comparing the total cases and the new cases of TB with the controls (OR = 5.57, 95% CI = 1.32–25.03, $p < 0.05$, and OR = 7.32, 95% CI = 1.61–33.36, $p < 0.01$, respectively). Significant differences in the allelic frequencies of MIF −794 CATT (5 + 6 vs. 7 + 8) were observed in the total cases (OR = 0.53, CI = 0.33–0.86, $p < 0.05$) and new cases of TB (OR = 2.09, CI = 1.23–3.56, $p < 0.01$) compared with the controls. The results suggested that MIF −173 G/C genotype (GC + CC) was associated with the total cases, new cases of TB, and retreatment cases of TB, and MIF −794 CATT genotypes (7/7 + 7/8) and alleles (7 + 8) were associated with both the total cases and new cases of TB. However, types of MIF −173 G/C allele were not related to TB susceptibility. Significant increases in the MIF serum concentrations in TB cases with the MIF −173 genotypes GG and (GC + CC), −794 genotypes (5/5 + 5/6 + 6/6) and (7/X + 8/X) were detected, compared with the healthy controls ($p < 0.05$), respectively. Moreover, the MIF levels in the retreatment cases were higher than in the new cases ($p < 0.05$). In contrast, there were no difference in the MIF concentrations between the MIF 173 G/C genotype GG and (GC + CC), and −794 CATT genotype (5/5 + 5/6 + 6/6) and (7/X + 8/X) within each TB group ($p > 0.05$).

Our results not only further confirm previous conclusions by other authors from studies, but also give a deeper and more extensive insight into the

associations between MIF −173 G/C single nucleotide polymorphisms (SNP) and susceptibility to active TB and between MIF −794 CATT5–8 microsatellite polymorphisms and susceptibility to active TB.

Keywords: Macrophage migration inhibitory factor, Gene promoter, microsatellite polymorphism, tuberculosis, *Mycobacterium tuberculosis*.

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LIST OF ABBREVIATIONS AND SYMBOLS

95%CI	95% confidence intervals
Alu-Ⅰ	*Alu*-Ⅰ enzyme
Bp	Base pair
ddH_2O	Double distilled water
DNA	Deoxyribonucleicacid
dNTP	Dexyribonucleoside triphophata
EB	Ethidium Bromide
EDTA	Ethylebediamine tetraacetic acid
ELISA	Enzyme-linked immuno sorbent assay
HWE	Hardy-Weinberg Equilibrium
MIF	Macrophage migration inhibition factor
mM	Millimole
MT	*Mycobacterium tuberculosis*
OD	Optical density
OR	Odds ratio
PCR	Polymerase chain reaction
P value	Significance probability
Q-PCR	Real time Quantitative PCR
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleicacid
rpm	Revolutions per minute
SNP	Single nucleotide polymorphism
Taq	Thermus aquaticus DNA polymerase
TB	Tuberculosis
TE	Tris-Hcl，EDTA
Tris	Trisaminomethane
uM	Micromole
WHO	World Health Organization

LIST OF PAPERS

1. Liu, A., Li, J., Bao, F., Zhu, Z., Feng, S., Yang, J., Wang, L., Shi, M., Wen, X., Zhao, H., Voravuthikunchai, S. P. 2016. Single nucleotide polymorphisms in cytokine MIF gene promoter region are closely associated with human susceptibility to tuberculosis in southwestern province of China. *Infect Genet Evol* 39:219-224.

2. Liu, A., Bao, F., Voravuthikunchai, S. P. 2018. CATT polymorphisms in MIF gene promoter is closely related to human pulmonary tuberculosis in a southwestern China population. *International Journal of Immunopathology and Pharmacology* 32:1-7.

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CHAPTER 1

INTRODUCTION

Tuberculosis (TB) has been in existence for thousands of years, but It is still a major public health and social problem which is widely concerned in the world. TB is a major infectious disease in developing countries, so it is of great practical significance and profound social significance to investigate the prevention, treatment, and pathogenesis of TB.

1. Pulmonary tuberculosis and its epidemic

Pulmonary tuberculosis (lung tuberculosis) is the most common chronic and delayed infectious disease of tuberculosis, accounting for 80% of tuberculosis. According to WHO reports in 2016, about 20~30 billions of individuals in the world were infected by *Mycobacterium tuberculosis* (Mtb) in 2015, and 5~15% of the infected persons eventually developed to tuberculosis. The number of newly occurring patients with tuberculosis was 10.40 millions, and the number of deaths reached 1.40 million, TB has become one of the ten top leading killers in the world. Sixty percent of the world's TB cases originate from six high-burden countries, with China ranking third only after India and Indonesia (WHO, 2016). Among TB patients, 80% of the patients are in the countryside, and 75% of the patients are young and young adults. Tuberculosis is one of the main diseases in the rural and remote poverty areas (WHO, 2016).

In recent years, with the development of multi-drug resistant strains of Mtb and the increase of the AIDS epidemic, TB is resurgence. It is extremely important to effectively control and prevent the prevalence of TB. In order to fight against TB epidemic, the systematical strategies should be implemented, including

health education, drug development, and vaccine immunization. Among them, vaccine prevention is best cost-effective. Bacillus Calmette Guerin (BCG) is the first and only vaccine to prevent tuberculosis in human use, but its immune preventive effect is not very ideal (Colditz et al., 1994; Bishai and Mercer, 2001). Therefore, it is very necessary to develop the new TB vaccines, and there are several research progresses of new TB vaccine in recent years (Kwon et al., 2018; Gong et al., 2018).

2. The main pathological mechanism of tuberculosis

After *Mycobacterium tuberculosis* (Mtb) invades the human lungs, it is phagocytized by epithelial cells, dendritic cells and macrophages in lungs tissues (active Mtb can be latent in macrophages for a long time, even survives for decades) which are used as antigen presenting cell to process Mtb antigen. Mtb antigen peptides are expressed on the surface of these cells, thus activating the T cells. $CD4^+$ T cells and $CD8^+$ T cells jointly activate macrophage and make it together with T cells to promote the formation of tuberculous granuloma, which can keep Mtb in its core and inhibits the spread of Mtb. Therefore, the immune function of the body plays a major role in controlling tuberculosis (Chaurasiya, S.K., 2018).

3. TB and human immunity

The immunity of the human body to Mtb is mainly cellular immunity. The cells that play non specific immunity to Mtb are mainly macrophages, and the cells mediating specific immunity against Mtb are lymphocytes. However, the immune response caused by tuberculosis is a sharp "double-edged sword", the appropriate response can help the body to kill Mtb, but the improper one will cause tissue damage to the body. When any part of T cells, important cytokines, or macrophages has a malfunction, Human immunity to Mtb will decrease, and result in active TB. During cellular immunity of the body to Mtb, the immune cells mainly form phagocytic bodies to phagocytize and then kill Mtb by oxygen dependent killing effect. Under the phagocytosis, the respiratory outbreaks occur, producing reactive oxygen intermediates (H_2O_2) and active nitrogen intermediates (mainly nitric oxide,

NO), and these substances have strong oxidation killing effects and cytotoxicity which both paly strong killing effects on Mtb (Van Crevel *et al*., 2002; Korbel *et al*., 2008; Cooper, 2009). Therefore, cellular immunity plays an important role in the occurrence, development and prognosis of tuberculosis.

4. TB, cellular immunity and cytokines

Tb is a disease regulated strictly by cellular immunity. Cytokines (CKs) is some kinds of proteins and small molecular polypeptides, which is synthesized and secreted by activated immune cells or some non immune cells and can regulate cell physiological function, mediate inflammatory reaction, participate in immune response and cause tissue repair. At present, cytokines can be roughly divided into six categories: interleukins (ILs), interferons (IFNs), colony stimulating-factors (CSFs), tumor necrosis factors (TNFs), growth factor (CF) and chemokines. In recent years, with the progress of molecular immunology, the role of CKs in the immunological pathogenesis of tuberculosis has been extensively and deeply studied, and a variety of cytokines, such as IL-1 beta, IL-6, IL-8, IL-10, IL-12, IL-18, IL-31, TNF-a, IFN- gamma, etc., have been found to associated with TB (Monina and Khadera,2014; Domingo-Gonzalez, et al., 2017). The relationship between CKs and TB has become the focus of attention. The roles of CKs are complex and multifaceted. They play different roles in different conditions of the body, and can be regulated by many factors. Some cytokines in response to Mtb infection sometimes protect the host and sometimes are harmful to the organism (Cooper, 2008). Systematic and comprehensive understanding of the roles of cytokines is necessary for the exploration to pathogenesis and prevention of tuberculosis, and also provides a basis for the diagnosis and treatment of this disease.

5. Macrophage migration inhibitory factor

In 1966, Bloom and David formally named a cytokine secreted by activated T lymphocytes to inhibit macrophage / mononuclear cell migration as macrophage migration inhibitory factor (MIF) (Bloom, 1966; David and Bennettm,

1966). MIF is mainly produced by macrophages, in addition, T lymphocyte, monocyte, blood dendritic cell, B lymphocyte, neutrophils, eosinophil, rod-shaped cell and basophil can express MIF. The main biological effect of MIF is to inhibit the migration of macrophages, promote accumulation, infiltration, hyperplasia, activation of macrophage, secretion of inflammatory cytokines (such as IL-1, TNF-α, IL-2, IL-6, IL-8, IFN-γ, etc.), release of NO, induction of COX-2. MIF may aggravate the inflammatory damage while it exerts an immune function (Bloom *et al*., 2016). Therefore, MIF plays a pivotal role in the chemotaxis and inflammation process of macrophages.

The human MIF gene is a single copy gene, which is located on the chromosome 22q11.2, containing 3 exons and 2 introns (Bloom, 1966; David and Bennettm, 1966). The polymorphisms (microsatellite polymorphism and single nucleotide polymorphisms) of the MIF gene in 4 loci are proved by high display liquid chromatogram analysis technique: there is a G/C polymorphism at the position of -173nt, C/G polymorphism in +656nt, C/G polymorphism in +254nt and CATT repeat sequence in -794nt, which are related to the occurrence and development of some diseases （Donn et al., 2001; Barton et al.,2003; Donn et al., 2004; Hizawa et al.,2004; Renner et al., 2006). The study showed that the serum MIF expression in the individuals who carry MIF -173C allele increased (Donn et al., 2002).

6. MIF, macrophage and host susceptibility to tuberculosis

TB, as an important chronic inflammatory respiratory infectious disease, has long disease course, long treatment course and causes serious harm, which brings a heavy economic burden to both the state and the individual. In recent years, the incidence of tuberculosis continues to increase, has shown a comeback trend, drug-resistant tuberculosis patients are increasing, so tuberculosis has attracted a more worldwide attention. The pathogenesis of tuberculosis in general is that Mtb bacteria mainly infect macrophages in the body, and causes a large number of macrophages to activate and proliferate, to move, gather, infiltrate and secrete a variety of cytokines to the infected site, mediating strong immune response and

causing tissue damage (Marakalala *et al*., 2018; Simmons *et al.*, 2018). The function of the macrophages is directly influenced by the level of MIF expression, and the amount of MIF expression is regulated by the polymorphisms of the promoter of MIF gene. Therefore, it can be seen that MIF, macrophages and tuberculosis may interrelate.

The inflammation that tuberculosis bacilli invade the human body to induce is often chronic process and forms a characteristic granulomatous lesion, which is most closely related to the macrophage. The main pathological changes are exudation, proliferation and degeneration of lung tissue. Macrophages play the central role in three pathological processes (Korb *et al.*, 2016). It can be concluded that macrophages play an important role in the development of tuberculosis.

As mentioned earlier, only 10% of Mtb-infected people develop tuberculosis. In the general population, about 20% of people are naturally resistant to Mtb infection. There are many susceptible factors of tuberculosis, but epidemiological data and experimental studies show that genetic factors play an important role (Alcais *et al.*, 2005). In the last 10 years, several genes related to susceptibility to tuberculosis have been found because of the development of molecular biology and the implementation of the human genome project. These genes susceptible to tuberculosis includes MCP-1 gene coding monocyte chemoattractant protein 1 (MCP-1), CD209 gene (coding DC-SIGN), SP110 gene, etc (Barreiro et al., 2006; Flores-Villianueva et al., 2005; Tosh et al., 2006; Vannberg et al., 2008).Therefore, TB can be seen as a multi gene genetic susceptibility disease (Abel *et al*., 2018). However, up to now, there are not many reports about the association between polymorphisms in the promoter region of MIF gene and host susceptibility to tuberculosis.

In vitro experiments have shown that human MIF can inhibit the propagation of Mtb in macrophages, suggesting that MIF plays an important role in anti-tuberculosis immunity. The study showed that the copy number of -794 CATT repeat sequence of the MIF gene promoter region was significantly different in the population. The number of CATT copies had a regulatory effect on the activity of the

MIF promoter, the more copies, the stronger the promoter activity and the higher the expression of MIF. Therefore, we hypothesized that the decreased MIF production caused by the low copy number of CATT may decrease the resistance to Mtb infection and increase the susceptibility to Mtb. In order to verify this hypothesis, we intend to study MIF at three different levels (DNA- mRNA-protein) to reveal the relationships among -794 CATT microsatellite polymorphism of the MIF promoter region, the single nucleotide polymorphisms of the -173 G/C locus, the expression level of MIF mRNA in the peripheral blood white cells, the protein concentration of MIF in the plasma and host susceptibility to the tuberculosis in the population, and to provides theoretical and practical basis for the pathogenesis and prevention of tuberculosis. Therefore, the study of the correlation between MIF gene polymorphism and susceptibility to tuberculosis has important theoretical significance and potential application value for tuberculosis prevention and treatment.

Objectives

1. To probe the relationship between human MIF -173G/C single nucleotide polymorphism and susceptibility of new cases of TB and retreatment cases of TB in Yunnan

2. To explore the relationship between human MIF -794 CATT 5-8 microsatellite polymorphism and susceptibility of new cases of TB and retreatment cases of TB in Yunnan

CHAPTER 2

MATERIALS AND METHODS

1. Study Population

The paired data study includes 200 TB patients-TB group, who are hospitalized at the Third Hospital of Kunming. Diagnosis of tuberculosis is made by positive results for acid-fast bacilli (AFB) in sputum samples and chest x-ray examination, patients with negative sputum culture results but positive by the purified protein derivative test (PPD test), x-ray examination, and clinical manifestations consistent with the diagnostic criteria of TB are confirmed as sputum culture negative TB. All patients respond to ant-imycobacterial treatment and are followed up.

According to WHO definitions of TB cases in Global Tuberculosis Report 2013, The cases of pulmonary TB group will be further categorized into sub-groups: new case of TB group and retreatment case of TB group, each group are composed of 100 patients.

The paired data study also includes 100 healthy control group, unrelated controls will be recruited from the physical examination population in CDC of Kunming City. The control group is chosen based on no history or clinical evidence of tuberculosis.

All subjects sign informed consent forms voluntarily and the research is approved by the Medical Ethics Commission of Kunming Medical University.

2. DNA level

2.1 Genomic DNA extraction

Peripheral blood samples anti-coagulated (EDTA) are used for genomic DNA extraction. A commercially-available Blood DNA Extraction Kit is

used according to the manufacturer's instructions. The extracted genomic DNA was stored at -20°C until further use.

2.2 MIF -173G/C genotyping

A 365 base pair (bp) fragment of the MIF gene was amplified by polymerase chain reaction (PCR) using genomic DNA as template. The forward primer was 5′-ACTAAGAAAGACCCGAGGG-3′, and the reverse primer was 5′-GGGGCACGTTGGTGTTTAC-3′. For MIF -173 genotyping, a 50 μL reaction mixture containing the reaction buffer, dNTPs, primers, Taq polymerase, and 100ng DNA was amplified in an iCycle PCR machine in the following conditions: denaturation at 95 °C for 10 min, followed by 35 amplification cycles of 95 °C for 45 s, 56.6 °C for 45 s, and 72 °C for 45 s, and a final extension at 72 °C for 7 min. The PCR products were digested with *AluI* restriction endonuclease for four hours at 37 °C, and then separated on 2.0% agarose gels stained with ethidium bromide and visualized using ultraviolet light. The 330 bp PCR products had a consistent *AluI* digestion pattern that corresponded with the -173 SNP. *AluI* digestion of MIF sequences with the GG genotype resulted in two fragments: 97 bp and 268 bp. The CC genotype comprised a second *AluI* cutting site, resulting in three fragments: 206 bp, 62 bp, and 97 bp. Likewise, the heterozygous GC genotype produced four bands upon *AluI* digestion: 268 bp, 206 bp, 62 bp, and 97 bp.

2.3 Genotyping MIF -794CATT5–8

A 346 bp fragment containing the microsatellite repeat sequence was amplified by PCR. The forward primer was 5′- TGCAGGAACCAATACCCATAGG-3′, and the reverse primer was 5′- AATGGTAAACTCGGGGAC-3′. The annealing temperature was 53.8 °C and all other amplification conditions were the same as described above. The PCR products were purified and sequenced using the same primers from PCR.

3. Protein level

Enzyme linked immunosorbent assay (ELISA) is used to quantify the concentration of MIF in serum samples. In brief, the purified anti-cytokine capture antibody is diluted to 1-4 µg/ml in binding solution. 100 µl of diluted antibody is added to the wells of an enhanced protein-binding ELISA plate. Seal plate to prevent evaporation. Incubate overnight at 4°C. Bring the plate to room temperature (RT), remove the capture antibody solution, and block non-specific binding by adding 200 µl of blocking buffer per well. Seal plate and incubate at RT for 1-2 hr. Wash 3 times with PBS/tween. Add standards and samples (diluted in blocking buffer/tween) at 100 µl per well. Seal the plate and incubate it for 2-4 hrs at RT or overnight at 4°C. Wash ≥ 4 times with PBS/tween. Dilute the biotinylated anti-cytokine detection antibody to 0.5-2 µg/ml in blocking buffer/tween. Add 100 µl of diluted antibody to each well. Seal the plate and incubate it for 1 hr at RT. Wash 4 times with PBS/tween. Dilute the streptavidin-Horseradish Peroxidase (HRP) or other enzyme conjugate to its pre-titered optimal concentration in blocking buffer/tween. Add 100 µl per well. Seal the plate and incubate it at RT for 30 min. Wash 5 times with PBS/tween use 3, 3', 5, 5'-tetramethylbenzidine (TMB) solution as a substrate according to the manufacturer's instruction. Thaw TMP substrate solution within 20 min of use. Add 100 µl of 3% H2O2 per 11 ml of substrate and vortex. Immediately dispense 100 µl into each well. Incubate at RT (5-10 min) for color development. Read the optical density (OD) for each well with a microplate reader set to 405 nm.

4. Statistical analysis

Hardy–Weinberg equilibrium was examined in both TB patients and healthy controls. The sequencing results were analyzed by Primer 6.0 software (http://www.primer-e.com). Chi-square was calculated with SPSS17.0 software to compare the allele frequencies between the tuberculosis patients and control-group individuals. Odds ratio (OR) with 95% confidence interval (CI) were calculated using SPSS 17.0 software (SPSS, USA). P-values < 0.05 were considered statistically significant.

CHAPTER 3

RESULTS

The paired data study included 200 cases of pulmonary TB who were hospitalized at the Third Hospital of Kunming, Yunnan, China. Diagnosis of pulmonary TB was made by positive results for acid-fast bacilli in sputum samples and chest x-ray examination. For the cases with negative sputum culture results but positive by the purified protein derivative test, also with consistent x-ray examination and clinical manifestations according with the diagnostic criteria of TB, diagnosis were confirmed as sputum culture negative TB. All cases were responded to anti-mycobacterial treatment and followed up. According to World Health Organization definitions of TB cases in Global Tuberculosis Report 2013, cases of pulmonary TB were further categorized into new cases of TB group and retreatment cases of TB group. Each sub-group is composed of 100 cases. The paired data study also included 100 healthy, unrelated controls who were recruited from people for the physical examination in Kunming CDC, Yunnan, China. The control group was chosen based on no history or clinical evidence of tuberculosis or other diseases (Table 1). All subjects signed informed consent forms voluntarily and the research project was approved by the Medical Ethics Commission of Kunming Medical University.

Table 1. Descriptive characteristics in the Chinese paired data study.

Cases (numbers)	Male	Female	OR (95% CI)	*P* value	Age (Mean±SD)	*P* value
Total cases of TB (200)	133	67	3.38 (2.05-5.58)	< 0.0001**	40.56 ± 17.39	0.30
New cases (100)	58	42	2.35 (1.33-4.15)	0.005**	40.64 ± 20.24	0.37
Retreatment cases (100)	75	25	5.11 (2.78-9.38)	< 0.0001**	40.52 ± 15.70	0.30
			0.46 (0.25-0.84)	0.01* [a]		0.96[a]
Controls（100）	37	63			38.65 ± 9.15	0.15

OR, odd ratio; 95% CI, 95% confidence intervals.

*$P < 0.05$,

**$P < 0.01$.

[a] For comparison between new cases of TB *vs.* retreatment cases of TB.

1. MIF −173 G/C single-nucleotide polymorphisms

A. *MIF −173 G/C genotyping is confirmed by restriction fragment length polymorphism (RFLP)*

We investigated the distribution of MIF −173 G/C polymorphisms in TB cases and controls by digesting the polymerase chain reaction (PCR) products and analyzing the fragments by electrophoresis. The sequence patterns of MIF −173 single nucleotide polymorphisms were showed in Figure 1.

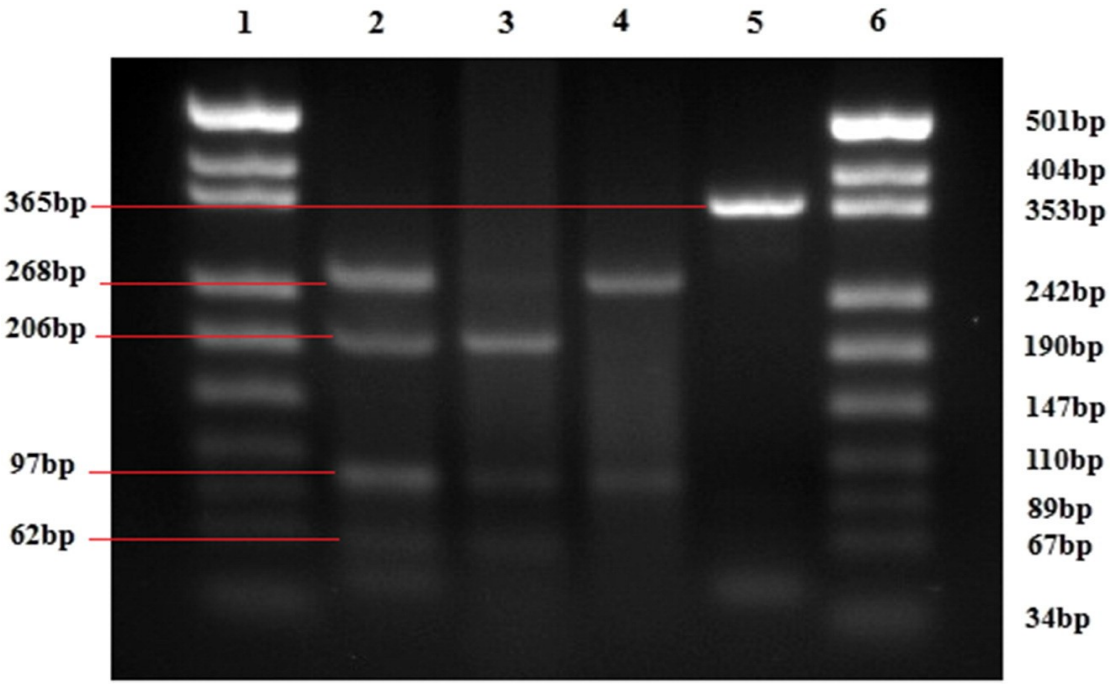

Figure 1. *AluI* digestion patterns of MIF -173 polymorphism. Lanes: (1, 6) Marker, (2) Genotype GC, (3) Genotype CC, (4) Genotype GG, and (5) MIF amplicon was 346 bp.

B. ***MIF −173 (GG vs. GC + CC) genotype polymorphisms are highly distributed in total cases of TB, but allele of −173 (C vs. G) polymorphism is not associated with TB***

Both the genotype and allele distribution of total cases of TB and controls were in Hardy–Weinberg equilibrium. Genotype distribution of MIF −173 G/C promoter polymorphisms in total cases of TB and controls was shown in (Table 2). Statistically significant difference in distribution for MIF −173 (GG vs. GC + CC) between total cases of TB and the controls was identified. The distribution of MIF −173 genotypes (GC + CC) was significantly higher in total cases of TB than in the controls (60% vs. 43%, $R = 0.503$ and $P < 0.01$). In contrast, no significant difference was observed in MIF −173 C alleles in total cases of TB, when compared with the controls (31% vs. 24.5%, $R = 0.722$ and $P > 0.05$).

Table 2. Comparison of Genotype and allele distribution of MIF -173 G/C polymorphisms between total cases of TB and controls.

MIF -173	Total cases of TB, n=200 (%)	Controls, n=100 (%)	OR (95% CI)	χ^2	*P* value
Genotype					
GG	80 (40.0)	57 (57.0)	1.00 (reference)		
GC+CC	116 + 4 (60)	37 + 6 (43.0)	0.503 (0.309-0.818)	7.765	0.005**
Allele					
G	276 (69.0)	151 (75.5)	1.00 (reference)		
C	124 (31.0)	49 (24.5)	0.722 (0.491-1.062)	2.745	0.098

OR, odd ratio; 95% CI, 95% confidence intervals.

*$P < 0.01$.

C. ***MIF −173 G/C genotypes (GG vs. GC + CC) are associated with new cases of TB, retreatment cases of TB, and total cases of TB***

In order to determine whether genotypic frequencies of MIF −173 G/C genotypes were associated with specific TB subgroups, genotypic frequencies of −173 G/C genotypes (GG vs. GC + CC) were calculated in total cases of TB (40% vs. 60%), new cases of TB (42% vs. 58%), retreatment cases of TB (38% vs. 62%), and controls (57% vs. 43%), the statistically significant differences of frequencies for MIF −173 (GG vs. GC + CC) were demonstrated when comparing total cases of TB (R = 1.99 and P < 0.01), new cases of TB (R = 1.83 and P b 0.05), and retreatment cases of TB (R = 2.16 and P < 0.05) to controls, respectively. In contrast, the frequencies of MIF −173 (GG vs. GC + CC) revealed no difference between new cases of TB and retreatment cases of TB (Table 3).

Table 3. Comparison of genotypic frequencies of MIF -173 G/C genotypes (GG vs. GC+CC) between tuberculosis cases and controls.

Cases	GG (%)	GC+CC (%)	OR (95% CI)	*P* value
Total cases of TB	80 (40.0)	120 (60.0)	1.99 (1.22-3.24)	0.007**
New cases	42 (42.0)	58 (58.0)	1.83 (1.05-3.21)	0.047*
Retreatment cases	38 (38.0)	62 (62.0)	2.16 (1.23-3.81)	0.011**
			0.96 (0.55-1.69)	1.000[a]
Controls	57 (57.0)	43 (43.0)	1.00 (reference)	

OR, odd ratio; 95% CI, 95% confidence intervals.

*$P < 0.05$,

**$P < 0.01$.

[a] For comparison between new cases of TB vs. retreatment cases of TB.

D. ***Allelic frequencies of MIF −173 G/C alleles (G vs. C) have no correlation with TB***

To determine whether allelic frequencies of MIF −173 G/C alleles (G vs. C) play a role on genetic susceptibility to TB, allelic frequencies of MIF −173 G/C alleles (G vs. C) were analyzed in total cases of TB, new cases of TB, retreatment cases of TB, and controls, and discovered no difference among these groups ($P > 0.05$) (Table 4).

Table 4. Comparison of allelic frequencies of MIF -173 G/C alleles (G *vs.* C) between tuberculosis cases and controls.

Cases	G (%)	C (%)	OR (95% CI)	*P* value
Total cases of TB	279 (69.0)	124 (31.0)	1.39 (0.94-2.04)	0.098
New cases	140 (70.0)	60 (30.0)	1.32 (0.85-2.06)	0.217
Retreatment cases	136 (68.0)	64 (32.0)	1.45 (0.94-2.25)	0.120
			1.10 (0.72-1.68)	0.746[a]
Controls	151 (75.57)	49 (24.5)	1.00 (reference)	

OR, odd ratio; 95% CI, 95% confidence intervals.

[a] For comparison between new cases of TB *vs.* retreatment cases of TB.

E. ***Serum MIF concentrations increased in TB cases, MIF expression in retreatment cases of TB is higher than that in new cases of TB***

To investigate the change of MIF concentration in TB cases, we compared the serum MIF concentrations of TB groups to controls. We found that MIF was significantly higher in total cases of TB (9.67 ± 7.24 ng/mL), new cases of TB (8.20 ± 4.62 ng/mL), and retreatment cases of TB (11.20 ± 8.97 ng/mL) than in controls (4.58 ± 1.89 ng/mL. $P < 0.01$). Similarly, MIF was significantly higher in retreatment cases of TB than in new cases of TB ($P < 0.01$) (Figure 2).

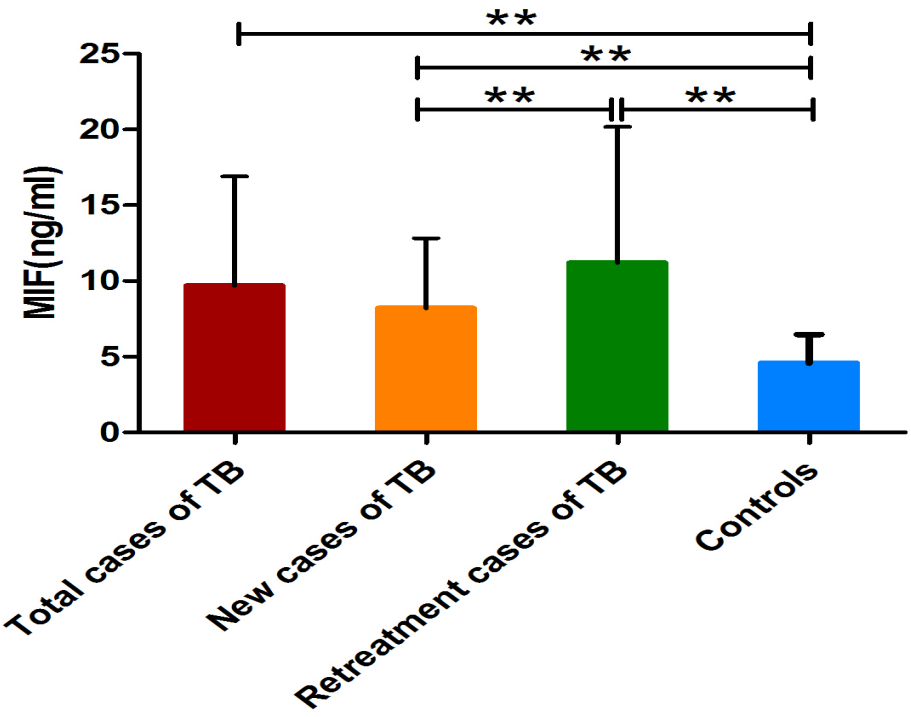

Figure 2. Comparison of serum macrophage migration inhibitory factor (MIF) concentration between tuberculosis cases and controls. $^{**}P < 0.01$.

F. ***MIF expression In TB cases with MIF −173 G/C genotype GG is highly increased. Similarly, MIF concentrations in retreatment cases of TB are higher than new cases of TB***

To figure out whether specified MIF −173 G/C genotype is related to the increased concentration of TB cases, we detected MIF concentration in different subgroups with MIF −173 G/C genotype GG. The results showed that new cases of TB (7.52 ± 4.15 ng/mL) and retreatment cases of TB (12.61 ± 7.24 ng/mL) with MIF −173 G/C genotype GG have a increased MIF when compared with the controls (4.46 ± 1.99 ng/mL). Interestingly, serum in retreatment cases of TB is significantly higher than in new cases of TB ($P < 0.01$) (Figure 3).

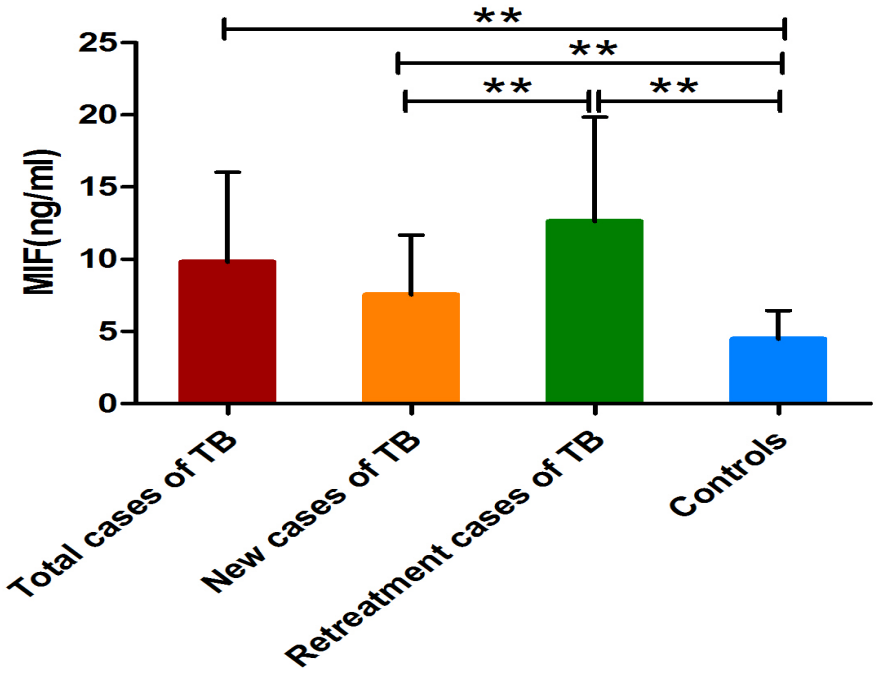

Figure 3. Comparison of serum macrophage migration inhibitory factor (MIF) concentration in MIF -173 G/C genotype GG between tuberculosis cases and controls. $^{**}P < 0.01$.

G. ***MIF expression in TB cases with MIF −173 G/C genotype (GC + CC) is highly increased. Furthermore, concentrations in retreatment cases of TB are higher than new cases of TB***

We quantified MIF concentration in different subgroups of MIF −173 G/C genotype (GC + CC) cases. The results indicated that MIF expression in new cases of TB (8.67 ± 4.91 ng/mL) and retreatment cases of TB (10.45 ± 9.75 ng/mL) with MIF −173 G/C genotype (GC + CC) increased when compared with the controls (4.61 ± 1.79 ng/mL) ($P < 0.01$). In addition, serum concentration in retreatment cases of TB is higher than that in new cases of TB ($P < 0.05$) (Figure 4).

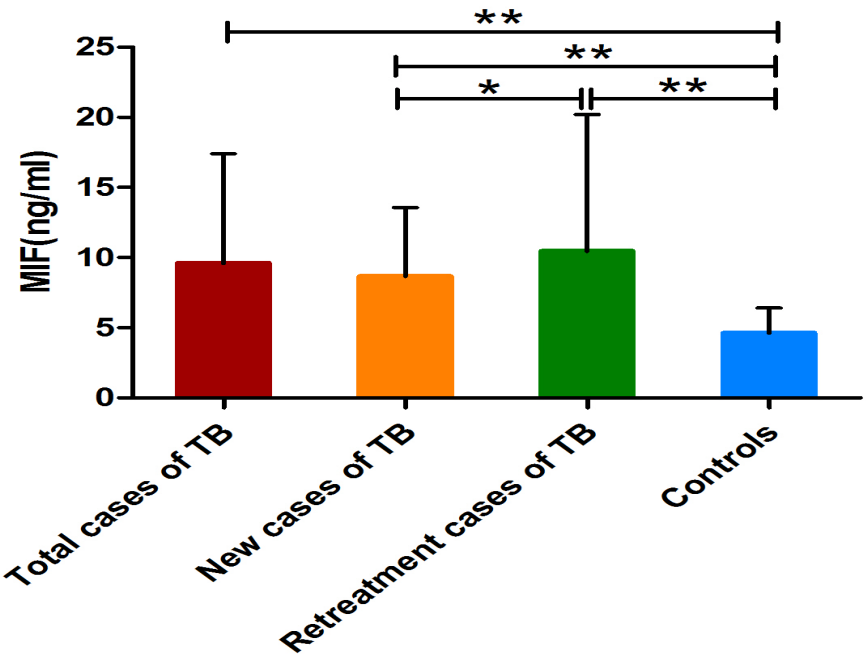

Figure 4. Comparison of serum macrophage migration inhibitory factor (MIF) concentration in MIF -173 G/C genotype (GC+CC) between tuberculosis cases and controls. *$P < 0.05$; **$P < 0.01$.

H. ***There is no difference of MIF expression in MIF −173 G/C genotype GG cases of TB and genotype (GC + CC) cases of TB within each same group***

In order to confirm that if MIF −173 G/C genotype plays a role on MIF production in TB cases, we studied serum MIF concentrations in MIF −173 G/C genotype GG cases and genotype (GC + CC) cases within each same group respectively, and found that there was no significant difference between MIF of genotype GG cases and that of genotype (GC + CC) cases within each same group (Figure 5).

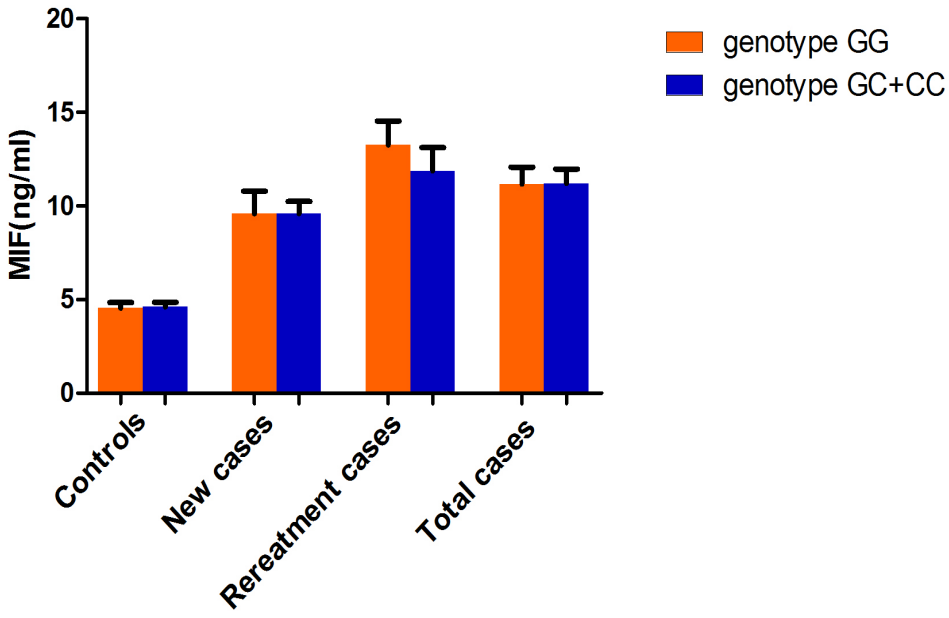

Figure 5. Comparison of serum macrophage migration inhibitory factor (MIF) concentration in MIF -173 G/C genotype (GC+CC) tuberculosis cases, genotype GG tuberculosis cases, and controls within each same group.

2. MIF −794 $CATT_{5–8}$ microsatellite polymorphism

A. *Genotype and Allele Distributions of MIF −794 CATT Polymorphisms*

Both the genotype and allele distributions of MIF −794 CATT microsatellite polymorphisms in the total cases of TB and healthy controls were in accordance with the Hardy–Weinberg equilibrium (Table 5). Statistically significant differences were observed in the distributions of both MIF genotypes −794 CATT 5/5 and CATT 7/8 between the TB patients and healthy controls. The distribution of MIF allele −794 CATT genotype 5/5 was significantly lower in the TB patients than in the controls (10.0% vs. 23%, OR = 0.37, 95% CI = 0.19–0.72, $p < 0.01$). Moreover, a significant decrease in MIF allele −794 CATT 5 was observed in the TB patients compared with the controls (31.5% vs. 43%, OR = 0.61, 95% CI = 0.43–0.87, $p < 0.01$). In contrast, significant increases in MIF −794 CATT, for both genotypes 7/8 (6.0% vs. 1.0%, OR = 6.32, 95% CI = 0.81–49.31, $p < 0.05$) and alleles CATT 8 (3.0% vs. 0.5%, OR = 6.15, 95% CI = 0.79–47.67, $p < 0.05$), were observed in the TB patients compared with the controls.

Table 5. Comparison of genotypic and allelic distributions of MIF -794 CATT polymorphisms between TB patients and controls.

MIF -794	Tuberculosis, (%)	Controls, n (%)	OR (95% CI)	χ^2	*P* value
Genotype					
CATT 5/5	20 (10.0)	23 (23.0)	0.372 (0.193-0.717)	9.176	0.002**
CATT 5/6	74 (37.0)	34 (34.0)	1.140 (0.689-1.886)	0.260	0.610
CATT 5/7	12 (6.0)	6 (6.0)	1.000 (0.364-2.748)	0.000	1.000
CATT 6/6	42 (21.0)	20 (20.0)	1.063 (0.586-1.931)	0.041	0.840
CATT 6/7	31 (15.5)	15 (15.0)	1.039 (0.532-2.030)	0.013	0.910
CATT 7/7	9 (4.5)	1 (1.0)	4.665 (0.583-37.35)	2.534	0.111
CATT 7/8	12 (6.0)	1 (1.0)	6.319 (0.810-49.31)	4.020	0.045*
Allele					
CATT 5	126 (31.5)	86 (43.0)	0.610 (0.429-0.865)	7.717	0.005**
CATT 6	189 (47.25)	89 (44.5)	1.117 (0.794-1.571)	0.406	0.524
CATT 7	73 (18.25)	24 (12.0)	1.637 (0.997-2.689)	3.843	0.050
CATT 8	12 (3.0)	1 (0.5)	6.155 (0.795-47.67)	3.931	0.047*

OR, odd ratio; 95% CI, 95% confidence intervals.

*$P < 0.05$,

**$P < 0.01$.

B. ***Genotypic Frequencies of MIF −794 CATT Genotypes (5/X + 6/X vs. 7/7 + 7/8) in Total Cases, New Cases, and Retreatment Cases of TB***

In order to determine the genotypic frequencies of the MIF −794 CATT genotypes in association with specific TB subgroups, the genotypic frequencies of the −794 CATT genotypes (5/X + 6/X vs. 7/7 + 7/8) were calculated for the total cases (89.5% vs. 10.5%), new cases (87.0% vs. 13.0%), and retreatment cases of TB (92.0% vs. 8.0%), and the controls (98.0% vs. 2.0%). Significant differences in the genotypic frequencies of MIF −794 CATT (5/X + 6/X vs. 7/7 + 7/8) were demonstrated when comparing the total cases and new cases of TB with the controls (OR = 5.57, 95% CI = 1.32–25.03, $p < 0.05$, and OR = 7.32, 95% CI = 1.61–33.36, $p < 0.01$, respectively). In contrast, no differences in the genotypic frequencies of the MIF −794 CATT (5/X + 6/X vs. 7/7 + 7/8) were observed between the retreatment cases and the controls or between the new cases and retreatment cases (Table 6). These results suggested that the MIF −794 CATT genotypes (7/7 + 7/8) were associated with new cases of TB and total cases of TB.

Table 6. Comparison of genotypic frequencies of MIF -794 CATT genotypes (5/X+6/X vs.7/7+7/8) between tuberculosis cases and controls.

Cases	5/X + 6/X (%)	7/7 + 7/8 (%)	OR (95% CI)	*P* value
Total cases of TB	179 (89.5)	21 (10.5)	5.57 (1.32-25.03)	0.01*
New cases	87 (87.0)	13 (13.0)	7.32 (1.61-33.36)	0.005**
Retreatment cases	92 (92.0)	8 (8.0)	4.26 (0.88-20.59)	0.101
			0.58 (0.23-1.47)	0.375[a]
Controls	98 (98.0)	2 (2.0)	1.00 (reference)	

OR, odd ratio; 95% CI, 95% confidence intervals.

*$P < 0.05$,

**$P < 0.01$.

[a] For comparison between new cases of TB vs. retreatment cases of TB.

C. ***Allelic Frequencies of MIF −794 CATT Alleles (5 + 6 vs. 7 + 8) in New Cases, Retreatment Cases, and Total Cases of TB, and Controls***

To determine whether the allelic frequencies of the MIF −794 CATT alleles (5 + 6 vs. 7 + 8) play a role in genetic susceptibility to TB, the allelic frequencies of the MIF −794 CATT alleles (5 + 6 vs. 7 + 8) were analyzed for the total cases (78.7% vs. 21.3%), new cases (77.0% vs. 23.0%), and retreatment cases of TB (80.5% vs. 19.5%), and the controls (87.5% vs. 12.5%). Significant differences in the allelic frequencies of MIF −794 CATT (5 + 6 vs. 7 + 8) were observed for the total cases of TB (OR = 0.53, CI = 0.33–0.86, $p < 0.05$) and new cases of TB (OR = 2.09, CI = 1.23–3.56, $p < 0.01$) compared with the controls. In contrast, the allelic frequencies of MIF −794 CATT (5 + 6 vs. 7 + 8) revealed no differences between the retreatment cases and the controls or between the new cases and the retreatment cases. These results suggested that the MIF −794 CATT alleles (7 + 8) were associated with both total cases and new cases of TB (Table 7).

Table 7. Comparison of allelic frequencies of MIF -794 CATT alleles (5+6 vs.7+8) between tuberculosis cases and control.

Cases	5 + 6 (%)	7 + 8 (%)	OR (95% CI)	*P* value
Total cases of TB	315 (78.7)	85 (21.3)	0.53 (0.33-0.86)	0.01*
New cases	154 (77.0)	46 (23.0)	2.09 (1.23-3.56)	0.009**
Retreatment cases	161 (80.5)	39 (19.5)	0.59 (0.34-1.02)	0.076
			1.23 (0.76-1.99)	0.463[a]
Controls	175 (87.5)	25 (12.5)	1.00 (reference)	

OR, odd ratio; 95% CI, 95% confidence intervals.

*$P < 0.05$,

**$P < 0.01$.

[a] For comparison between new cases of TB vs. retreatment cases of TB.

D. ***Serum MIF Levels in TB Cases with MIF −794 Genotypes (5/5 + 5/6 + 6/6) and (7/X + 8/X)***

To investigate whether the MIF −794 CATT genotype is related to the increase in serum MIF concentrations in TB cases, we quantified the MIF concentrations in different subgroups with the MIF −794 CATT genotypes (5/5 + 5/6 + 6/6) and (7/X + 8/X). The results demonstrated an increase in MIF concentrations for the new cases (8.38 ± 4.25 ng/mL) and retreatment cases of TB (10.85 ± 8.54 ng/mL) in TB patients with the MIF −794 CATT genotype (5/5 + 5/6 + 6/6), compared with the controls (4.50 ± 1.98 ng/mL). Furthermore, the serum MIF levels were significantly higher for the retreatment cases than for the new cases ($p < 0.05$) (Figure 6). Similarly, a significant increase in serum MIF concentrations was observed in TB patients with the MIF −794 CATT genotype (7/X + 8/X), with serum MIF concentrations of 9.78 ± 8.06 ng/mL and 11.92 ± 9.95 ng/mL being detected for the total cases and retreatment cases, respectively, compared with the controls (4.80 ± 1.59 ng/mL) ($p < 0.01$; Figure 7). In addition, the serum MIF concentrations for the retreatment cases were significantly higher than those for the new cases ($p < 0.05$). In contrast, no significant increase in MIF expression was observed in the new cases of TB (7.84 ± 5.32 ng/mL), compared with the control group ($p > 0.05$).

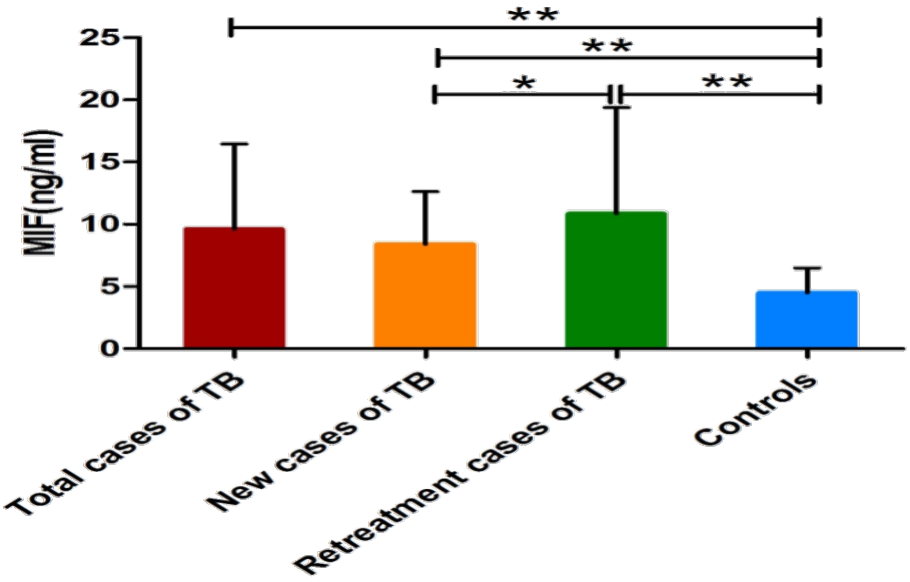

Figure 6. Comparison of serum MIF concentration in MIF -794CATT genotype (5/5+5/6+6/6) between tuberculosis cases and controls. *$P < 0.05$; **$P < 0.01$.

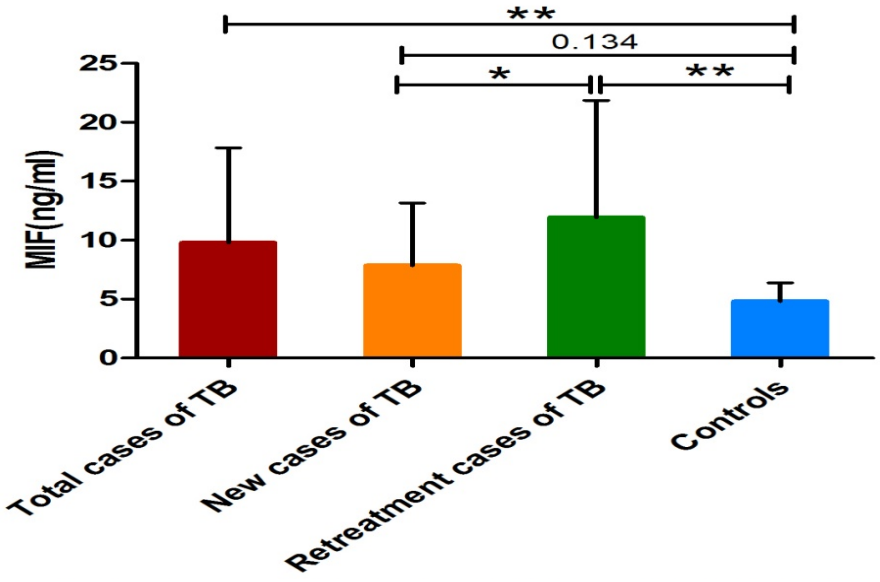

Figure 7. Comparison of serum MIF concentration in MIF -794 CATT genotype (7/X+8/X) between tuberculosis cases and controls. *$P < 0.05$; **$P < 0.01$.

E. ***No Differences Were Found between Serum MIF Concentrations in MIF −794 CATT Genotypes (5/5 + 5/6 + 6/6) and (7/X + 8/X) Cases within Same TB Group***

In order to determine whether the MIF −794 CATT genotype plays a role in MIF production in TB cases, we studied the serum MIF concentrations for the MIF −794 CATT genotype (5/5 + 5/6 + 6/6) and genotype (7/X + 8/X) cases within each group. No significant differences in MIF concentrations between genotype (5/5 + 5/6 + 6/6) and (7/X + 8/X) cases within the same group were observed (Figure 8).

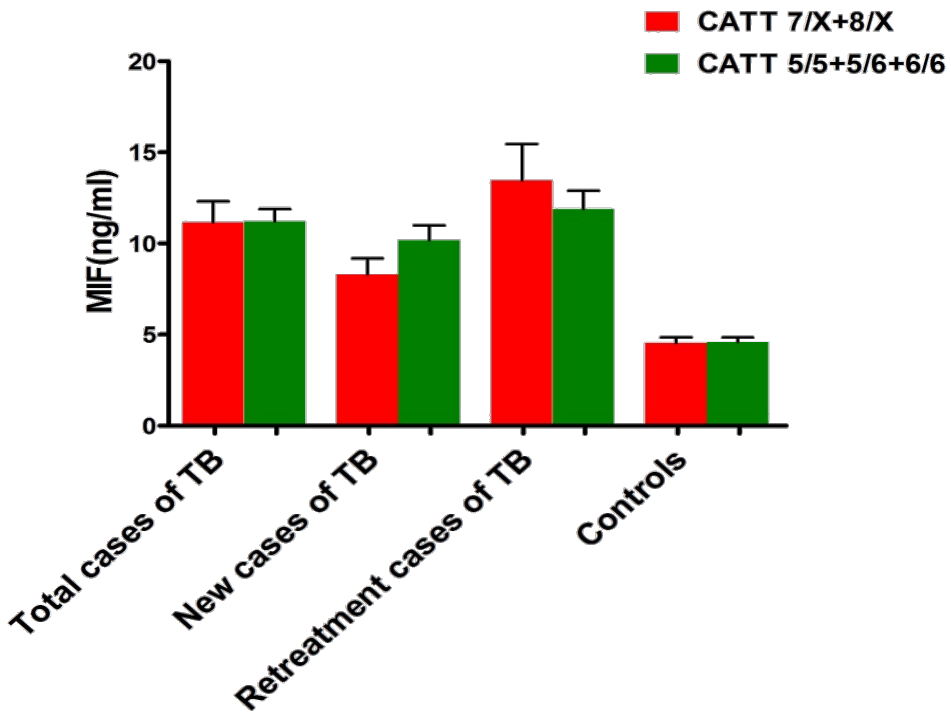

Figure 8. Comparison of serum MIF concentration in MIF -794 CATT genotype (7/X+8/X)) tuberculosis cases, genotype (5/5+5/6+6/6) tuberculosis cases, and controls within same tuberculosis group.

CHAPTER 4

DISCUSSION

In recent years, several studies have provided evidences that gene polymorphisms in the human host are closely related to the pathogenesis and individual prognosis of tuberculosis. Genetic variations of some immunity-related genes have been proved to be associated with human TB susceptibility, including such as natural resistance associated macrophage protein-1 (Nramp1), human leukocyte antigen (HLA), SP110 nuclear body protein gene (SP110), nitric oxide synthase 2 gene (NOS2A), mannose binding lectin (MBL), IFN-γ (IFNG), vitamin D receptor (VDR), mannose receptor (MR, CD206), dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN, CD209), Dectin-1, toll-like receptors(TLRs), complement receptor 3 (CR3, CD11b/CD18), nucleotide oligomerization domain 1 (NOD1) and NOD2, tumor necrosis factor (TNF), IL-8, monocyte chemoattractant protein 1 (MCP-1), RANTES, CXCL10, inducible nitric oxide synthase (iNOS) and solute carrier protein 11A1 (SLC11A1) (Azad *et al.*, 2012; Meyer and Thye, 2014; O'Garra *et al.*, 2013).

Many studies have reported that MIF is not only a T-cell-derived cytokine but could also be released from other cells such as monocyte/macrophage cells and anterior pituitary cells [Kleemannt and Bucala, 2010]. Because of its wide expression in various cells, MIF is considered to be a versatile cytokine. As a proinflammatory cytokine, MIF may function as a double-edged sword that contributes to detrimental tissue inflammation but may also be important for controlling infection. MIF has been proposed to be a protective host cytokine against TB-causing bacteria. Oddo et al. demonstrated growth inhibition of *M. tuberculosis* by MIF (Odd *et al.*, 2005). MIF deficient mice succumbed much faster with higher numbers of the organisms burdened, increased lung pathology, and decreased innate cytokine production. Furthermore, MIF deficient animals exhibited an increase in

pulmonary neutrophil accumulation with a suppressed adaptive immune response [Das *et al.*, 2013]. A number of clinical investigations have reported higher MIF protein expression levels (Kuai et al., 2016; Li *et al.*, 2012a; Li *et al.*, 2012b; Liu et al., 2016; Wang et al., 2016), as well as elevated serum MIF levels in patients with pulmonary TB (Li *et al.*, 2012b; Tong *et al.*, 2017; Yamada *et al.*, 2002).

Research results have been gathered to support the opinion that MIF gene and its expression product are associated with tuberculosis. A study by Kibiki et al. showed that the mean levels of circulating MIF concentration were significantly higher in those with pulmonary tuberculosis than in the healthy controls (Yamada *et al.*, 2002). An earlier study pointed to Colombian population with TB found that the MIF −173 C allele was associated with TB (Gómez *et al.*, 2010). The study from Khalid Sadki group identified a statistically significant increase of the MIF −173 C homozygote genotype and MIF −173 C allele frequencies in pulmonary TB cases when compared with healthy controls in Moroccan population (Sadki *et al.*, 2010). In a case–control study, Deng et al. investigated the association between human MIF promoter region polymorphisms and tuberculosis in Chongqing (a municipality located in Southwestern China directly under the central government of China) population, and found that the frequency of MIF −173 genotype (GC + CC) in TB cases and controls was of statistically significant difference, and MIF −173 C allele frequency in TB cases is also statistic ally significant increasing when compared with healthy controls (Li *et al.*, 2012a). MIF −794 CATT5–8 microsatellite polymorphism also has been preliminarily proved to be related to genetic susceptibility to active TB in the southwestern Han Chinese population (Li *et al.*, 2012b). Similarly, a significant association was found between genotypes carrying MIF -794 CATT 7 or 8 and susceptibility to active lung TB (Kuai et al., 2016). In contrast, no allele in the MIF −794 CATT microsatellite was associated with TB risk in the northwestern Colombian population (Gómez *et al.*, 2010). MIF −794 CATT5–8 microsatellite polymorphism has been found to be associated with the alteration of MIF gene transcription levels. The CATT repeat number regulates the activity of the MIF gene promoter; higher CATT repeat numbers lead to stronger activity of the promoter (Jie *et al.*, 2006; Temple *et al.*, 2008).

However, the previous studies, which probed the association between MIF gene promoter region SNPs or CATTs and human susceptibility to TB, just divided studied population into TB patient group and control group. In order to obtain more detailed information, and investigate the association between MIF gene promoter region SNPs or CATTs and human susceptibility to different TB types, we arranged the studied population into three groups: control group, cases of pulmonary TB were further categorized into new cases of TB group and retreatment cases of TB group, which was according to World Health Organization definitions of TB cases in Global Tuberculosis Report 2013. Therefore, our study further explored the related topics.

At the same time, in the case–control study presented here, we probed the association among human MIF gene promoter region polymorphisms, serum MIF concentration, and tuberculosis in the population of Yunnan. Yunnan Province, which is located in Southwestern China, has a long border with three other countries, has higher tuberculosis prevalence, and a higher altitude when compared to other provinces of China, the reasons of higher tuberculosis prevalence are unknown. So a research on the association between MIF gene promoter region SNPs and human susceptibility or between CATTs and human susceptibility to TB in this province, respectively, may still be valuable.

1. MIF −173 G/C single-nucleotide polymorphisms

In our current study, OR of MIF −173 (GC + CC vs. GG) was compared between total cases of TB and controls. We also observed a moderate difference of MIF −173 C allele in TB cases compared with healthy controls. A statistically significant difference of frequencies for the MIF −173 (GC + CC vs. GG) was identified between new cases of TB and controls, also between retreatment cases of TB and controls, which confirmed the conclusions of three earlier results (Li et al., 2012; Gómez et al., 2010; Sadki et al., 2010). The frequencies of MIF −173 genotypes (GC + CC) were also significantly higher in new cases of TB than in controls, the frequencies of MIF −173 genotypes (GC + CC) were significantly high in retreatment

cases of TB than in controls. Meanwhile, we compared frequencies for the MIF −173 (GC + CC vs. GG) between new cases of TB and retreatment cases of TB, and found no difference. We analyzed the frequencies of MIF −173 G/C alleles (G vs. C) in new cases of TB, retreatment cases of TB, total cases of TB and controls, found no difference among these three groups.

These results indicated that the MF −173 (GC + CC) genotype is associated with an increased risk of tuberculosis in Yunnan population. However, types of MIF −173 G/C allele are not related to TB susceptibility, which is inconsistent with two earlier studies (Li et al., 2012; Gómez et al., 2010; Sadki et al., 2010).

In the MIF protein level, we found that the concentrations of MIF cytokine are significantly higher in total TB group, new cases of TB, and retreatment cases of TB than in controls. Serum MIF in retreatment cases of TB is significantly higher than in new cases of TB with same genotype which provides an evidence to support previous findings (Yamada et al., 2002). However, we detected serum MIF concentrations in MIF −173 G/C genotype GG cases and genotype (GC + CC) cases respectively, and found that there was no significant difference between of genotype GG cases and that of genotype (GC + CC) cases. These results showed that elevation of MIF concentration in TB cases is not related to specified MIF −173 G/C genotype.

We found that MIF −173 allele frequencies in Yunnan population of China were different from those reported from the Colombian population, Moroccan population, and Chongqing population of southwestern China (Li *et al*., 2012; Gómez *et al*., 2010; Sadki *et al*., 2010). Thus, the different distributions of the MIF promoter single nucleotide polymorphism were dependent not only on specific differences in environ- mental or geographic factors, but also on ethnic groups and genetic background.

Promoter sequence analysis has indicated that the MIF −173 C allele creates a potential activator protein 4 transcription factor binding site, and the levels

of MIF expression were significantly different among MIF −173 G/C genotypes in a cell type-specific manner (Temple et al., 2008). In addition, MIF −173 C allele has been shown to significantly increase the promoter activity and production of MIF (Donn *et al.*, 2002). Although there is no proved association between MIF −173 G/C allele and TB susceptibility, we found an increased serum MIF in TB cases and MIF −173 genotypes (GC + CC) have correlation with TB.

2. MIF −794 $CATT_{5–8}$ microsatellite polymorphism

Our current study has demonstrated a significant increase in MIF −794 CATT, for both genotypes 7/8 and alleles CATT 8, in TB patients compared with healthy controls. On the other hand, no significant difference for either CATT 5/5 or CATT 5 was found for the total TB cases compared with the control group. These results suggested that the genotypic frequencies of the MIF −794 CATT genotypes (7/7 + 7/8) and the allelic frequencies of the MIF −794 CATT alleles (7 + 8) were associated with the new cases and total cases of TB.

The MIF serum concentrations in TB cases with the MIF −794 genotype (5/5 + 5/6 + 6/6) and genotype (7/X + 8/X) were found to be greatly increased compared with the healthy controls. Moreover, the MIF concentrations in retreatment cases of TB were higher than those in new cases of TB. No differences were observed in the MIF concentrations between the MIF −794 CATT genotype (5/5 + 5/6 + 6/6) cases and genotype (7/X + 8/X) cases of TB within each individual group.

We speculate that the specific CATT repeat number at position −794 in the MIF gene promoter is closely associated with high MIF expression, resulting in increased susceptibility to TB. Our results not only further confirm previous conclusions by other authors from studies on the Han Chinese population but also give a deeper and more extensive insight into the association between MIF −794 CATT5–8 microsatellite polymorphisms and susceptibility to active TB.

In conclusion, in recent years, the roles of MIF in pathogenesis of infection and inflammation have been the focused subject of research (Greven et al., 2010; Grieb *et al*., 2010). Several research groups thought that MIF may be a protective cytokine against TB pathogens: MIF released by infected human macrophages inhibits the growth of virulent *Mycobacterium tuberculosis* MIF is a key initiator of the immune response to *Mycobacterium tuberculosis* infection (Oddo et al., 2005; Das et al., 2013). But MIF may play a two-edged sword role on bacterial infection, which provide protection against infection and also contributes to detrimental inflammation. Our focus on the investigation of new cases and retreatment cases of TB may have a significant impact on controlling drug-resistant TB.

CHAPTER 5

CONCLUSION

1. We proved that MIF -794CATT5-8 microsatellite polymorphism is associated with TB susceptibility. The higher repeat numbers of MIF -794 CATT microsatellite, the stronger the activity of the promoter.

2. We also found that MIF -173 single nucleotide polymorphisms are closely associated with human susceptibility to tuberculosis in a southwestern province of Yunnan.

3. The pathogenic mechanism which specified MIF genotype/allele is related to the susceptibility to TB remains to be completely illuminated, but exploring the relationship between MIF gene promoter polymorphisms and the susceptibility to TB infection, progression, and prognosis may help to identify new molecular Targets for TB diagnosis, prevention, and prognosis.

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APPENDIX

APPENDIX

Supplementary information

1. Genomic DNA extraction

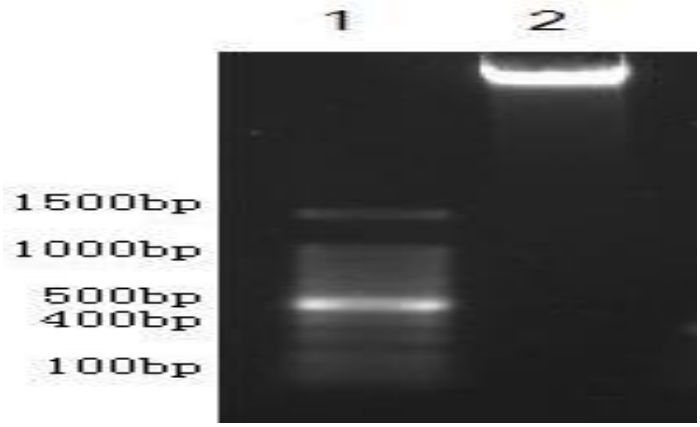

Figure 1. Genomic DNA extraction. Lanes: (1).Marker, (2) Genomic DNA.

2. PCR amplify MIF gene promoter -794 CATT repeat sequence microsatellite polymorphism region

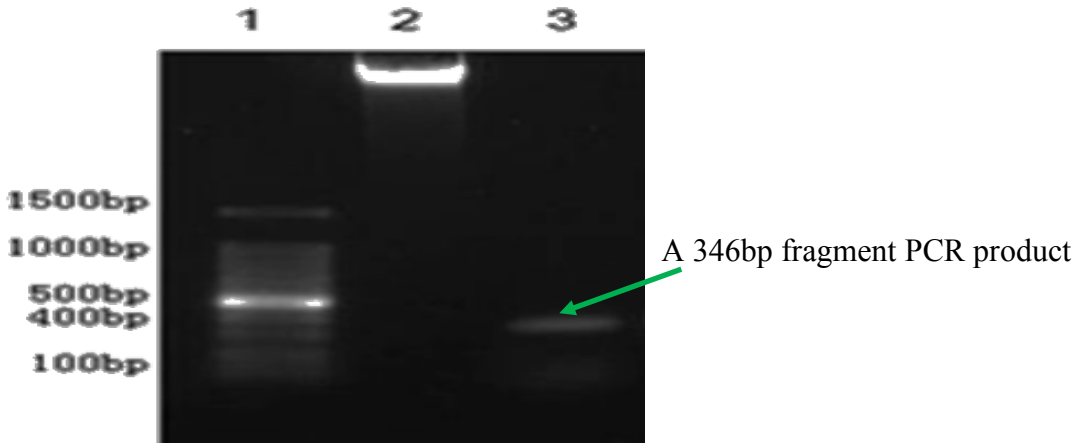

Figure 2. Genomic DNA extraction. Lanes: (1) .Marker, (2) Genomic DNA, (3) PCR product(346bp).

3. Purification of PCR products

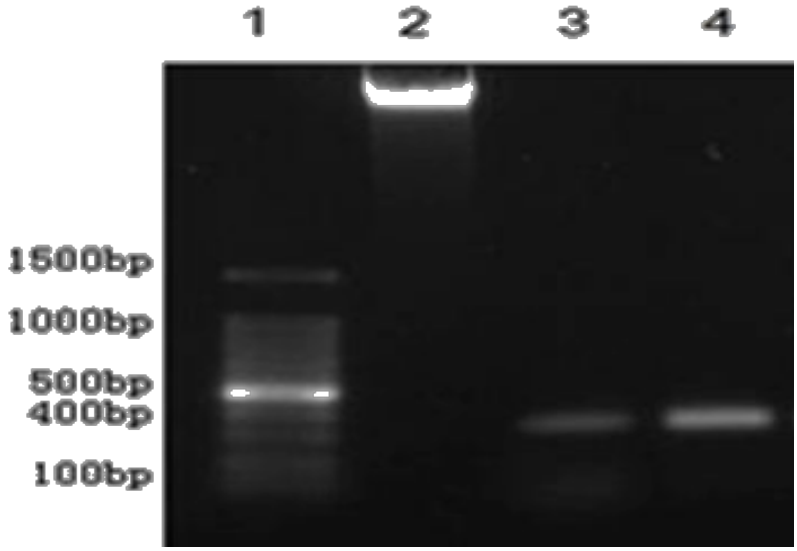

Figure 3. Genomic DNA extraction. Lanes: (1).Marker, (2) Genomic DNA, (3) PCR product, (4) PCR product after purification.

4 Sequence of MIF gene promoter -794 CATT repeat sequence microsatellite polymorphisms

A. $CATT_{5\text{-}7}$ sequence diagrams of forward

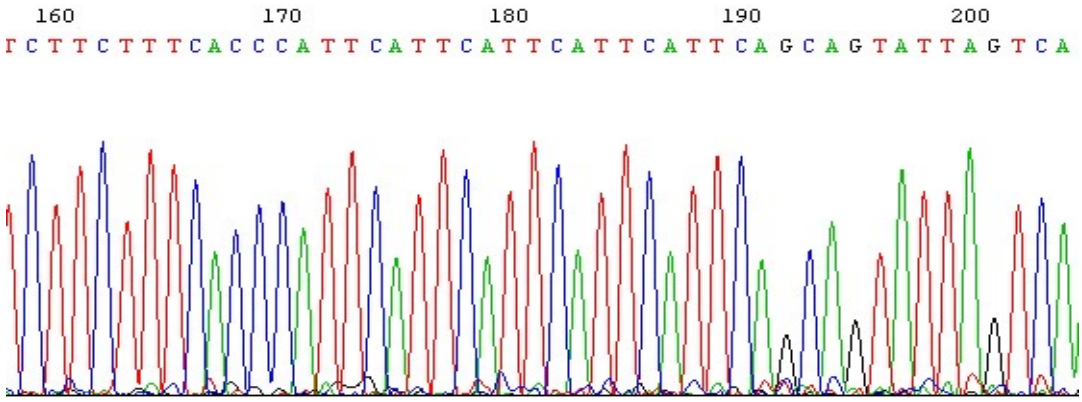

Figure 4. Sequencing diagram of allele MIF -794 $CATT_{5}$

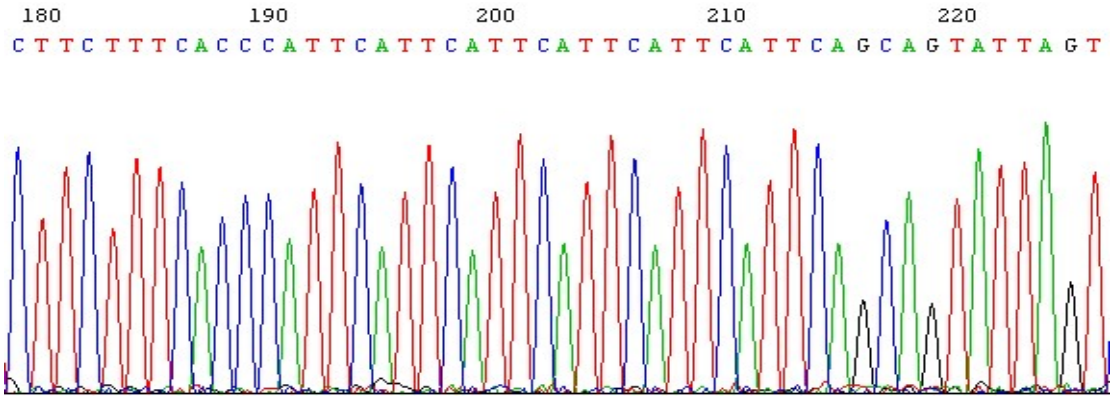

Figure 5. Sequencing diagram of allele MIF -794 $CATT_{6}$

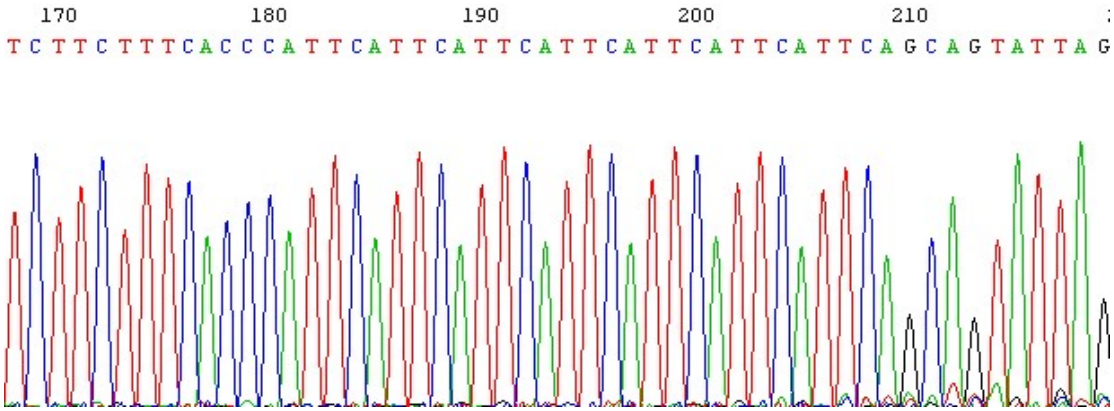

Figure 6. Sequencing diagram of allele MIF -794 $CATT_{7.}$

B. AATG5-8 sequence diagrams of reverse

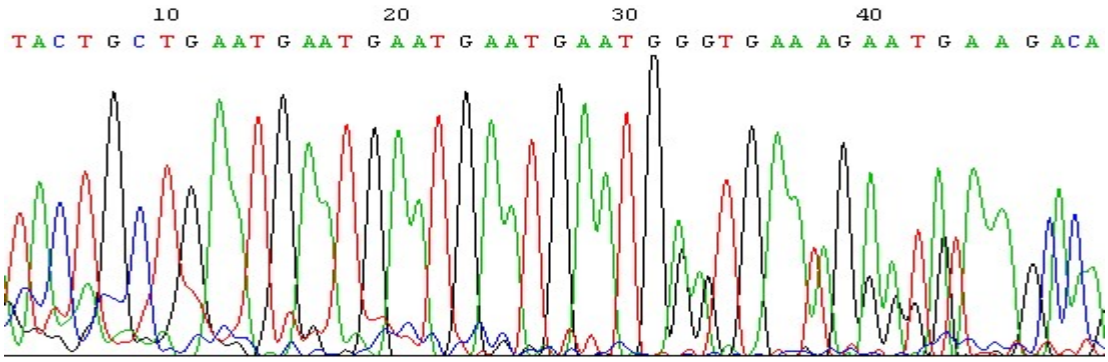

Figure 7. Sequencing diagram of the reverse sequences $AATG_5$ of allele MIF -794 $CATT_5$.

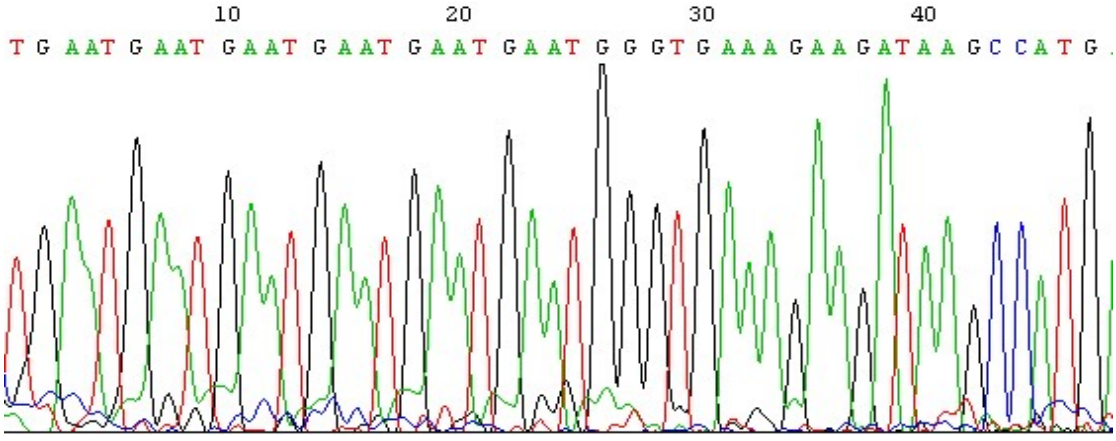

Figure 8. Sequencing diagram of the reverse sequences $AATG_6$ of allele MIF -794 $CATT_6$.

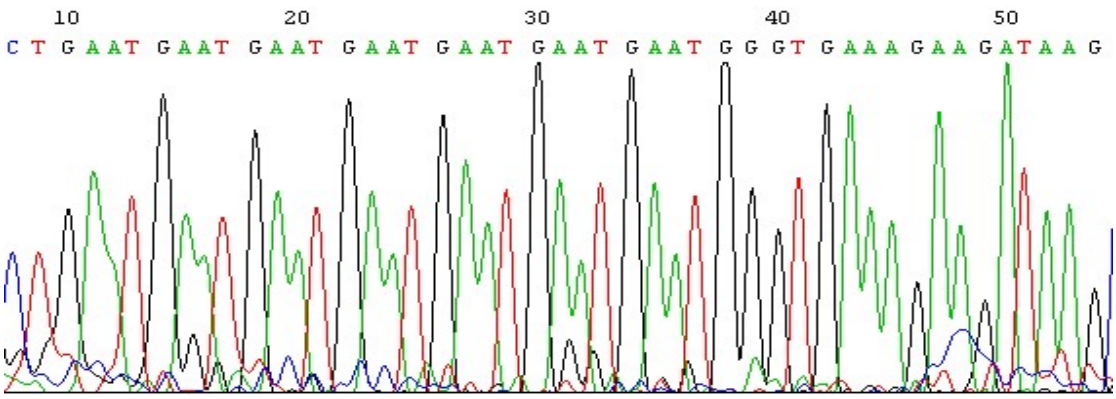

Figure 9. Sequencing diagram of the reverse sequences $AATG_7$ of allele MIF -794 $CATT_7$.

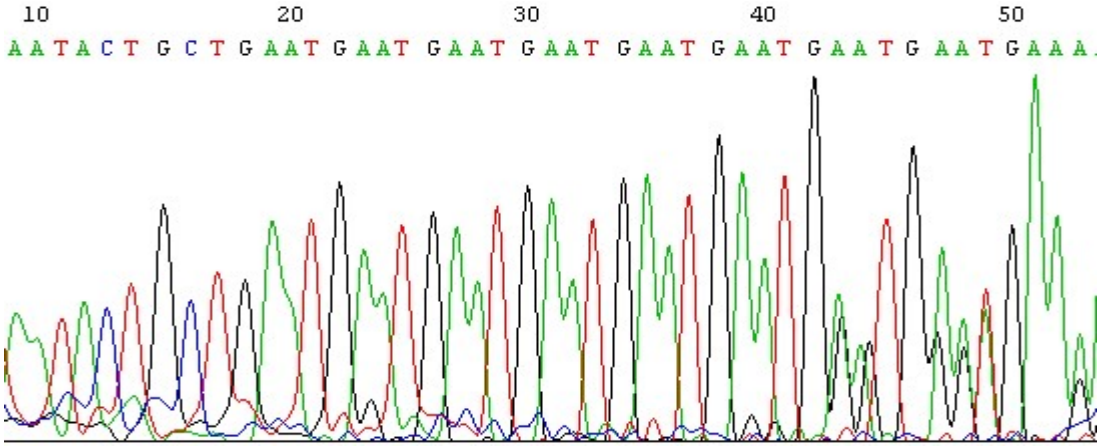

Figure 10. Sequencing diagram of the reverse sequences $AATG_8$ of allele MIF -794 $CATT_8$.

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Liu, A., Bao, F., Voravuthikunchai, S. P. 2018. CATT polymorphisms in MIF gene promoter is closely related to human pulmonary tuberculosis in a southwestern China population. International *Journal of Immunopathology and Pharmacology* 32:1-7.

International conference

Liu, A., Cui, Y., Chi, S., Voravuthikunchai, S. P. 2014. Anti-*Streptococcus mutans* activity of six plant extracts. The 5th International Conference on Natural Products for Health and Beauty, 6th-8th May 2014, Phuket, Thailand. pp 292. (Poster presentation)