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การเปรียบเทียบความหลากหลายของยีน CXCL9 ระหว่างผู้ป่วยวัณโรคปอด และผู้ที่มีสุขภาพดี ในภาคตะวันออกเฉียงเหนือของประเทศไทย

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Comparison of CXCL9 Polymorphism between Pulmonary Tuberculosis Patients and Healthy Controls in Northeast Thailand

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หลักการและเหตุผล: วัณโรคเป็นหนึ่งในโรคติดเชื้อที่สำคัญ ผลของความหลากหลายทางพันธุกรรมตำแหน่งต่าง ๆ ของยีน (หรือ สนิปส์) ต่อความไวในการติดเชื้อและป่วยเป็นวัณโรคนั้นยังไม่ทราบแน่ชัด CXCL9 เป็นคีโมไคน์ที่มีบทบาทสำคัญในการเรียกเซลล์ภูมิคุ้มกันมาชุมนุมกันในช่วงแรกของการติดเชื้อวัณโรค สนิปส์ของยีน CXCL9 อาจส่งผลต่อความไวในการติดเชื้อวัณโรค อย่างไรก็ตามยังไม่มีการศึกษาความสัมพันธ์ระหว่างความหลากหลายของยีน CXCL9 กับการเกิดวัณโรคมาก่อน การศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาความสัมพันธ์ระหว่างสนิปส์ของยีน CXCL9 ตำแหน่ง rs2276886 ต่อความไวในการติดเชื้อวัณโรคในประชากรภาคตะวันออกเฉียงเหนือของประเทศไทย

วิธีการศึกษา: การศึกษาเชิงสังเกตและวิเคราะห์แบบมีกลุ่มเปรียบเทียบ กลุ่มตัวอย่างเป็นอาสาสมัครตามเกณฑ์คัดกรองประกอบไปด้วย ผู้ป่วยวัณโรคปอด 49 ราย และผู้ที่มีสุขภาพดีโดยไม่เคยมีประวัติสัมผัสกับวัณโรคมาก่อน 40 ราย ที่มารับบริการที่โรงพยาบาลศรีนครินทร์ จังหวัดขอนแก่น เก็บข้อมูลพื้นฐานโดยใช้แบบสอบถามและสกัดดีเอ็นเอจากเลือดของอาสาสมัครแต่ละกลุ่ม ทำการศึกษาสนิปส์ยีน CXCL9 (rs2276886) โดยใช้ tetra-primer ARMS-PCR และ วิเคราะห์

Background and objective: Tuberculosis (TB) is one of the most prominent infectious diseases. The genetic polymorphism or single nucleotide polymorphisms (SNPs) for prediction of susceptibility to *Mycobacterium tuberculosis* (*Mtb*) infection and TB development is still unclear. Chemokine (C-X-C motif) ligand 9 (CXCL9) plays an important role in recruiting immune cells during the early event of *Mtb* infection. The SNPs of CXCL9 gene may contribute to the susceptibility of *Mtb* infection. However, the association between the SNPs of this gene and the susceptibility to TB has yet been investigated. This study aims to determine the association between the SNPs of CXCL9 (rs2276886) and TB susceptibility among the population of Northeast Thailand.

Methods: Observational analytic studies: Case-Control study. The enrolled subjects, according to the inclusion criteria, comprised of 49 active pulmonary TB (ATB) patients and 40 healthy controls (HC) with no known risk to TB exposure who received healthcare services at Srinagarind Hospital, Khon Kaen. The questionnaires were collected. Genomic DNA from venous blood of each

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เปรียบเทียบผลอัลลีลและจีโนไทป์ของสนิปส์ระหว่างกลุ่มผู้ป่วยวัณโรคและผู้ที่มีสุขภาพดี

ผลการศึกษา: สัดส่วนเพศชายต่อเพศหญิงระหว่างผู้ป่วยวัณโรคกับผู้ที่มีสุขภาพดีเท่ากับ 2:1 และ 1:2 ตามลำดับ และอายุเฉลี่ยเท่ากับ 50.9 ± 14.7 และ 40 ± 15 ตามลำดับ ผลการศึกษาพบว่าความถี่อัลลีล โดย A เทียบกับ G (OR=0.847, 95% CI=0.395-1.815) และความถี่จีโนไทปน์ของสนิปส์ยีน CXCL9 โดย AA+GA เทียบกับ GG (OR=0.762, 95% CI=0.328-1.770) AA เทียบกับ GA+GG (OR=0.804, 95% CI=0.153-4.221) AA เทียบกับ GG (OR=0.724, 95% CI=0.133-3.948) และ GA เทียบกับ GG (OR=0.769, 95% CI=0.318-1.862) ระหว่างผู้ป่วยวัณโรคและผู้ที่มีสุขภาพดี ไม่พบว่ามีความแตกต่างอย่างมีนัยสำคัญทางสถิติ

สรุป: ผลจากการศึกษานี้ไม่สนับสนุนความสัมพันธ์ระหว่างความหลากหลายของยีน CXCL9 (rs2276886) กับการเกิดวัณโรคในกลุ่มประชากรภาคตะวันออกเฉียงเหนือของประเทศไทย การศึกษาสนิปส์เพิ่มเติมของยีน CXCL9 หรือ ยีนคีโมไคน์อื่น ๆ อาจทำให้ได้ข้อมูลที่ชัดเจนมากยิ่งขึ้น

group of participant was extracted. The tetra-primer ARMS-PCR method was used to define the CXCL9 (rs2276886) SNPs in individuals. Genotype and allele between ATB and HC were analyzed and compared.

Results: The male/female ratio in ATB and HC group were 2:1 and 1:2, respectively. The average age of ATB and HC were 50.9 ± 14.7 years and 40 ± 15 years, respectively. It was found that the allele distribution of A vs G (OR=0.847, 95% CI=0.395-1.815) and genotypic distributions of CXCL9 including AA+GA vs GG (OR=0.762, 95% CI=0.328-1.770), AA *vs* GA+GG (OR=0.804, 95% CI=0.153-4.221), AA vs GG (OR=0.724, 95% CI=0.133-3.948) and GA vs GG (OR=0.769, 95% CI=0.318-1.862) between ATB and HC were not significantly different.

Conclusion: The association between the CXCL9 (rs2276886) polymorphisms and TB susceptibility in the population of Northeast Thailand was not supported by the result in this study. Other genetic variations within this gene or other chemokine related genes might be further investigated for clearer information.

Keywords: CXCL9, Northeast Thailand, Single nucleotide polymorphisms, Susceptibility, Tuberculosis

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Introduction

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (*Mtb*). It is estimated that one-third of the world population is infected by *Mtb* and 5-10% of infected individuals will develop the active disease. There were several factors contributing to the susceptibility of *Mtb* infection and TB development. The prevalence of TB is varied among geography, race and ethnicity which suggests that there is a genetic predisposition to TB susceptibility. Many studies investigated the single nucleotide polymorphisms (SNPs) that associate with TB susceptibility[1-3]. However, the specific genetic variations that indicate the susceptibility to TB and *Mtb* infection remain unclear.

Chemokine (C-X-C motif) ligand 9 (CXCL9) is a chemokine subset of CXCR3 ligands family, which is produced by IFN-γ in a dependent manner[4]. Several studies demonstrated the association between CXCL9 level and the development of TB. An early granuloma formation was regulated by neutrophils via CXCL9 signaling pathway and anti-CXCL9 treated wild type mice showed an impaired granuloma formation[5]. CXCL9 was significantly increased during pulmonary TB and might indicate that CXCL9 participates in T cells and other effector cells trafficking to the site of infection[6, 7]. Recently, a significant decrease in CXCL9 level proved to be a useful biomarker for monitoring with *Mtb*-infected animal undergoing treatment therapy[8, 9]. Furthermore, the significantly lower CXCL9 level was found in TB relapse in mice with good treatment response[10]. CXCL9 combined with other cytokines and chemokines had shown to be useful markers for TB diagnosis[11-13].

Similarly, the cytokine level of CXCL9 also improved the diagnostic performance for latent TB infection screening among healthcare workers[14]. Previously, CXCL9 was highly expressed among disease patients[15]. CXCL9 polymorphism (rs2276886) was associated with susceptibility to seasonal allergic rhinitis and A allele was associated with protection against pediatric Crohn's disease[16, 17].

To date, the association between CXCL9 polymorphism and TB susceptibility has never been studied. Based on the information above, CXCL9 is a good candidate gene to explore in the study of host genetics-TB association. We hypothesize that the genetic variations in CXCL9 is associated with TB susceptibility. Therefore, we aim to determine the association between CXCL9 (rs2276886) and TB susceptibility in the Northeast Thailand population.

Material and Methods

Study population and setting

The study population consists of 89 participants, including 49 active TB patients (ATB) who received the medical treatment at the tuberculosis clinic and 40 healthy controls with no known risk of TB exposure who received health check-up at Srinagarind Hospital, Khon Kaen, Thailand. The participants were enrolled between September 2012 and March 2014. The blood samples and questionnaires were collected. Demographic data and information regarding underlying diseases were collected. Chest X-ray and anti-HIV test were performed on all TB patients. This study was approved by the Committee of Human Research (Ethic number HE551100) and informed consent was obtained from all participants.

Inclusion and exclusion criteria

ATB patients who had TB symptoms such as chest X-ray lesion, chronic cough and weight loss, and were tested positive for microbiological test, i.e., acid fast bacilli (AFB), Xpert MTB/RIF (Cepheid, Sunnyvale, USA) or TB culture, were defined as TB patients. HC without signs and symptoms of TB with normal chest X-ray results, and no history of TB infection or TB exposure were defined as healthy controls. The participants with HIV or received immunosuppressive agent were excluded from the study.

DNA sample preparation

Venous blood samples were collected in EDTA tubes and stored at -20°C until use. The genomic DNA was extracted using an isopropanol-fractionation with concentrated NaI and SDS protocol[18].

Amplification-Refractory Mutation System (ARMS)

CXCL9 polymorphism (rs2276886) was genotyped using the tetra-primers ARMS-PCR method[19]. Polymerase chain reaction (PCR) was performed using PCR master mix (Life Technologies, USA) in a total volume of 25 mL, containing 1X PCR buffer, 1.5 μM $MgCl_2$, 0.2 μM dNTP mix, 0.1 μM of inner-forward and outer reverse-primer, 0.2 μM and 0.3 μM of outer forward and inner reverse primer respectively, 1U Taq polymerase (Life Technologies, USA) and 100 ng/mL DNA samples. The primer sequences used in the PCR were shown in Table 1. The PCR was performed using Bio-Rad C1000 Thermal cycler (Bio-Rad, USA). The amplification condition was 5 min at 95°C followed by 35 cycles of 30 sec at 95°C, 30 sec at 54°C and 36 sec at 72°C with a final step at 72°C for 10 min. The amplified products were analyzed by 2% gel electrophoresis, stained by ethidium bromide and visualized using Gel Doc™ XR+ System (Bio-Rad, USA). The samples representative of each particular genotype were submitted for DNA sequencing (Bioneer, South Korea).

Data analysis

Statistical analysis was performed using SPSS version 16 (SPSS Inc, Chicago, IL, USA). The allele and genotype distribution between group of ATB patients and HC group were analyzed using Chi-square test. The comparisons of demographic data between groups were performed using Chi-square and student t's test. The risk estimation (OR) of both univariate and multivariate analysis were performed using logistic regression. P-values less than 0.05 were considered statistically significant.

Table 1 Primers used for CXCL9 (rs2276886) genotyping

Primers	Primer sequence
Outer forward primer	5'-ATA TGC CAT ACA TTG TGT AGC-3'
Outer reverse primer	5'-ATA CAG GAG TGA CTT GGA AC-3'
Inner forward primer (G allele)	5'-CAC TGA GAA GCT TTT ATG ACT AAC-3'
Inner reverse primer (A allele)	5'-CTG CAT TGA AGA GTA ATA TTG GA-3'

Results

Characteristics of participants

The demographic data between ATB patients (n=49) and HC (n=40) groups were described in Table 2. The male/female ratio in the TB and control groups were 2:1 and 1:2, respectively. The average age of TB patients and healthy controls were 50.9 ± 14.7 years and 40 ± 15 years, respectively. The BMI in TB patient group was 20.6 ± 3.2 (kg/m^2) and in healthy control group was 22.6 ± 3.4 (kg/m^2) (Table 2).

Genotypic determination of CXCL9 (rs2276886) using tetra-primers ARMS-PCR

Two external primers generated the control band (594 bp) and other two inner-primers generated the allele specific products, i.e., 232 bp for G allele and 408 bp for A allele (Fig 1). To verify the genotypic results, 20% of the samples were randomly selected to repeat the PCR and the result was 100% concordant. In order to validate the SNP genotyping result, sequencing was performed. The sequencing result and genotypes based on the tetra-primers ARMS-PCR was in concordance (data not shown).

Genotypic comparison between ATB patient and HC group

The association between host-genetic polymorphism and TB susceptibility were determined. The genotypes and allele distribution of CXCL9 (rs2276886) in ATB (n=49) and HC (n=40) are shown in Table 3. There is no significant difference of the genotype frequencies of CXCL9 (rs2276886) in ATB vs HC, which were 59.2% vs 52.5% for homozygous GG (wild type), 34.7% vs 40% for heterozygous GA and 6.1% vs 7.5% for homozygous AA (mutant), respectively. No significant association was found between allelic frequencies of CXCL9 (rs2276886) between ATB and HC group, A vs G, (OR=0.847, 95% CI=0.395-1.815, p=0.669). The multivariate regression analysis adjusted by age and gender was performed. However, there is still no significant association between allelic frequencies of CXCL9 to the TB disease (data not shown). In addition, there was no significant difference in all possible comparative genetic models when compared between case and control groups as shown in Table 4.

Table 2 Demographic characteristics of TB patients and healthy controls.

Characteristics	TB patients (n=49)	Healthy controls (n=40)
Sex (male/female ratio)	2:1	1:2
Age (yr.) (mean±SD)	50.9.1 ± 14.7	40 ± 15
BMI (kg/m^2) (mean±SD)	20.6 ± 3.2	22.6 ± 3.4

Discussion

CXCL9 is associated with the development of *Mtb* infection and TB in many aspects including early pathogenesis[6, 7, 9], granuloma formation[20, 21] and diagnosis[11-14]. There are many evidences that support the association between chemokine polymorphism such as MCP-1 and CCL-5 and TB susceptibility[2, 22].

Table 3 Genotype and allele distribution of CXCL9 polymorphism in case and control

CXCL9 (rs2276886)	TB patients, n (%)	Healthy controls, n (%)
Genotype		
GG	29 (59.2)	21 (52.5)
GA	17 (34.7)	16 (40)
AA	3 (6.1)	3 (7.5)
Allele		
G	46 (69.7)	37 (66.1)
A	20 (30.3)	19 (33.9)

Table 4 Comparative genetic model of CXCL9 SNP (rs2276886)

Genetic models	OR	95% CI	p-values
A vs G	0.847	0.395-1.815	0.669
AA+GA vs GG	0.762	0.328-1.770	0.527
AA vs GA+GG	0.804	0.153-4.221	1.000
AA vs GG	0.724	0.133-3.948	1.000
GA vs GG	0.769	0.318-1.862	0.561

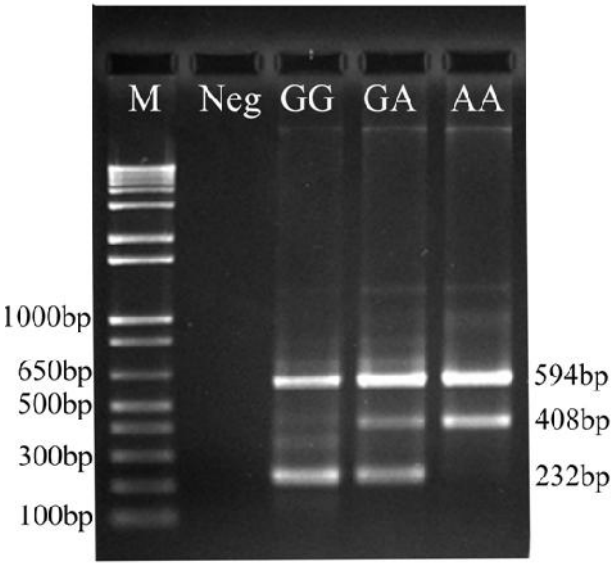

Fig 1 The electrophoresis patterns of tetra-primer ARMS-PCR for CXCL9 polymorphism (rs2276886) genotyping. The representative pattern of each genotype was demonstrated. Note: M is DNA marker, Neg is Negative control, GG, GA and AA are genotypes of CXCL9 (rs2276886).

However, none of them involved CXCL9 genetic polymorphism and development of TB. Base on the reasons stated above, we selected CXCL9 gene as a candidate gene for the study of host genetic-TB association in the population from Northeast Thailand. To our knowledge, this is the first study exploring the association between CXCL9 SNPs and TB susceptibility.

Our study did not find any significant difference in CXCL9 SNP rs2276886 genotype and allele frequencies between TB patients and healthy controls. It can be interpreted that CXCL9 SNP rs2276886 is not associated with the susceptibility of *Mtb* infection and development of TB in the population from Northeast Thailand. On the other hand, the CXCL9 polymorphism might actually be associated with TB susceptibility and the negative result might be due to many reasons. First, the SNP that we have selected is specific to autoimmune and inflammatory disease. We selected the SNP at position rs2276886 to study because this SNP contain good variation among genotypes in Asian population and it was reported to be associated with seasonal allergic rhinitis and pediatric Crohn's disease[16, 17]. Systemically, the genetic analysis of the SNPs from the whole sequence of CXCL9 might provide the clearer results. Second, this study might be limited by the relatively small sample size that affects the power of statistical analysis. Third, the ethnicity and study population can influence the result of the disease susceptibility due to controversial results of particular SNP from different population around the world[1]. Ideally, the global sampling of population that covers a broad spectrum of ethnicity and region for the study of genetic polymorphisms, such as chemokine genes and CXCL9, could provide a more significant and representative result.

The controls that we used to compare were healthy persons with no known risk of TB exposure. The healthy control group might not be the perfect control group for the TB susceptibility study because a healthy person might harbor the susceptible genetic background but remain uninfected due to no exposure of TB. Even that, majority of the studies exploring the genetic susceptibility of TB, prefer the use of healthy controls with no known risk of TB exposure as the control group[2, 3, 23]. This confounding factor might be stronger in low TB prevalence area where it has less likelihood of exposure to TB in the community. Therefore, the genetic distribution from improper control group can affect the analysis and result. In our setting, Thailand is one of the high TB burden countries. Hence, the healthy control group in our population should provide a certain confidence of resistance phenotype to *Mtb* infection. The latent TB infection status can be measured using the screening test such as the interferon gamma release assays (IGRA)[24, 25]. Nonetheless, this test measures the

memory T cell respond to *Mtb* antigen and cross-reacts with certain species of mycobacteria. In our study, the status of *Mtb* infection based on the screening test was performed in the healthy control and found just only 6 IGRA positive cases (data not shown). The comparison between TB patients and healthy controls, excluding these 6 cases, still provide the same insignificant association to the CXCL9 SNP. Ideally, resistance of TB could be inferred from a control group that has exposure to TB in significant durations, for example, the healthcare workers and the households that have close contact with the TB patient, but remained uninfected. Notably, the resistance phenotype of individual to TB might have the certain level of infectious dose too. These confounding factors could affect the result and interpretation, and should be considered for study design and analysis.

In summary, this is the first study exploring the association between CXCL9 polymorphism and TB susceptibility. Our findings showed that CXCL9 (rs2276886) polymorphism is not associated with *Mtb* infection and development of TB. However, other genetic variations within this gene or other chemokine related genes could be further investigated for its association with TB susceptibility.

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