Allele C of the *IL-12p40 1188A/C* Single Nucleotide Polymorphism Associates with the Increased Susceptibility to Tuberculosis

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Abstract

Background: Tuberculosis (TB) represents a major public health problem worldwide. Wide variation occurs in the incidence of TB in different races and ethnicities suggesting a role for genetic factors.
Aims: This study aimed to find out the relationship between single nucleotide polymorphism (SNP) in interleukin-12 (*IL-12p40 1188A/C*) with TB and serum levels of IL-12 in Iraqi patients.
Subjects and Methods: This case/control study involved 55 TB patients and30 age-matched unrelated healthy controls. Blood samples were obtained from the study population. DNA was extracted from leukocytes, and *IL-12p40 1188A/C* gene was amplified with sequence specific primer polymerase chain reaction (SSP-PCR) using a specific set of primers. Serum levels of IL-12 was estimated using enzyme-linked immunosorbent (ELISA*)* technique.
Results: The *IL-12p40 1188A/C* SNP appeared in three genotypes: AA, AC and CC. These genotypes represented 236.37%, 40% and 23.64% respectively among patients and 56.37%, 36.7% and 6.6% among control, with significant difference for the heterozygous genotype (OR= 5.525, 95%CI=1.090-28.003, *P*=0.039), and insignificant difference for the homozygous mutant genotype (OR=3.250, 95%CI=0.621-17.013, P=0.163**).** Carriers of CC genotype had higher level of IL-12p40 (219.45± 91.567 pg/ml) than either AC genotype carriers (166.705± 76.211 pg/ml) or AA genotype carriers (107.321± 68.358 pg/ml) with significant differences among the three groups (*P*<0.05)
Conclusion: The C allele of *IL-12p40 1188A/C* SNP can be considered as a risk factor for TB in Iraqi patients. Moreover, this allele causes increase production in IL-12p40.

Keywords: tuberculosis, single nucleotide polymorphism, *IL-12p40 1188A/C*

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Introduction

Tuberculosis, caused by *Mycobacterium tuberculosis*, represents a major public health problem worldwide and results in approximately 8.6 million new cases of TB and approximately 1.3 million deaths in 2012 (WHO, 2013). The incidence of TB differs among

particular races, ethnicities, and families which suggests a genetic factors affecting TB susceptibility (Azad et al., 2012). These genetic factors explain, at least in part, why some people are more or less susceptible to infection.

IL-12 is important cytokine for adaptive immunity with a crucial role in the defense against intracellular micro-organisms (Nakahira et al., 2002). It acts as a modulator for immune response through the stimulation IFN-γ producing cells. Boisson-Dupuis et al. (2011) have demonstrated the critical importance of CD4-mediated immunity and of the interleukin (IL)-12/IFN-γ loop in baseline resistance to *M. tuberculosis*.

Structurally, IL-12 is a 70-kDa heterodimeric protein (IL-12 p70) composed of two covalently-linked subunits, IL-12p35 (35 kDa) and IL-12p40 (40 kDa), each of which is expressed on different chromosomes. IL-12p40 is produced predominantly by activated monocytes, macrophages, neutrophils, and dendritic cells (DCs) Interleukin-12 plays The biological activities of IL-12 are mediated through binding to a membrane receptor complex which is composed of two subunits: IL-12Rβ1 and IL-12Rβ2 where the first subunit is required for high-affinity binding to IL-40p subunit (Hamza et al., 2010).

Several molecular and genetic studies have demonstrated the association between *IL-12B* gene polymorphisms and risk of infections (Cupic et al., 2014; Houldsworth et al., 2015) and different types of malignancies (Chang et al., 2015). The association of *IL-12p40 1188A/C* with TB is a controversial issue. An association of this polymorphism with the incidence of TB among Russian and Gambian populations (Freidin et al., 2006; Morris et al., 2011). However no such association was found among Indian population (Anand et al., 2007). This study aimed to investigate the association of *IL-12p40 1188A/C* SNP with the incidence of pulmonary TB and serum levels of IL-12 among Iraqi patients.

Subjects and Methods

A total of 55 patients with confirmed pulmonary tuberculosis (31 males and 24 females, age range 7-85 years, mean 69.6 ±9.76) who were attending Al-Hilla Consultant Clinic for Respiratory Disease/ Babylon Province/Iraq during the period from December 2013 to April 2014 were rercriuted in this case/control study. The specific criteria for enrollment were defined as the presence of at least one of the following: (1) clinical and radiological findings that indicate the presence of pulmonary TB, and at least one positive *M. tuberculosis* culture from three separate sputum examination, or one bronchial washing specimen obtained from bronchial scopy, (2) improvement in suspected pulmonary TB with empirical anti-TB therapy as indicated via clinical and radiological findings, (3) positive result for Xpert test which is a modern test for molecular detection of the causative bacteria in body fluid and (4) pathological evidence of TB as indicated from pleural or lung biopsy.

Family unrelated, apparently healthy 30 individuals from workers of the same hospital and from College of Medicine/ Babylon University were employed to represent the control group. The mean age of control was 66.68±8.29 years. Exclusion criteria were defined as the presence of at least one of the following: (1) fever greater than 38.5 ^{o}C, (2) significant weight loss according to BMI calculation, (3) productive cough and night sweat for more than two

weeks, (4) pregnancy or nursing an infant and (5) receiving an immuno-suppressive drug or cancer-related therapy. Informed consents from patients as well as control were taken.

Samples

Five ml of venous blood was collected from each participant; 2 ml of which was kept in EDTA tube and the other 3ml in plan tube. The latter was undergone centrifugation where the serum was obtained and preserved at -20 ^{o}C until be used.

DNA Extraction

DNA was extracted from these samples using ready kit (Favor prep DNA extraction mini kit/ Favor Gene Biotechnologies/ Taiwan) according to the manufacturer's instructions**.**

PCR

Sequence-specific primers PCR (SSP-PCR) method was used for amplification and genotyping of *IL-12* gene. Three primers were used for this purpose which were Consensus F: ATCTTGGAGCGAATGGGC, R1: TTGTTTCAATGAGCATTT AGCATCT and R2: GTTTCAATGAGCATTTAGTATCG. *TLR2 Arg753Gln* set of primers (F: GCCTACTG GGTGGAGAACCTT and R: CCAGTTCATACTTGCACCACT) was used as internal control. The cycling conditions were an initial denaturation for 5 min at 95 ºC, followed by 30 cycles of denaturation at 95 ºC for 30 sec , annealing at 61 ºC for 40 sec, extension at 72 ºC for 1 min, followed by final extension at 72 ºC for 7 min with an expected fragment length of 780 bp for IL-12 gene and 199 bp for *TLR2 Arg753Gln* gene.

A ready 50 µl PCR master mix (Bioneer/Korea) was used for preparing the PCR reaction. Template DNA (10 ng) from each sample and primers (5 ng from each) were added to each master mix tube. The mixture then put in shaker and spinner for 10 cycles for better mixing. Then, the mastermix tubes were transferred to the thermocycler (MyGenie 32 thermal block/Bioneer/Korea) which is previously programmed with the above protocols according to the gene to be amplified.

A 2% gel was prepared, and 10 µL aliquot of PCR product from each PCR tube was mixed with 2 µL loading dye and loaded into the wells of the gel. After 1 hour of electrophoresis, the gel was stained with ethidium bromide (Biobasic/Canada) (0.5 g/mL) for 20 min and examined using U. V. transilluminator with camera. The amplified products were determined by comparison with a commercial 1000 bp ladder (Kappa Biosystem/USA).

Enzyme-Linked Immunosorbent Assay

A commercial kit (Cusabio/China, Catalog Number CSB-E07362r) was used to measure serum levels of IL-12p40. Breifly, One hundred µl of Standard and serum samples was poured for the first and the rest wells of the plate respectively. The plate was covered with adhesive strip and incubated for 2 hrs at 37℃. The liquid was removed from the wells without washing, and 100 µl of Biotin-antibody was added to each well. The plate was again covered with a new adhesive strip and incubated for 1 hr at 37℃. The content of each well was aspirated and the plate was washed. This process was repeated three times, after which each well was filled with 200 µl of Wash Buffer using multichannel pipette. The plate was left to stand for 2 min, after which the liquid was removed and the plate was inverted against clean filter paper. HRP-avidin (100 µl) was added to each well and the plate was covered

with a new adhesive strip and incubated for 1 hr at 37℃. Ninety µl of TMP Substrate was added to each well, and the plate was incubated for 30 min at 37℃ protected from light. Stop solution (50 µl) was added to each and the plate was gently taped to ensure thorough mixing. The optical density was determined for each well within 5 min using microplate reader (Diagnostic Automation Inc, USA) set at 450 nm. Optical densities were converted into concentrations using the standard curve.

Statistical Analysis

Data were expressed as a mean± standard deviation. The Statistical Package for the Social sciences (SPSS, version 14) was used for statistical analysis. Risk association between the genotype and TB susceptibility was estimated by the calculation of adjusted odd ratio and 95% confidence intervals using multivariate logistic regression. For this analysis, subjects who were homozygous for the wild type allele were considered as reference, and polymorphisms as dependent variables. Chi- square was used for testing the deviation from Hardy- Weinberg equilibrium, distribution of different alleles between patients and control, and to compare serum levels of IL-12. A *p*-value < 0.05was considered statistically significant.

Results

Sequence Specific Primer PCR

The SNP IL-12p40 1188A/C had three genotypes which were AA, AC and CC. These genotypes represented 20(36.37%), 22(40%) and 13 (23.64%) respectively among patients and 17(56.37%), 11(36.7%) and 2 (6.6%) among control, with significant difference for the heterozygous genotype (OR= 5.525, 95%CI=1.090-28.003, *P*=0.039), and insignificant difference for the homozygous mutant genotype (OR=3.250, 95%CI=0.621-17.013, P=0.163) (Figures 1 and 2, Table 1).

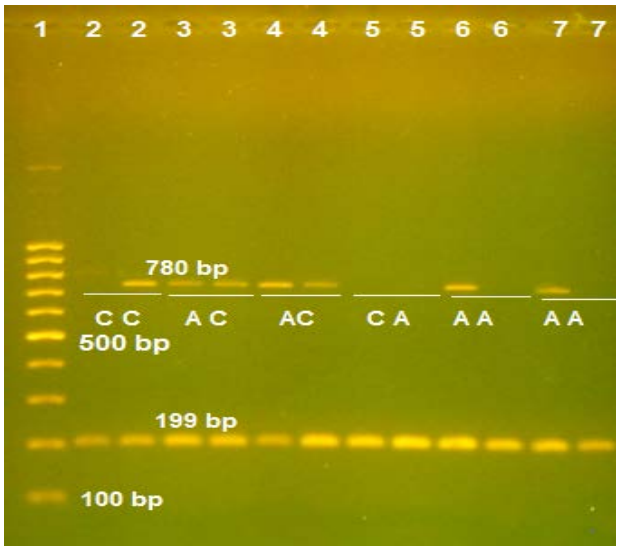

Figure (1): Agarose gel electrophoresis of PCR products of *IL-10p401188A/C* primer with product size of 780 bp. Lane 1: DNA molecular size marker (100-2000 bp), lane The 780 bp represents the amplification of *IL-12p40* 1188A/C, while the 199 bp represent the amplification of internal control.

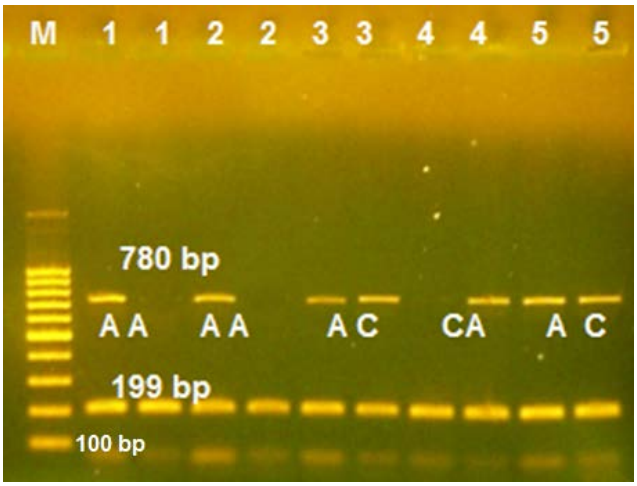

Figure (2): Agarose gel electrophoresisof PCR products of *IL-10p401188A/C* primer with product size of 780 bp. Lane 1: DNA molecular size marker (100-2000 bp). The 780 bp represents the amplification of *IL-12p40* 1188A/C, while the 199 bp represent the amplification of internal control (*TLR2* gene).

Chi –sequare test showed that allele frequency in both patients and control met Hardy Weinberg Equilibrium. Allele C of the SNP IL-12p40 1188A/C had significant role as a risk factor for TB since the frequencies of this allele among patients and control were 43.64% and 25% respectively (*P*=0.016) (table 1).

Table (1): Genotypes and alleles of SNP IL-12p401188

Variables	Cases N=55	Control N=30	*P*-value	OR(95%CI)
IL-12p40 1188A/C				
AA	20(36.37%)	17 (56.7%)	0.103	1.0
AC	22(40%)	11(36.7%)	0.039	5.525(1.090-28.003)
CC	13(23.64%)	2(6.6%)	0.163	3.250(0.621-17.013)
Alleles			0.016	
A	62(56.36%)	45(75%)		1.0
C	48(43.64%)	15(25%)		2.323(1.159-4.655)

N: number, OR: odds ratio, CI: confidence interval

Serum levels of IL-12p40

Figure (3) shows mean serum levels of IL-12p40 in TB patients and control. TB patients showed significantly higher IL-12p40 (183.774±74.103 pg/ml) than control group (97.972±31.251 pg/ml).

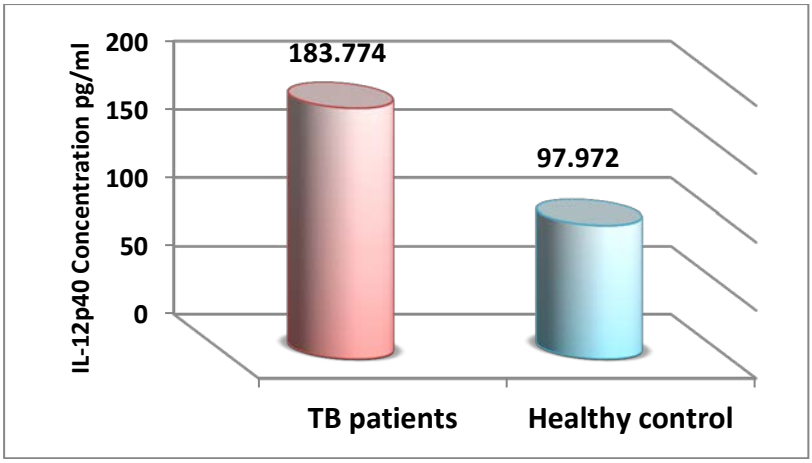

Figure 3: serum levels of IL-12p40 in TB patients and control.

Analysis of serum levels of IL-12p40 among the three genotypes (AA, AC and CC) in both TB patients and control group revealed that CC genotype carriers had higher level of IL-12p40 (219.45± 91.567 pg/ml) than either AC genotype carriers (166.705± 76.211 pg/ml) or AA genotype carriers (107.321± 68.358 pg/ml) with significant differences among the three groups (P<0.05) (Figure 4).

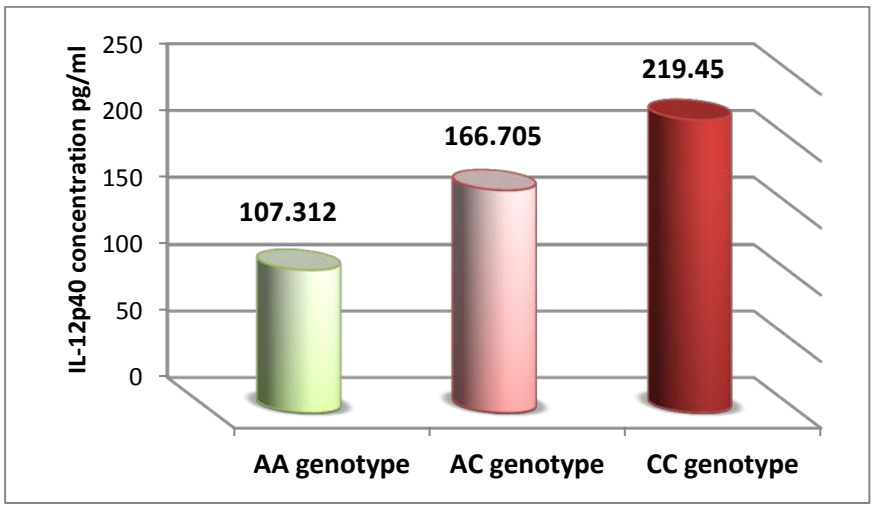

Figure 4: serum levels of IL-12p40 in the three different genotypes.

Discussion

The present study revealed that allele C of the SNP *IL-12B1188A/C* (rs3212227) is associated with an increasesd susceptibility to infection with *M. tuberculosis*. This result was previously obtained by Alvarado-Navarro et al. (2008).

The 3' untranslated region (3'UTR), although does not encode for a protein, it can influence the amount of translated protein through several mechanisms including effects on mRNA stability as well as on transcriptional activity (Matoulkova et al., 2012). The SNP 1188A/C lies in exon 8 in the 3'UTR of the transcript. Thus it possibly affects exon silences and could regulate the level of IL-12B mRNA expression (Kaarvatn et al., 2012). Peresi et al. (2013) found that AA genotype of this SNP is associated with lower plasma level of IL-12 in normal control and in TB patients after 3 months of anti-TB treatment.

Morahan et al. (2001) and Seeger et al. (2002) observed that the rs3212227AA genotype was associated with a significantly elevated expression of IL-12, while the rs3212227 A variant was shown to be correlated with reduced levels of IL-12p40 in several other researches (Seeger et al., 2002; Wong et al., 2012). Thus, it seems that there is a contradiction because increased production of IL-12 is supposed to enhance cell-mediated immune response and increase the resistance to *M. tuberculosis* not the reverse.

There are three explanations for such discrepancy. The first one is the increased production of IL-12 does not mean that IL-12p70 is overproduced. Rather, the induction involves only the IL-p40 subunit. The homodimers of this subunit antagonizes IL-12p70 activity by binding to the β1 subunit of the IL-12 receptor (Ling et al., 1995). Therefore, the increased production of this subunit, in fact, causes reduction in the activity of IL-12 and hence reduces the efficiency of CMI response and increases the susceptibility to *M. tuberculosis*.

The second explanation referred to the influence of other genetic polymorphisms affecting the IL-12B gene expression or due to linkage disequilibrium of the 1188C allele with other common polymorphisms in IL12B (Eskandari-Nasab et al., 2013).

The third explanation referred to the effect of IL-12p40 homodimers on the activity of IL-23 as these homodimers have high affinity to IL-23 receptor and hence abolish IL-23 role in immune response (Shimozato et al., 2006). The main role of IL-23 involves the stimulation of Th17 cells to produce IL-17 (Aggarwal et al., 2003) which has a critical role in the resistance to TB (Torrado and Cooper, 2010)

Neither AC nor CC genotypes of the SNP rs3212227 had significant association with susceptibility to TB, while at allele level, such significant appeared. The advantage of allelic frequencies over the genotypic frequencies is that genotype breaks down to alleles when gametes are formed, and it is alleles rather than the genotypes that pass from one generation to another. Thus, it is the allele that has continuity over time and the gene pool evolves through changes in the frequencies of alleles (Bergholdt et al., 2004).

In order to confirm the hypothetical association of the SNP rs3212227 with the incidence of TB, serum levels of IL-12p40 were measured. The result indicated higher serum levels of this subunit in TB patients compared with control group. Furthermore, IL-12p40 values were grouped according to the three genotypes (AA, AC and CC). The significance higher levels of IL-12p40 subunit in CC genotype carriers compared with either AC genotype carriers or AA genotype carriers indicates that the SNP rs3212227 causes an increase in the production of this subunit in an allele-dependent manner. However, the high levels of IL-12p40 could not only attribute to the mutant allele. Rather, the infection with *M. tuberculosis* had also caused increase in these levels as evidenced by previous study (Deveci et al., 2005).
According to the current results it can be concluded that the C allele of the *IL-10p401188A/C* could be considered as a risk factor for TB, and causes increases IL-p40 production.

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