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Genetic association between a chemokine gene *CXCL-10* (IP-10, interferon gamma inducible protein 10) and susceptibility to tuberculosis

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ABSTRACT

Background: Previous studies showed that activation of CXCL-10 and other chemokines were prominent in many infectious diseases. These chemokines are components of innate immune response to respiratory tract pathogens. We examined the promoter variants of CXCL-10 and their role in predisposition to tuberculosis (TB).

Methods: The promoter 1.8 kb of CXCL-10 was sequenced in 24 healthy Chinese individuals to identify genetic polymorphisms. Three tagging SNPs in CXCL-10 promoter (−1447A>G, −872G>A, −135G>A) were selected, and genotyping were performed in 240 TB patients and 176 healthy Chinese subjects. Disease associations were examined by χ^2 and Fisher exact test.

Results: A promoter SNP (−135G>A) with minor allele frequency of 0.1 showed a moderate association with TB both in genotype analysis ($p = 0.01$) and allelic analysis ($p = 0.03$); other tagging SNPs (−1447A>G, −872G>A) were not associated with TB. The odd ratio of the protective allele −135G>A was 0.51(C.I 0.29 − 0.91) for homozygotes and heterozygotes carriers of the A allele.

Conclusion: A new potential protective SNP (−135G>A) for TB is identified in the promoter of chemokine gene, *CXCL-10*. Interestingly, the exact same allele has been shown to enhance IP-10 transactivation and susceptibility to Hepatitis B virus infection in a recent publication. This SNP, located at 14 bp upstream of a NF-kB binding site, might also account for the susceptibility to TB. Our results expanded the clinical significance of this SNP in CXCL-10 promoter.

1. Introduction

Tuberculosis is a significant health problem in the world. WHO recently estimated that about one-third of the world population has been infected with tubercle bacilli [1,2]. It is also a prevalent infectious disease in Hong Kong, with >6000 new cases and 200 tuberculosis (TB)-related deaths every year [3]. Although chemotherapy is fairly effective, TB is still the topmost killer of adults among infectious diseases [2]. It is expected that TB will continue to be a major health issue for Hong Kong and the world in the coming decades [4].

Host susceptibility is an important risk factor for infection. It has been estimated that although one-third of the population has been exposed to *Mycobacterium tuberculosis* (MTB), only 10% of the exposed will develop clinical disease and symptoms [5,6]. Early studies revealed a higher concordant rate between monozygotic twins than dizygotic twins; this supported an underlying genetic effect of susceptibility to TB [7,8].

This between-individual variation in disease predisposition is determined largely by a single-base variant in our genome, known as single nucleotide polymorphisms (SNPs). Several susceptibility genes of strong effect (high penetrance) have been defined by familial linkage study in highly susceptible families. The familial form of disseminated atypical mycobacterial infection and disseminated BCG infection are both caused by defects in one of the following genes involved in interferon-gamma effector pathway: interferon-gamma receptor-1 (IFNGR1), interferon-gamma receptor-2 (IFNGR2), interleukin-12B (IL12B), interleukin-12 receptor β (IL12RB1) and signal transducer and activator of transcription-1 (STAT1). However, these familial forms

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Table 1
Sequencing primers. Five pairs of sequencing primers covered 1.8 kb promoter regions of IP-10. Each segment overlapped approximately 100 bp with the latter one.

No.	Primer	Sequence	Product length (bp)
1	Forward	GGAAGGATCCCTCCATTGTC	414
	Reverse	GGTCAGGGAATGGAAAAGCT	
2	Forward	TTTGAGGATCCAAGTTTTATGTG	399
	Reverse	AGCTAGCAGTAGAAAGCACATGA	
3	Forward	ATTTGCAAAGAACACAACCAA	404
	Reverse	GGGTTATTGTAAAAAACAAAGGG	
4	Forward	GCTCAATGGCCAACACTTTT	441
	Reverse	GGCCTGCTTTGACAGGGTC	
5	Forward	TGGTGTTATGAATGATCAAAGCA	455
	Reverse	CCATGTTGCAGACTCGAAGG	

of predisposition are rare. On the other hand, the great majority of patients infected by *M. tuberculosis* are sporadic due to the interplay between genetic and environmental risk factors. The objective of this study is to identify the genetic risk factors leading to the sporadic form of TB.

Several genes contributing to the susceptibility to TB at the population level have been identified, including *SLC11A1* (formerly known as *NRAM1*) and it was recently reviewed [9]. Other predisposition genes found in sporadic cases included vitamin D receptor, IL12B [10–13]. It is evident that genetic determinants of the rare familial form of TB and the prevalent sporadic cases may be different. However, limited information is known for the latter which requires large-sampled genetic association studies on a dense map of genetic variations/SNPs.

Recent studies from ourselves and others showed that chemokines, in addition to cytokines, were important early inflammation mediators which determine the host response after exposure to pathogens [14,15]. In the case of lung infection, CXCL-10 (IP-10) is an early secreted chemokine that determines the extent and degree of host response [16,17]. Furthermore, CXCL-10 has been found to play important roles in the immune response to hepatitis [19], and the innate immune response to respiratory tract pathogens, including SARS coronavirus [17] and TB [18].

The biological action of CXCL-10 includes recruitment of effector cells (chemotaxsis) to the site of infection. As the extent of host response to lung infection was partly determined by the expression level of CXCL-10 and its blood concentration [18], we hypothesized that genetic variation in the gene encoding for CXCL-10 might be a cause of the between-individual difference of CXCL-10 expression. It is also expected that genetic variations affecting the transactivation of chemokine genes, particularly those in the promoter region of the gene, might influence the extent of gene expression after stimulation, and thus determine the susceptibility to infection. There are many examples of disease predisposition polymorphisms in promoter regions of various genes [20,21]. Here, we used a genetic association approach to study whether the promoter variations of CXCL-10 were associated with predisposition for TB.

Table 2
List of promoter SNPs identified by DNA sequencing.

	NCBI accession No.	Position[a]	rs no.	Variants	Heterozygosity reported in dbSNP[b]	MAF[c]
1	1440885	−1502	Novel	(T/C)	Nil	0.167
2	1440844	−1489	rs4386624	(G/C)	0.426	0.021
3	1440802	−1447	rs4508917	(C/T)	N.D.	0.4
4	1440686	−1331	rs4309862	(T/C)	0.432	0.021
5	1440632	−1227	Novel	(T/C)	Nil	0.021
6	1440390	−1053	rs4257674	(T/C)	0.398	0.021
7	1440227	−872	rs4256246	(C/T)	N.D.	0.48
8	1439490	−135	Novel	(G/A)	Nil	0.167

[a] Relative position (bp) to the transcription starting site of IP-10.
[b] SNP data that were not reported in dbSNP were marked as "Nil".
[c] MAF was calculated with 24 Chinese samples.

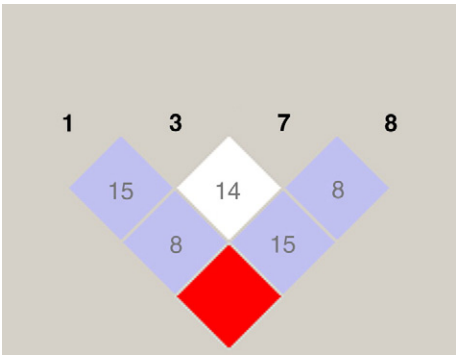

Fig. 1. LD plot of common SNPs in the promoter of CXCL10 (IP-10) with the R^2 values. SNPs with MAF of 5% or more were analyzed, (1) – SNP-1502, (3) – SNP-1447, (7) – SNP-872, and (8) – SNP-135. SNP nos. 2, 4, 5, and 6 (see Table 2) were excluded because their MAF were below 0.05, indicating that they are rare SNPs. On the other hand, rs1440885 and rs1439490 (SNPs 1 and 8) were in complete LD; both of them have MAF of 0.167.

2. Materials and methods

We sequenced a 1.8 kb segment of genome DNA upstream of the first exon of CXCL-10 and sequence variants were identified among 24 healthy individuals. Tagging SNPs were then identified by Haploview program which captures genetic variation by R^2>0.8. To test whether CXCL-10 promoter polymorphisms might contribute to human TB susceptibility, the genotype frequencies of the three tagging SNPs in CXCL-10 promoter (−1447A>G, −872G>A, −135G>A) were examined in 240 TB patients and 176 Chinese cord blood control population.

2.1. A sample of Chinese TB patients [N=240]

Patients attending the territory-wide Chest Clinic of the Tuberculosis and Chest Service in Hong Kong with confirmed TB were invited to participate in the study. All patients (Hong Kong Chinese) had to fulfil the inclusion criteria for established TB: (1) smear positive for MTB and/or (2) culture positive for MTB and/or (3) clinical TB with the diagnostic criteria of the International Union against tuberculosis and lung diseases with clinical-radiological and histological grounds and a clinical response to treatment. Patients with HIV and patients with diabetes or other causes of immunodeficiency were excluded. The study had received ethical approval from the University and Department of Health, and informed written consent was obtained from all patients. A 10-ml blood

Table 3
Frequency distribution of SNPs −1447A>G, −872G>A, and −135G>A genotypes and alleles in control and tuberculosis disease samples.

	Control no. (%)		TB disease no. (%)		χ^2(*p*-value)
Genotype					
−1447A>G					
AA	31	(25)	62	(25.9)	NS
AG	66	(53.2)	122	(51.1)	
GG	27	(21.8)	55	(23.0)	
−872G>A					
GG	50	(34)	78	(32.9)	NS
GA	75	(51)	124	(52.3)	
AA	22	(15)	35	(14.8)	
−135G>A					
GG	124	(78)	207	(87.4)	0.018*
GA	35	(22)	28	(11.8)	
AA	0	(0)	2	(0.80)	
Allelic analysis					
−1447A>G					
A	128	(52)	246	(51)	NS
G	120	(48)	232	(49)	
−872G>A					
G	175	(64)	280	(74)	NS
A	97	(36)	97	(26)	
−135G>A					
G	283	(89)	442	(93)	0.035
A	35	(11)	32	(7)	

* The genotype association was determined by Fisher's exact test for −135G>A under a dominant model (i.e., GG vs GA and AA).

Table 4
Odd ratio and exact test (*p*-value) calculation of the 3 tagging SNPs.

	Odd ratio	95% C.I		Exact test (*p*-value)
		Lower	Upper	
−1447A>G				
AA vs GG	0.92	0.53	1.61	NS
AG vs GG	1.10	0.61	1.98	NS
−872G>A				
GA vs GG	0.94	0.58	1.53	NS
AA vs GG	0.98	0.49	1.95	NS
−135G>A				
AA/GA vs GG	0.51*	0.29	0.91	0.018

*$p<0.05$.

sample was collected from each patient for DNA extraction. Clinical parameters about the extent and severity of disease were collected in clinical worksheet, and X-ray findings were also recorded.

2.2. Sequencing to identify promoter SNPs for tagging SNPs

PCR of the 1.8 kb promoter segment was performed by 5 pairs of primers (Table 1) which yielded overlapping PCR products. Sequencing of PCR products were performed by BigDye Terminator Cycle sequencing kits with an ABI-3100 autosequencer (Applied Biosystems, Foster City, CA) in a reaction mixture containing the purified PCR product, sequencing primer, and reaction mixture, including the terminator dye, and buffer. The sequencing reaction cycles were performed under the manufacturer's instructions. In brief, the PCR product was first purified by an Exo-SAP protocol (Affymetrix Inc., Santa Clara, CA), where Exonuclease I and shrimp alkaline phosphatase were added and incubated for 15 min. The mixture was then inactivated by heating to 80 °C for 15 min. Thermocycling was carried out in a Bio-Rad or ABI GeneAmp thermocycler for 25 cycles according to the standard temperature protocol. The sequencing product was then injected into auto-sequencer and the sequence read was retrieved by the ABI sequence analysis software.

2.3. Genotyping protocols for tagging SNPs

DNA was extracted from the peripheral blood of Chinese patients, and polymorphisms were analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). In brief, the standard PCR and RFLP protocol was as follows: PCR was performed in 25 ul reactions comprising 0.25 umol/l of each primer pair, 2 mmol/l MgCl2, 1 Uof Ampli Taq Gold Polymerase (Applied Biosystems) and PCR buffer (10 mmol/l Tris–HCl, pH 8.3; 50 mmol/l KCl). Reaction mixture was initially heated at 96 °C for 15 min to activate the polymerase and DNA amplification was achieved by 35 cycles of 96 °C for 30 s, annealing for 45 s and 72 °C for 45 s. The final elongation step was 72 °C for 7 min. For restriction enzyme digestion, 7 ul of the PCR product was digested by the required enzyme in the presence of the accompanying buffer, in a final volume of 14 ul incubated at the temperature with optimal activity of the enzyme for overnight. Genotype calling was made by separating the DNA in a 4% agarose gel and stained with ethidium bromide.

For SNP (−1443 A>G), primers TTGGTCAGGGAATGGAAAAG and CGGTTTCCCA-CAGCTAATTC amplified a PCR product of 290 bp and this was digested with 10 units of SacI. The mutant PCR product was cleaved to produce a fragment of 145 bp while the wild-type remained uncut. Genotyping of SNP (−872G>A) was performed by mismatched PCR-RFLP with primers: TGAAATTAAGTTTTGCCACGA and GGAAACAGGTT-GATTTACcATG. The PCR product is 155 bp and the A allele forms a restriction site for NcoI. After digestion, a shorter fragment (133 bp) was produced from the G allele. For SNP (−135 G>A), we used primers CcGTTCATGTTTTGGAAAGTGA and GGGAAGTCC-CATGTTGCAGAtT to perform the PCR, and the product of G allele was 123 bp that had a restriction site for BstBI.

3. Statistical analysis

Haploview was used to examine the LD pattern among SNPs in the promoter and to identify the tagging SNPs [22]. The chi-square analysis was used to compare the distribution of genotypes between the case and control groups. Fisher's exact test was used when a cell count was <5. Logistic regression was also performed to determine the odds ratio (OR) and their corresponding 95% CI to examine associations between the polymorphisms and TB.

4. Results

4.1. SNPs and linkage disequilibrium of IP-10 promoter region

A total of 8 SNPs including 3 novel SNPs not reported in dbSNP, were identified in the promoter region, and minor allele frequencies (MAF) were calculated (Table 2). Linkage disequilibrium (LD) analysis showed that only four SNPs had MAF of 5% or more (Fig. 1). As SNPs (−1502 T>C and −135G>A) were in complete LD, we selected SNP (−135G>A) and the other 2 prevalent SNPs (−1447A>G and −872G>A) (MAF 0.4 and 0.48) to be the tagging SNPs. The mean age of patients was 48.3 years (SD 19.4 years). Male sex was predominant, as it accounted for 67% of the sample. Eighty-eight percent of the patients were classified as new cases, and only Chinese subjects were included in this study.

4.2. Association between promoter SNPs and TB

The genotype distributions of all 3 tagging SNPs followed the Hardy-Weinberg equilibrium in both case and control groups. SNPs (−1447A>G and −872G>A) did not show any association with TB.

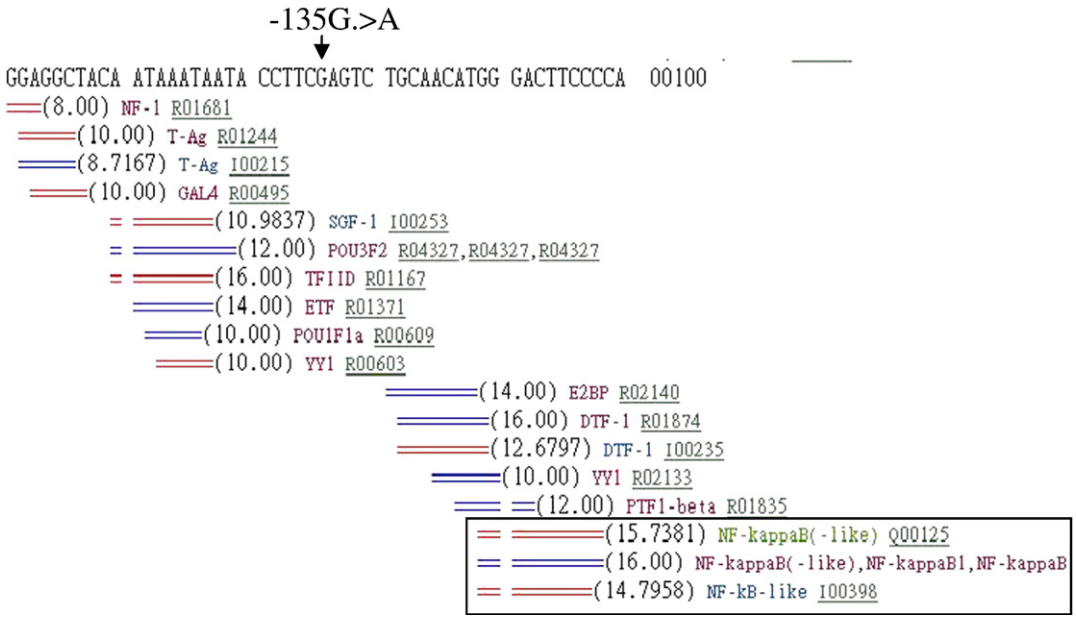

Fig. 2. TESS results. SNP −135G>A (arrow pointed) located 14 bp upstream of NF-kB binding sites (highlighted in box) in the promoter region of CXCL-10.

Interestingly, an SNP (−135G>A) with minor frequency of 0.167 showed an association with TB in both genotype analysis ($p = 0.01$ by Fisher's exact test] and allelic analysis ($p = 0.03$) (Table 3).

The minor allele appeared as a protective allele against TB with an odds ratio of 0.51 (95% C.I.: 0.29–0.91) (Table 4). The association was also confirmed by permutation (non-parametric) analysis. Analysis of haplotype association by Haploview showed that the haplotype "−1447G to −872A to −135G" was significant ($p = 0.013$) and accounted for the protective effect.

The potential transcription factor binding site was revealed by Transcription Element Search System (TESS) (Computational Biology and Informatics Laboratory at the University of Pennsylvania) in the sequence of the region covering −135G>A (Fig. 2). It was found that 14 bp adjacent to this SNP was a binding site for nuclear factor-κB (NF-kB).

5. Discussion

Previous studies showed that infection of macrophages by *Mycobacterium tuberculosis* (MTB) induced expression of specific chemokines [23]. It was mediated through the up-regulation of expression of chemokines encoding genes, including CXCL-10. Higher mRNA expressions of 18 chemokines were found in granuloma of TB patients [24], while other chemokines were suppressed [25].

Among those chemokines studied by Qiu et. al., CXCL-10 showed a significant increase during the early recruitment period [24] and was chemotactic specifically for natural killer cells, monocytes and activated T-lymphocytes [14,26]. It suggests that an important network response is induced by MTB exposure and is partly mediated through CXCL-10. Furthermore, CXCL-10 also plays an essential role in the formation of granuloma [27].

As it is a key player in granulomation inflammation, the genetic variation of CXCL-10 was explored here for the association with susceptibility to TB infection. It has been shown that genetic polymorphisms affected the intensity of cytokine response to MTB antigens [28]. There are also many other examples of disease predisposition (both immunological and chronic diseases) determined by polymorphisms located to promoter region of cytokine genes [29,30]. For example, Barreiro et al showed that two SNPs in the DC-SIGN encoding gene (*CD209*) were associated with predisposition to TB [31,32]. Therefore, we hypothesized that genetic variants in the CXCL-10 promoter would also affect the extent of CXCL-10 stimulation against the mycobacterial pathogens, and host immune reaction against the mycobacterial pathogens.

We showed here that the minor allele of −135G>A was a protective allele for TB. This association was also confirmed by permutation analysis and suggested that haplotype GAG (at the position −1447/−872/−135) accounted for most of the protective effect. Interestingly, Deng et al. [ref needed] found that −135G>A was also highly associated with susceptibility to liver disease progression (cirrhosis or liver cancer) among HBV carriers. The −135G>A polymorphism regulated the expression of CXCL-10 [19], which suggested that this SNP was important in transactivation of CXCL-10. The A allele had both strong nuclear protein binding affinity on EMSA and an increased transactivation capacity on luciferase reporter assay.

The sequence around this SNP (−135G>A) (upstream by 20 bp) was predicted by TESS to contain a potential transcription factor binding site to NF-kB. Tchou-Wong et al. found that, after infection by *Mycobacterium tuberculosis*, NF-kB was activated and interleukin-2 receptor-α (IL-2Rα) expression level was increased [33]. We postulate that this SNP (−135G>A) may also affect the transactivation effect of NF-kB on CXCL-10 expression.

In summary, we identified a new potential protective SNP (−135G>A) of *CXCL-10* gene for the predisposition of TB. More extensive study on neighboring genes and other chemokine genes in immunological reaction pathways will be required to fully elucidate the mechanism of immune response of CXCL-10 in TB.

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