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Microbiology Section

Correlation of Single Nucleotide Polymorphism 35-Kb Upstream of HLA-C and Clinical Profile of Multidrug-Resistant Tuberculosis

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ABSTRACT

Introduction: The SNP HLA-C-35 kb (rs9264942) may contribute to the host immune defense mechanism by affecting the cell surface expression pattern of HLA-C and antigen presentation to CD8+ cytotoxic cells. Thus, this SNP may contribute to intracellular multidrug-resistant (MDR)-tuberculosis (TB) infection.

Aim: To examine the association between the SNP HLA-C-35 kb (rs9264942) and the clinical profile of MDR-TB infection.

Settings and Design: MDR-TB-positive patients were followed from May 2012 to December 2013 to observe the progression of MDR-TB infection. Non-TB individuals and non-MDR-TB individuals were also recruited as controls.

Materials and Methods: The patients' HLA-C-35 kb (rs9264942) status was determined by PCR.

Results: The C allele was slightly more frequent in the MDR-TB patients than in the non-MDR TB patients (OR= 1.28; 95% CI: 0.701 – 2.328). The C allele was found to be more frequent in the MDR-TB patients exhibiting pulmonary fibrosis (OR= 2.13; 95% CI: 0.606 – 7.480) or pulmonary infiltrates (OR= 3.17; 95% CI: 0.690 – 14.598) and among the MDR-TB patients who were classified as underweight (OR= 8.00; 95% CI: 1.261 – 50.770). The CC genotype was associated with the treatment after failure of category II group (OR= 4.17; 95% CI: 1.301 – 13.346).

Conclusion: The C allele SNP HLA-C-35 kb (rs9264942) may contribute to the clinical profile in MDR-TB infection.

Keywords: Human leukocyte antigen C, Major histocompatibility complex class I, Rs9264942

INTRODUCTION

Multidrug-resistant tuberculosis (MDR-TB) is an infection caused by *Mycobacterium tuberculosis* that is resistant to at least two of the most effective anti-TB drugs, isoniazid and rifampicin [1]. The number of MDR-TB-infected patients appears to be increasing. Because MDR-TB results in a high probability of treatment failure and it can easily spread from person to person via the same mode of transmission as drug-sensitive TB [1]. The management of MDR-TB has become even more complex because the second-line drugs display high toxicity and require a longer treatment period [2]. Among the MDR-TB patients who received treatment, only 48% were successfully treated [1]. Therefore, determining the factors that affect the progression and severity of MDR-TB infection is important for understanding the treatment outcome.

Immunological and genetic factors in the patients may also contribute to the prevalence of MDR-TB [3]. MDR-TB patients tend to exhibit impaired T helper (Th) 1 response, which is characterized by reduced interferon-gamma (IFN-γ) production, enhanced interleukin-10 (IL-10) production, and increased circulating T regulatory cell (Treg) levels [4,5]. This impaired Th1 response may be crucial for promoting the progression of MDR-TB [4].

Major histocompatibility complex class I (MHC-I) is known to contribute in intracellular infection by presenting the cytosolic peptides to CD8+ T cells [6]. The MHC-I molecules can also present the exogenous peptides through a process called the cross-presentation by macrophages and dendritic cells (DCs) which will enhance the cytotoxic activity of CD8+ T cells [6,7]. MHC-I consists of two groups, the highly polymorphic classical molecules [human leukocyte antigen (HLA)-A, -B, and -C] and the conserved non-classical molecules (HLA-E, -F, and –G) [7].

HLA-C is one of the polymorphic classical molecule which is located on chromosome 6 of the MHC-I locus [7]. HLA-C tends to display low expression on the cell surface, however, found to be expressed in a higher level in antigen presenting cells (APCs) such as macrophage [7,8]. Host genetic variations in HLA-C may contribute to the host immune defense mechanism by affecting the HLA-C expression pattern on the cell surface. A polymorphism in the HLA-C gene has been reported to enhance the expression of HLA-C on the cell surface. The variant rs9264942 is located 35-kb upstream of the HLA-C gene (-35C/T), and individuals carrying the C allele (-35C) display higher HLA-C expression on the cell surface [9]. The single nucleotide polymorphism (SNP) HLA-C-35C is known to confer a better outcome for intracellular infections due to the increased expression of HLA-C on the cell surface, which promotes antigen presentation and recognition by CD8+ cytotoxic T cells and natural killer (NK) cells [9,10].

CD8+ cytotoxic T cells play an important role in cellular immunity against intracellular *M. tuberculosis* [11]. The class I major histocompatibility complex (MHC), which presents mycobacterial antigens on the cell surface, can activate CD8+ cytotoxic T cells that recognize and kill *M. tuberculosis*-infected macrophages. This mechanism is important for the regulation of the growth and clearance of *M. tuberculosis* [12]. However, the cytotoxic activity of CD8+ cytotoxic T cells has been reported to be decreased in MDR-TB patients because of their enhanced Th2 profile [4]. Because of the difference in the expression of HLA-C and as a result, in antigen presentation to CD8+ cytotoxic T cells [9,10], HLA-C-35 kb (rs9264942) may contribute to the progression of MDR-TB infection. However, to our knowledge, no study has examined the association between this polymorphism and MDR-TB infection. Moreover, by understanding the contribution of the SNP HLA-C-35 kb to MDR-

TB infection, we may also improve our understanding of the genetic contribution of the host to the progression and severity of MDR-TB infection. Therefore, the aim of our study was to examine the association between the SNP HLA-C-35 kb (rs9264942) and the clinical profile of MDR-TB infection. We are the first to report data regarding the SNP HLA-C-35 kb (rs9264942) in the Indonesian population.

MATERIALS AND METHODS

Participants

A cohort study was performed using 52 MDR-TB patients. The MDR-TB-positive patients, TB-infected patients showing resistance to at least isoniazid and rifampisin by the drug susceptibility testing (DST), were followed from May 2012 to December 2013 to assess the clinical characteristics of these patients, including body mass index (BMI) and complete blood count (haemoglobin, haematocrit, erythrocyte, leukocyte, and thrombocyte levels). The CD4+ T-helper cell counts and the levels of IFN-γ and IL-10 were measured and thorax imaging was performed on the MDR-TB patients. All of the MDR-TB patients suffered from bacteriologically confirmed TB and were classified as pulmonary tuberculosis and HIV-negative. A total of 52 MDR-TB patients were subjected to genotypic analysis of the SNP HLA-C-35 kb (rs9264942). To determine the association between HLA-C-35 kb (rs9264942) and disease susceptibility, genotypic analysis was also performed on 66 non-MDR TB patients and 34 healthy individuals. The non-MDR TB patients were previously confirmed to suffer from non-MDR-TB during the study and were used as a control group. Approval was obtained from the Institutional Review Boards of the Faculty of Medicine of Sebelas Maret University and Dr. Moewardi General Hospital, Surakarta, Indonesia. Written informed consent was obtained from all participants in this study. All of the procedures were conducted according to the principles of the Declaration of Helsinki.

DNA isolation and molecular determination of the SNP HLA-C-35 kb: Genomic DNA was isolated from whole blood using the High Pure PCR Template Preparation Kit (Roche Applied Science, Mannheim, Germany). Then, the genomic DNA was used as a template for amplifications that were conducted using a common forward primer (5′-GCC CAT ACC TGT TTA TAC ATC CA-3′) and allele-specific reverse primers (5′-CAG AAA GTC CCA CAG TGC CTG-3′ for detecting the C allele and 5′-CAG AAA GTC CCA CAG TGC CTA-3′ for detecting the T allele) [13]. Fast Start HiFi PCR System dNTPack (Roche Applied Science) polymerase was used in the reaction, which was performed under the following conditions: an initial denaturation step at 95ºC for 2 minutes followed by 40 cycles of 95ºC for 30s, 60ºC for 30s, and 72ºC for 30s. At the end of these cycles, a final extension step was performed at 72ºC for 7 minutes. For data interpretation, we conducted electrophoresis using a 1.5% agarose gel (100 V for 30 minutes), which was visualized using a UV transilluminator. All samples were tested at least twice.

Flow cytometry analysis

The CD4+ T-helper cell count was performed on fresh whole-blood samples that were collected in an ethylenediaminetetraacetic acid (EDTA)-containing tube. This assay was conducted using a BD FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. All samples were tested at least twice.

Cytokine assays

The concentration of the cytokines IFN-γ and IL-10 in the plasma samples were evaluated via sandwich enzyme-linked immunosorbent assay (ELISA) using the Quantikine® ELISA Human IFN-γ Immunoassay and the Quantikine® ELISA Human IL-10 Immunoassay (R&D Systems, Minneapolis, MN, USA), respectively. These assays were performed according to the protocols provided in the kits and were conducted in duplicate for each sample.

STATISTICAL ANALYSIS

The statistical analyses were performed using IBM SPSS Version 20 software (IBM Corporation, United States). All of our categorical data were analysed using the Pearson Chi-Square test and Fisher's exact test. Results displaying a p-value < 0.05 were considered to be significant. All of the odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated using bivariate models. The data for the rs9264942 genotype was evaluated for Hardy-Weinberg equilibrium using the Chi-Square test.

RESULTS

Clinical characteristics: In the group of 52 MDR-TB patients, 55.8% (29/52) were females. The mean age of the 52 MDR-TB patients was 40.0 (±10.4) years, which ranged from 19 to 62 years. A total of 74.5% (38/51) of the patients were classified as underweight [Table/Fig-1]. None of the MDR-TB or non-MDR TB patients were classified as overweight. A greater percentage of the MDR-TB patients {74.5% (38/51)} than the non-MDR TB patients {58.5% (38/65)} were classified as underweight (OR= 2.08; 95% CI: 0.933 – 4.622).

Association of HLA-C-35 kb with the clinical course of MDR-TB: In the present study population (152 individuals), 48 (31.6%) participants carried the CC genotype, 92 (60.5%) participants carried the CT genotype, and 12 (7.9%) participants carried the TT genotype. A comparison of the two alleles (C/T) of rs9264942 was performed between the non-MDR TB patients and the healthy individuals and between the MDR-TB patients and the non-MDR TB patients to evaluate the association between the SNP HLA-C-35 kb (rs9264942) and the susceptibility to MDR-TB infection. The frequency of the C and T alleles for each group was as follows: 91.2% (31/34) and 79.4% (27/34), respectively, in the healthy individuals; 95.5% (63/66) and 74.2% (49/66), respectively, in the non-MDR TB patients; and 88.5% (46/52) and 53.8% (28/52), respectively, in the MDR-TB patients. Upon comparison of the non-MDR TB patients with the healthy individuals, no significant association was observed between the HLA-C-35 kb allele and disease susceptibility (C allele: OR= 2.03; 95% CI: 0.388 – 10.657; p= 0.406; T allele: OR= 0.75; 95% CI: 0.276 – 2.027; p= 0.566). When comparing the MDR-TB patients with the non-MDR TB patients, an association was observed between the T allele and MDR-TB infection (OR= 0.41; 95% CI: 0.186 – 0.879; p= 0.021). However, the C allele was only slightly more frequent than the T allele in the MDR-TB patients (OR= 1.28; 95% CI: 0.701 – 2.328).

Among the non-MDR TB patients, 25.8% (17/66) carried the CC genotype, 69.7% (46/66) carried the CT genotype, and 4.5% (3/66) carried the TT genotype. In the non-MDR TB patients, the frequency of each genotype was not consistent with Hardy-Weinberg equilibrium (p< 0.001). Among the healthy individuals, 20.6% (7/34) carried the CC genotype, 70.6% (24/34) carried the CT genotype, and 8.8% (3/34) carried the TT genotype. In the healthy individuals, the frequency of each genotype was not consistent with Hardy-Weinberg equilibrium (p= 0.012). No significant association was found between the rs9264942 genotype and BMI among the non-MDR TB patients. Furthermore, no association between the rs9264942 genotype and BMI was observed among the healthy individuals.

In the MDR-TB patients, the frequency of each genotype corresponded to Hardy-Weinberg equilibrium (p= 0.780). A higher frequency of the C allele was found among the MDR-TB patients who were classified as underweight {94.7% (36/38)} than among those who were classified as normal weight {69.2% (9/13)} (OR= 8.00; 95% CI: 1.261 – 50.770; p= 0.031). No significant association was found between the complete blood count, the CD4+ T-helper cell count, the CD4+ T-helper cell percentage, the cytokine levels, or the presence of a pulmonary cavity and the rs9264942 genotype [Table/Fig-1,2].

	Genotype			Total (%)
	CC Frequency (CC patients/total patients)	CT Frequency (CT patients/total patients)	TT Frequency (TT patients/ total patients)	
Sex				
Female	54.2 (13/24)	54.5 (12/22)	66.7 (4/6)	55.8 (29/52)
Male	46.2 (11/24)	45.5 (10/22)	33.3 (2/6)	44.2 (23/52)
BMI (kg/m²)†				
< 18.5	75.0 (18/24)	85.7 (18/21)	33.3 (2/6)	74.5 (38/51)
18.5 - 24.99	25.0 (6/24)	14.3 (3/21)	66.7 (4/6)	25.5 (13/51)
≥ 25	0.0 (0/24)	0.0 (0/21)	0.0 (0/6)	0.0 (0/51)
Thorax imaging†				
Pulmonary fibrosis	47.8 (11/23)	5.0 (1/20)	50.0 (3/6)	30.6 (15/49)
Pulmonary cavity	43.5 (10/23)	60.0 (12/20)	50.0 (3/6)	51.0 (25/49)
Pulmonary infiltrates	100.0 (23/23)	85.0 (17/20)	66.7 (4/6)	89.8 (44/49)
Patient classification				
New	4.2 (1/24)	4.5 (1/22)	0.0 (0/6)	3.8 (2/52)
Relapse	16.7 (4/24)	36.4 (8/22)	16.7 (1/6)	25.0 (13/52)
Treatment failure of category I	16.7 (4/24)	27.3 (6/22)	50.0 (3/6)	25.0 (13/52)
Treatment failure of category II	62.5 (15/24)	31.8 (7/22)	16.7 (1/6)	44.2 (23/52)

[Table/Fig-1]: The characteristics and the HLA-C-35 kb (rs9264942) genotype of the 52 MDR-TB patients
†This result contains missing data BMI: body mass index

	Genotype			Total (%)
	CC Frequency (CC patients/total patients)	CT Frequency (CT patients/total patients)	TT Frequency (TT patients/ total patients)	
CD4+ T-helper cell count (cells/mm³)				
< 500	29.2 (7/24)	27.3 (6/22)	16.7 (1/6)	26.9 (14/52)
500 – 1200	66.7 (16/24)	68.2 (15/22)	83.3 (5/6)	69.2 (36/52)
> 1200	4.2 (1/24)	4.5 (1/22)	0.0 (0/6)	3.8 (2/52)
CD4+ T-helper cell percentage (%)				
< 31	41.7 (10/24)	59.1 (13/22)	33.3 (2/6)	48.1 (25/52)
31 – 60	54.2 (13/24)	40.9 (9/22)	66.7 (4/6)	50.0 (26/52)
> 60	4.2 (1/24)	0.0 (0/22)	0.0 (0/6)	1.9 (1/52)
Leukocyte count (10³ cells/uL)†				
< 4.5	0.0 (0/22)	4.8 (1/21)	0.0 (0/6)	2.0 (1/49)
4.5 – 11	68.2 (15/22)	57.1 (12/21)	83.3 (5/6)	65.3 (32/49)
> 11	31.8 (7/22)	38.1 (8/21)	16.7 (1/6)	32.7 (16/49)
Thrombocyte count (10³ cells/uL)†				
< 150	0.0 (0/22)	0.0 (0/21)	0.0 (0/6)	0.0 (0/49)
150 – 450	72.7 (16/22)	66.7 (14/21)	83.3 (5/6)	71.4 (35/49)
> 450	27.3 (6/22)	33.3 (7/21)	16.7 (1/6)	28.6 (14/49)
Erythrocyte count*†				
Low	31.8 (7/22)	38.1 (8/21)	66.7 (4/6)	38.8 (19/49)
Normal	59.1 (13/22)	61.9 (13/21)	33.3 (2/6)	57.1 (28/49)
High	9.1 (2/22)	0.0 (0/21)	0.0 (0/6)	4.1 (2/49)
Haemoglobin level†**				
Low	54.5 (12/22)	61.9 (13/21)	66.7 (4/6)	59.2 (29/49)
Normal	40.9 (9/22)	38.1 (8/21)	33.3 (2/6)	38.8 (19/49)
High	4.5 (1/22)	0.0 (0/21)	0.0 (0/6)	2.0 (1/49)
Haematocrit*†**				
Low	40.9 (9/22)	57.1 (12/21)	66.7 (4/6)	51.0 (25/49)
Normal	50.0 (11/22)	42.9 (9/21)	33.3 (2/6)	44.9 (22/49)
High	9.1 (2/22)	0.0 (0/21)	0.0 (0/6)	4.1 (2/49)

[Table/Fig-2]: The haematological status and the HLA-C-35 kb (rs9264942) genotype of the 52 MDR-TB patients
*Normal range for males: 4.7 to 6.1 million cells/uL; normal range for females: 4.2 to 5.4 million cells/uL.
**Normal range for males: 13.8 to 17.2 g/dL; normal range for females: 12.1 to 15.1 g/dL.
***Normal range for males: 40.7 to 50.3%; normal range for females: 36.1 to 44.3%.
†This result contains missing data

Based on the treatment history, the CC genotype was associated with the treatment after failure of category II group {65.2% (15/23)} (OR= 4.17; 95% CI: 1.301 – 13.346; p= 0.014) [Table/Fig-1].

Among the MDR-TB patients who exhibited pulmonary fibrosis, 27.9% (12/43) carried the C allele, and 15.4% (4/26) carried the T allele. The C allele frequency was higher than the T allele frequency among the MDR-TB patients exhibiting pulmonary fibrosis, but this difference was not significant (OR= 2.13; 95% CI: 0.606 – 7.480; p= 0.232). Among the MDR-TB patients who exhibited pulmonary infiltrates, 93.0% (40/43) carried the C allele, and 80.8% (21/26) carried the T allele. The C allele frequency was higher than the T allele frequency among the MDR-TB patients exhibiting pulmonary infiltrates, but this difference was not significant (OR= 3.17; 95% CI: 0.690 – 14.598; p= 0.123).

DISCUSSION

A total of 152 individuals were subjected to genotypic analysis of rs9264942 to determine the association between this polymorphism and the susceptibility to MDR-TB infection. We found that 92.1% (140/152) of the present study population carried the C allele and that 68.4% (104/152) of the present study population carried the T allele. The frequency of the C allele in our study population was slightly higher than that of a study population in central China {C allele= 80.6% (261/324); T allele= 69.4% (225/324)} [14].

The C allele was only slightly more frequent than the T allele in the MDR-TB patients when comparing with the non-MDR TB patients (OR= 1.28; 95% CI: 0.701-2.328). The results showed that the C allele did not significantly contribute to the susceptibility to MDR-TB infection. In a previous study, the C allele of the SNP HLA-C-35 kb led to a better outcome of intracellular infection which was thought to be caused by increased antigen presentation by HLA-C via the elevated expression of HLA-C on the cell surface [9,10,15]. Consequently, individuals carrying the C allele exhibit a more effective immune response (CD8+ cytotoxic T cells and NK cells) than those not carrying the C allele [9,10]. However, we did not find a significant association between the C allele and MDR-TB disease susceptibility. This result suggests that the SNP HLA-C-35 kb may not significantly contribute to the susceptibility to MDR-TB infection.

Among the MDR-TB patients exhibiting pulmonary fibrosis, the C allele frequency was higher than the T allele frequency (OR= 2.13; 95% CI: 0.606 – 7.480; p= 0.232). A previous study reported that the C allele is associated with increased expression of HLA-C on the cell surface [9]. This elevated expression of HLA-C may increase antigen presentation by HLA-C, leading to enhanced recognition by and activation of CD8+ cytotoxic T cells. Therefore, this allele promotes cellular apoptosis induced by CD8+ cytotoxic T cells, which results in cell death due to the increased expression of the Fas ligand (FasL) [16]. Increased cell death also promotes tissue remodeling, which may contribute to the formation of fibrotic tissues. However, other factors should also be considered, as this polymorphism was not the primary contributor to pulmonary fibrosis [17].

Pulmonary infiltrates are a radiological finding suggestive of *M. tuberculosis* infection [18]. Pulmonary infiltrates can occur in re-infection cases or in adult-type TB, which is caused by an impaired immune response [18]. We found the C allele frequency was higher than the T allele frequency among the MDR-TB patients exhibiting

pulmonary infiltrates, but this difference was not significant (OR= 3.17; 95% CI: 0.690 – 14.598; p= 0.123).

Being underweight (BMI<18.5) is associated with disease severity, poor prognosis, and the treatment outcomes of relapse and death for MDR-TB infection [19,20]. In present study, a greater percentage of patients were classified as underweight among the MDR-TB patients {74.5% (38/51)} than among the non-MDR TB patients {58.5% (38/65)} (OR= 2.08; 95% CI: 0.933 – 4.622), and these underweight patients were associated with the C allele (OR= 8.00; 95% CI: 1.261 – 50.770; p= 0.031). The C allele was previously known to be associated with a better outcome in intracellular infection [9,10].

Having a history of treatment failure had been reported with increased risk for developing MDR-TB infection [21]. Interestingly, we found the CC genotype was more common in the treatment after failure of category II group (OR= 4.17; 95% CI: 1.301 – 13.346; p= 0.014), suggesting the CC genotype may have bigger risk for developing TB treatment failure.

CONCLUSION

The SNP HLA-C-35C (rs9264942) is not significantly associated with the susceptibility to MDR-TB infection. However, this SNP may contribute to the clinical profile of MDR-TB infection, in which the C allele was more frequent among the MDR-TB patients who were classified as underweight (BMI < 18.5), who exhibited pulmonary fibrosis, and who exhibited pulmonary infiltrates. Further research should be conducted to understand the role of the SNP HLA-C-35 kb (rs9264942) in a larger cohort that is representative of a wider population to avoid the possibility of bias in the data from this sample.

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