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Original Article

Correlation between NRAMP1 gene polymorphism and susceptibility of pulmonary tuberculosis in Chinese Tibetan population

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Abstract: Tuberculosis is one widely distributed infectious disease worldwide, and severely threatens public health. Lung tuberculosis has relatively higher incidence within Tibetan region of China, probably due to unique genetic background of Tibetan Population and certain susceptibility genes. NRAMP1 participates in host resistance against mycobacterium tuberculosis, although its correlation with tuberculosis prevalence in Tibetan is still unclear. This study aimed to investigate the possible correlation between NRAMP1 polymorphism and tuberculosis in Tibetan Population. A total of 292 Tibetans diagnosed with lung tuberculosis were recruited in parallel with 290 healthy Tibetan volunteers as the control group. Venous blood samples were collected for separating genomic DNA of blood cells. PCR-RFLP sub-typed NRAMP1 gene and grouped patients based on genotype. Sample representativeness, chi-square test and OR were examined for investigating the correlation between NRAMP1 and lung tuberculosis. Genomic DNA extracted had satisfactory quality for sequencing and PCR-RFLP by electrophoresis. NRAMP1 showed polymorphism at multiple sites, fitting Hardy-Weinberg equilibrium. 190 (64.6%), 100 (34.0%) and 4 (1.4%) of patients had intact TGTG/TGTG, TGTG/TGTG deletion, and homozygous TGTG deletion genotypes, respectively. Control group had 230, 58 and 2 cases of these genotypes, with significantly higher heterozygous mutation (TGTG/TGTG deletion) ratio (χ^2=6.80, p=0.0042). Polymorphism of NRAMP1 gene 3'UTR in Tibetan Population might be related with tuberculosis susceptibility as it lacked equilibrium linkage of heritance. Those Tibetan people carrying TGTG deletion alleles might be more susceptible for tuberculosis.

Keywords: Genetic polymorphism, NRAMP1, 3'UTR, tuberculosis, disease susceptibility

Introduction

Tuberculosis is one widely distributed infectious disease worldwide, and severely threatens public health [1]. The prevention and treatment of tuberculosis are thus of critical importance [2, 3]. About 5%~10% of total population are carriers of tuberculosis [4, 5], and this rate is especially higher in Tibet, China [6], probably due to prevalence of susceptible genes [7].

The susceptibility towards tuberculosis has variance across ethic groups [8, 9]. Moreover, different hosts presented different sensitivities for tuberculosis, probably related with polymorphism of various genes [10] such as NRAMP1, human leukocyte antigen, mannose binding lectin [11, 12], all of which are closely correlated with tuberculosis susceptibility.

Incidence of tuberculosis is especially high in Tibet, China. NRAMP1 gene has been reported to be related with resistance against mycobacterium tuberculosis [13, 14]. The correlation between NRAMP1 and Tibetan population, however, is still unclear yet.

This study aimed to investigate the relationship between NRAMP1 and tuberculosis, as NRAMP1 is probably related with disease susceptibility. We found that polymorphism of NRAMP1 gene showed imbalance of linkage, indicating TGTG-deleted allele might be tuberculosis susceptible gene in Tibetan people, indicating that those people with TGTG deleted

Table 1. General information of research subjects (x±s)

Parameter	Experiment	Control	P
Age (year)	53.1±19.1	57.1±18.2	0.441
SOFA	5.0±1.2	8.7±2.6	0.002
APECHE II	10.6±6.4	17.3±5.5	0.021
CRP	10.6±6.4	11.1±7.2	0.896
WBC	13.8±7.4	14.3±6.4	0.806

allele might be more susceptible for tuberculosis.

Materials and methods

Research subject

Inclusive criteria: Early stage pulmonary tuberculosis or mild disease, with fever, cough, septum, uncomfortable or pain in upper stomach, frequently accompanied with sour regurgitation, belching and fatigue. Other symptoms include anorexia, nausea, vomiting, diarrhea, all of which fitted pathology and toxicology features.

Exclusive criteria: Accompanied with pneumonia, pulmonary abscess, fungal infection, primary/metastatic lung cancer, pulmonary cyst and other benign lung lesions.

A total of 292 Tibetan patients who were diagnosed based on abovementioned criteria from June 2014 to June 2016 in Naq Qu, Tibet, China were recruited as the experimental group. Another cohort of 290 healthy Tibetan volunteers were recruited as the control group. All diagnosis was in accordance with newly developed diagnostic and subtyping criteria stipulated by China in 2013. All research subjects were native Tibetan habitants without biological relevance. All participants have singed informed consents of this study [15].

Sample collection

Blood samples were collected as previously reported [16, 17]. Anti-coagulated venous blood samples were separated and extracted for genomic DNA of blood cells. DNA was specifically isolated from leukocytes.

PCR-restriction fragment length polymorphism (RFLP) analysis

PCR was performed for subtyping [18]. Grouping analysis was performed on all groups based on genotypes. Gene-bank was accessed and primers were designed (5'-GCATC TCCCC AATTC ATGGT-3', and 5'-AACTG TCCCA CTCTA TCCTG-3') [19]. Samples were collected, purified and sequenced in BGI (China). Lengths and sequence of amplified fragments were confirmed. Samples DNA with different genotypes were used as the template for PCR amplification. Products were then purified and sequenced. PCR reaction system adopted previously reported protocols [18].

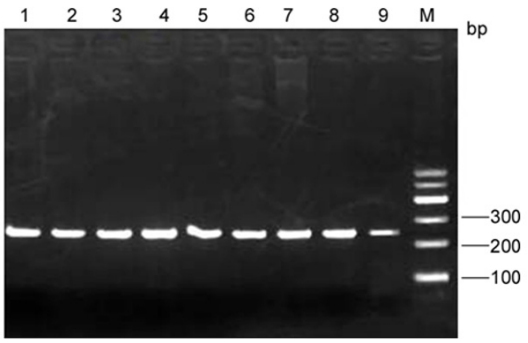

Figure 1. PCR results of 3'UTR of NRAMP1 gene. Lane M, DNA marker; lane 1-9, PCR products of samples.

Restriction enzyme digestion

PCR products were identified by enzyme digestion. Restriction enzyme FokI was mixed with PCR products for 37°C 2 h incubation, and was treated at 55°C for 5 min to inactivate restriction enzymes. 6× loading buffer was added into enzyme digestion products, which were examined in agarose gel electrophoresis as routine methods [20]. 1% agarose gel was firstly prepared, and enzyme digested products were loaded into wells of agarose gel (6 μL per well). DNA marker was added for 120 V electrophoresis for 25 min, Analysis was performed in gel imaging apparatus after electrophoresis.

Statistical processing

SPSS 13.0 software was used for statistical analysis as previously documented [16, 17]. A statistical significance was defined when $p<0.05$.

Results

General information of research subjects

A total of 292 Tibetan patients who were diagnosed based on above mentioned criteria from

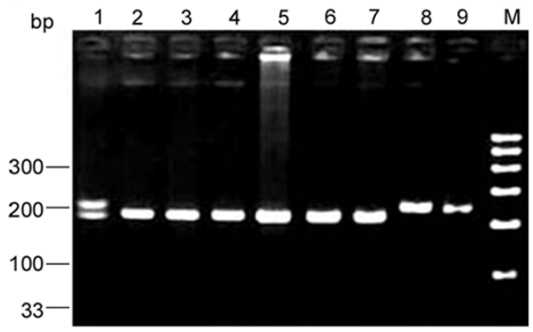

Figure 2. Enzyme digestion and identification of PCR products of NRAMP1 3'UTR. Lane M, DNA marker; lane 1, TGTG+/deletion (del) genotype; lane 8-9, del/del genotype; lane 2-7, TGTG+/TGTG+ genotype.

June 2014 to June 2016 in Naq Qu, Tibet, China were recruited as the experimental group. Another cohort of 290 healthy Tibetan volunteers were recruited as the control group. All diagnosis was in accordance with newly developed diagnostic and subtyping criteria stipulated by China in 2013. Averaged age of patients was 55.3±18.4 years (18~88 years), and averaged age of healthy volunteers was 58.3±17.4 years (25-83 years). No significant difference existed between two research groups (p=0.86, **Table 1**). Pathology and cytology features of tuberculosis patients fitted inclusive criteria, presenting as significant difference of sepsis-related organ failure assessment (SOFA), APECHE II, C reaction protein (CRP) and white blood cell (WBC) number between experimental and control groups.

Electrophoresis results

Genomic DNA extracted in this study generally showed satisfactory quality as shown in electrophoresis, and can be used for sequencing and PCR-RFLP analysis. As shown in **Figure 1**, PCR results of 3'UTR of NRAMP1 showed good quality and about 240 bp length.

Restriction enzyme digestion

In order to assess the accuracy of the PCR production of NRAMP1, enzyme digestion was performed using FokI enzyme. As shown in **Figure 2**, polymorphism was observed in the 3'-UTR of NRAMP1 with heterozygotes having a big molecular weight (Lane 1 and 8-9) and homozygotes having small molecular weight (Lane 2-7).

Sequencing and confirmation of NRAMP1 polymorphism

Sequencing of polymorphism of 3'UTR of NRAMP1 was shown as in **Figure 3**. Results indicated genetic polymorphism of NRAMP1 gene at multiple loci.

Genotype and allele frequency of NRAMP1 polymorphism

All research subjects fitted Hard-Weinberg equilibrium. In experimental group, 190 (64.6%) patients showed TGTG/TGTG genotype at 3'UTR of NRAMP1, 100 (34.0%) patients showed TGTG/TGTG-del heterozygous genotype, and 4 cases (1.4%) showed TGTG-del/TGTG-del homozygous mutant forms. In normal control group, there were 230 (79.3%), 58 (20.0%) and 2 (0.7%) cases showing TGTG/TGTG, TGTG/TGTG-del, and TGTG-del/TGTG-del phenotypes. The ratio of TGTG/TGTG-del phenotype was higher in experimental group compared to control group (χ^2=6.80, p=0.0042). Results showed 85% allele frequency of TGTG, and 15% frequency of TGTG-del allele (**Table 2**).

Discussion

Tuberculosis induced by mycobacterium tuberculosis is one widely distributed infectious disease in worldwide, and severely threatens public health. Incidence of tuberculosis in Tibet, China is especially high, probably due to unique genetic background and certain susceptible genes of Tibetan Population [21, 22].

NRAMP1 is known to be related with host resistance against mycobacterium tuberculosis. However, the correlation between NRAMP1 and disease onset in Tibetan Population is still inconclusive. This study aimed to investigate possible correlation between NRAMP1 polymorphism and tuberculosis of Tibetan Population. Currently there have been various studied reporting the correlation between NRAMP1 gene polymorphism and tuberculosis [23, 24]. Previous study showed that polymorphism of 3'UTR site was probably susceptible factor causing pulmonary tuberculosis in Han Population in Northern China [25]. Another study in Thailand, however, rejected the correlation between NRAMP1 gene and susceptibility of lung tuberculosis [26]. Another study among 147 tuberculosis patients and 145

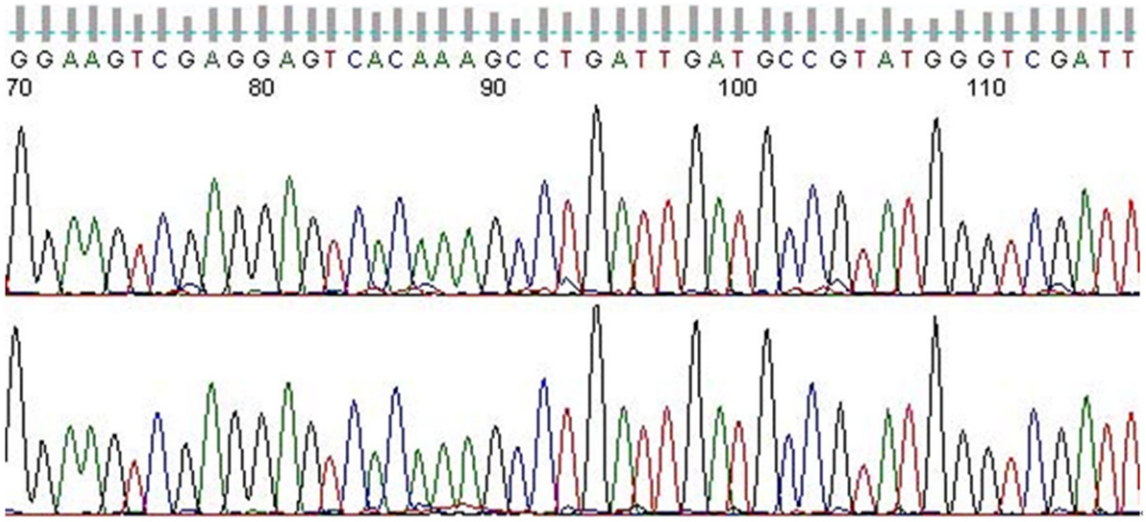

Figure 3. Sequencing results of NRAMP1 polymorphism. TGTG-del site was indicated by underscored lines, containing Fok I digestion site (GGATG), whilst TGTG-del loci was identified by black arrows, without Fok I digestion site.

Table 2. Genotype/allele frequency of NRAMP1 polymorphism

Genotypes	Patients (292)	Control (290)	χ^2	P	Risk factor
TGTG+/+	290	230	6.80	0.0042	1
TGTG+/-	100	58	6.72	0.0015	2.09
TGTG+/+	4	2	5.81	0.0026	2.42

healthy controlled individuals revealed correlation between NRAMP1 and tuberculosis.

Previous study showed no significant difference of single intron 4 (INT4) and D543N gene polymorphism between tuberculosis patients and healthy individuals. Combined analysis, however, showed strong correlation between lung tuberculosis and those two factors. A study in Taiwan, China compared INT4, D543N, 3'UTR and 5'(CA)n regions of NRAMP1 gene between 105 local and 110 Han Population tuberculosis patients and found correlation between polymorphism at INT4 and 5'(CA)n regions of NRAMP1 with disease susceptibility in local residents, whilst Han patients did not present correlation with NRAMMP1 polymorphism and tuberculosis susceptibility [26]. Another study in Japan showed no significant difference of INT4 or D543N genotype between normal controlled people and tuberculosis patients, although correlation was observed in those patients with vacuole formation [27]. In Uighur ethnic group of Xinjiang, China, polymorphism at 3'UTR of NRAMP1 was correlated with tuberculosis susceptibility, whilst polymorphism at Fok1 and Taq1 loci of VDR gene was not correlated with tuberculosis of Uighur ethnic population. These studies [28] indicated that VDR-ff was one candidate of susceptible genes.

Certain limitations, however, existed in current study, mainly due to the relatively smaller samples size, and the lack of mechanism study showing involvement of NRAMP1 in susceptibility of lung tuberculosis.

Conclusion

NRAMP1 polymorphism is probably correlated with lung tuberculosis. NRAMP1 polymorphism shows imbalanced linkage, indicating that TGTG-del allele might be one susceptible gene for tuberculosis in Tibetan Population, in which those carrying TGTG-del genotype are more susceptible for tuberculosis.

Disclosure of conflict of interest

None.

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