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Contribution of *NRAMP1* gene expression and protein level in pulmonary and latent TB infection in Indonesia

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ABSTRACT

Natural resistance-associated macrophage protein 1 (*NRAMP1*) gene is regarded to play an important role in human vulnerability against various infections, including mycobacterial infection. This research purpose was to investigate the correlation between *NRAMP1* gene expressions and protein level with susceptibility to tuberculosis (TB) infection and their contacts. *NRAMP1* gene expression and protein level from 30 active TB patients, 27 latent TB infection household contacts, and 33 healthy subjects as control were examined by the real-time polymerase chain reaction and enzyme-linked immunosorbent assay method. *NRAMP1* gene expression value in the healthy group was 4.61 times higher than active TB patients ($p = 0.025$) and the *NRAMP1* expression value in the healthy group was 4.02 times higher than in the latent TB group ($p = 0.117$). The mean protein level of *NRAMP1* was 196.41, 199.27, and 224.70 ng/ml in the active TB, latent TB, and healthy groups, respectively ($p > 0.05$). *NRAMP1* expression was downregulated and protein level was lower in active TB patients than latent TB patients and healthy individuals.

INTRODUCTION

Human genetics that regulates infectious disease vulnerability has been widely researched (Canonne-Hergaux *et al*., 1999). These research studies are expected to contribute to identifying the high-risk populations being exposed to infectious diseases, so that prevention and diagnosis can be carried out properly as early as possible. In addition, the study also clarified the pathomechanism of infection and how the host defends against microbes. Several human genes have been studied that correlated to the susceptibility of tuberculosis (TB) infection (Qidwai *et al*., 2012).

Natural resistance-associated macrophage protein 1 (*NRAMP1*) gene that is also called *SLC11A1* (solute carrier 11a1) has been known to play a role in human susceptibility to various infections, including mycobacterial infection. The human *SLC11A1* gene is arranged on chromosome 2q35 and codes the

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NRAMP1, an integral protein-membrane consisting of 550 amino acids that are widely expressed in the lysosomal compartments of monocytes and macrophages (Dow, 2015). *NRAMP1* is located in the phagosome membrane of macrophages and is recruited rapidly to the phagosome membrane after the phagocytosis process. It appears to work as a divalent cation transporter [Mn (II) and Fe (II)] across the phagosomal membrane of macrophages, otherwise known as phagosome waste pumps (Hanikenne *et al*., 2009; Murray, 2013). The antimicrobial effect of *NRAMP1* is due to the ability of *NRAMP1* to deplete the essential metal nutrients for bacteria in the phagosomes (Zhao and Enns, 2012). In addition, *NRAMP1* also increases the acidity of phagosomes to levels that are lethal to bacteria (Dow, 2015; Flaherty, 2012). For patients with *NRAMP1* deficiency, their phagolysosome pH approaches pH 6.8, which promotes bacterial growth. Mutations in the *NRAMP1* gene are thought to affect alveolar macrophage phagosome function (Flaherty, 2012). NRAMP gene polymorphisms have been reported to be related to tuberculosis (Meilang *et al*., 2012). However, we did not find any research in patients with TB on the expression of the *NRAMP1* gene.

Understanding the role of human genetic factors in the immune system controlling infectious disease susceptibility/resistance is essential for tuberculosis research because it will enable genetic dissection of antimycobacterial immunity and open up new possibilities for preventive and therapeutic measures. This study aimed to investigate the expression and protein level of *NRAMP1* in patients with pulmonary tuberculosis and household contacts who are dormant infected with TB, to contribute to understanding the role of genetic and immunology factors in susceptibility to tuberculosis.

MATERIALS AND METHODS

Sample recruitment

In this research, 90 participants were selected and divided into three categories, each with 30 samples of the new smear-positive pulmonary tuberculosis group from the Community Center for Lung Health Makassar or Balai Besar Kesehatan Paru Masyarakat (BBKPM) Makassar as active TB group; 33 subjects of household contact with TB patients and were actively screened using QuantiFERON TB Gold Plus TB test (Qiagen, Germany) to determine their exposure to *Mycobacterium tuberculosis* (Mtb). The group with positive Immunoglobulin Release Assay (IGRA) was categorized as a latent group. The third group included 27 healthy subjects who were also subjected to an IGRA examination first and had negative IGRA results. Positive sputum samples were decontaminated and then continued with the culture process at the Tuberculosis Unit of the Hasanuddin University Medical-Research Center (HUM-RC) Laboratory, Makassar, Indonesia. Blood samples were centrifuged for separation plasma samples at 3,000 rpm for 15 minutes at 4°C. Plasma samples and clotted blood samples were stored at −80°C before ELISA and RNA extraction.

Ethical approval

This research had ethical approval by the Research Ethics Commission of the Faculty of Medicine, Universitas Hasanuddin, Makassar, South Sulawesi, Indonesia (No. 1125/UN4.6.4.5.31/PP36/2020), and all research subjects had signed the consent.

NRAMP1 gene expression measurement

Total RNA was extracted from blood samples using RNA Isolation Kit RNeasy Mini Kit (Qiagen, USA) according to the manufacturer's instructions. In short, the samples were lysed and homogenized first. In order to have optimal binding conditions, ethanol was added to the lysate. The lysate was then placed onto the RNeasy silica membrane. RNA binds, and all contaminants were efficiently washed away. Pure concentrate RNA was eluted in 50 µl RNAse free water and used in complementary DNA (cDNA) synthesis.

The iScript cDNA Synthesis Package (Bio-Rad Laboratories, Inc., CA) was used to synthesize the cDNA from 100 ng of total RNA. A mixture of 20 microliters was prepared with 4 µl 5× iScript Reaction blend, 1 µl of iScript reverse, and nuclease-free water. The cycling requirement for the synthesis of cDNA was priming for 5 minutes at a temperature of 25°C, followed by 20 minutes of reverse transcription at 46°C and 1 minute of RT inactivation at 95°C.

For the target genes, the relative quantification was rendered using 12.5 µl Fast Evagreen® qPCR Master Mix (Biotium, Inc., Fremont, CA), 0.5 µl cDNA, and 0.5 µl (0.5 µM concentration) of each NRAMP forward and reverse primers for *NRAMP1* (forward 5′-GCATCTCCCCAATTCATGGT-3′ and reverse 5′-AACTGTCCCACTCTATCCTG-3′) and glyceraldehyde-3 phosphate dehydrogenase (*GAPDH*) (forward 5′-CCTGCACCACCAACTGCTTA-3′ and reverse 5′-GGCCATCCACAGTCTTCTGGG-3′) and amplified in real-time PCR (Bio-Rad Lab. Inc., Hercules, CA) with cycling conditions at 95°C for 30 seconds (initial denaturation and activation of enzyme) followed by 45 cycles of 95°C for 5 seconds and annealing at 60°C for 30 seconds. The amplicons have been heated at 65°C–95°C with a temperature rise of 0.5°C at 5 seconds per phase, and the melting curve analyses have been completed.

All analyses were performed using Bio-Rad CFX™ Manager Software (version 3.1, Bio-Rad Lab. Inc., Hercules, CA). The level of messenger ribonucleic acid (mRNA) expression was normalized by using GAPDH mRNA as a housekeeping gene, and the relative fold induction was determined using the $2^{(-\Delta\Delta Ct)}$ calculation, where ΔCt is Ct(target)−Ct (GAPDH), and Ct is the cycle at which an arbitrary detection threshold is crossed.

Quantification of *NRAMP1* protein level

NRAMP1 protein level was measured using the Human Natural Resistance-associated Macrophage Protein 1 ELISA Kit (Cat. No. E4411Hu, Bioassay Technology Laboratory, Shanghai, China) according to the manufactory instruction. A linear curve was used to measure the level of *NRAMP1* protein from a standard curve. The standard curve range of the ELISA kit is 15–3,000 ng/l.

Statistical analysis

All experimental data were analyzed using Bio-Rad CFX™ Manager Software (version 3.1, Bio-Rad Lab. Inc., Hercules, CA) and Statistical Package for the Social Sciences (version 21.0). A statistical analysis using one-way analysis of variance differential test was carried out in order to assess the discrepancy in the expression of the *NRAMP* 1 genes in the three study classes. p-values < 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

Of the 90 subjects studied, the number of male respondents was 38 (42.22%) and the number of female respondents was 52 (57.78%). In the active TB group, 17 out of 30 subjects were male (56.67%), and the rest were women (43.33%). In contrast to the latent group and healthy group, female subjects were more than men, as described in Table 1.

The comparison of *NRAMP1* protein level of active TB, latent TB, and healthy groups is shown in Figure 1. Based on the ELISA result, the mean protein level of *NRAMP1* was 196.41, 199.27, and 224.70 ng/ml in the TB, latent TB, and healthy groups, respectively. However, statistical test results (Kruskal-Wallis test) obtained a *p*-value of > 0.000 (0.96) which showed that there was no significant difference in *NRAMP1* protein levels in either active TB, latent TB, or healthy group. It can be stated that the amount of *NRAMP1* protein will decrease if the patient has TB, compared to those who have latent TB only and the amount was even higher in normal people although it was not statistically different.

The expression of *NRAMP1* mRNA genes in the three sample groups can be seen in Figure 2. The mean result of the mRNA expression of the *NRAMP1* genes of the active TB group was the lowest, followed by the latent TB and healthy group. The differences in mRNA expression of the *NRAMP1* gene of active TB, latent TB, and healthy groups can be seen in Table 2. *NRAMP1* gene expression value in the healthy group was 4.61 times higher than active TB patients ($p = 0.025$) and the *NRAMP1* expression value in the healthy group was 4.02 times higher than those in the latent TB group ($p = 0.117$).

Table 1. Characteristics of subjects in the active TB, latent TB, and healthy participant groups.

	Active TB *n* = 30	Latent TB *n* = 27	Healthy participants *n* = 33	Total
Gender				
Male	17 (56.7%)	10 (37%)	11 (33.3%)	38 (42.2%)
Female	13 (43.33%)	17 (63%)	22 (66.7%)	52 (57.8%)
Age group				
17–29	7 (23.33%)	7 (25.9%)	5 (15.2%)	19
30–44	10 (33.3%)	13 (48.1%)	12 (36.4%)	35
45–60	11 (36.7%)	6 (22.2%)	14 (42.4%)	31
>60	2 (6.7%)	1 (3.7%)	2 (6.1%)	5

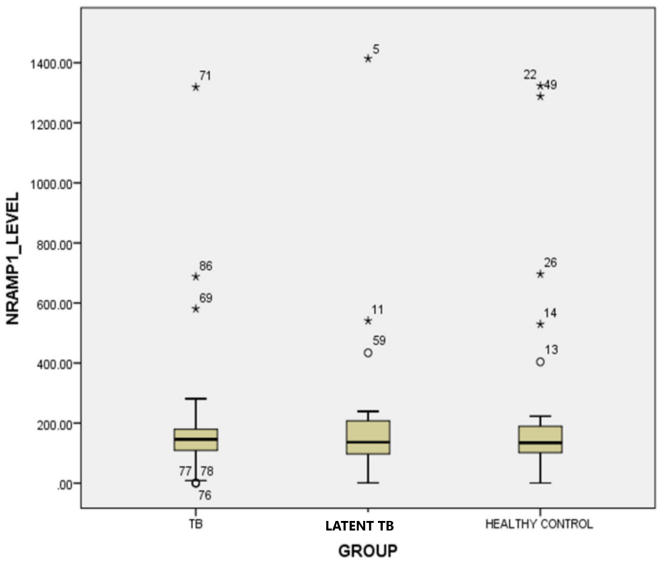

Figure 1. Comparison of the mean from *NRAMP1* protein level in the active TB, latent TB, and healthy groups.

The study involved 90 research subjects. Of the 90 subjects, it was found that active Tb patients were 56.7% male and 43.3% female. This is consistent with Uplekar's study in which 64% more men were diagnosed with TB with symptoms and positive acid-fast bacillus (AFB) screening than women which was only 34% (Uplekar *et al*., 2001). This can happen because more men are working outdoors compared to women; thus, the risk of being exposed to Mtb from TB patients TB is considerably large. Active pulmonary TB is more commonly found in working men than women (Htet *et al*., 2018). However, the biological distinctions between sexes should also be taken into account in the prevalence and diagnosis of TB (Thorson, 2015).

In this study, the active TB and latent TB samples were largely distributed in the age range of 31–45 years, with the active TB category was 33.3% and TB latent 48.1%. This was similar to research conducted by Coppola *et al*. (2020) and Hao *et al*. (2020) in which patients diagnosed with active pulmonary TB 51% and latent TB 48.6% in the age range of 27–40 years. The productive age group is more predominantly exposed to Mtb germs because the outdoor activities are higher, and the immunity factor at a productive age is better than the immunity of the child or elderly.

Immunoglobulin Release Assay (IGRA) has become the primary method for clinical diagnosis of a person's exposure to current *M. tuberculosis* infection (Xu *et al*., 2018). IGRA positiveness rates are higher for individuals who have a history of close contact with active pulmonary TB patients than those in contact with pulmonary disease other than TB (Eom *et al*., 2018). In our study, it was found that 9 out of 30 healthy people were positive for IGRA and were included in the latent TB group with a total of 27 people with a percentage of 63% more occurring in women. This is in line with the research conducted by Htet *et al*. (2018) where as much as 59.2% of people with a history of active pulmonary TB contact will suffer from TB. This is consistent with

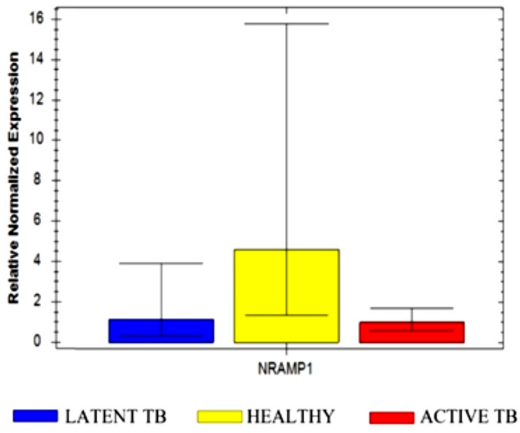

Figure 2. Graph of the mean value of *NRAMP1* mRNA expression in the active TB, latent TB, and healthy groups.

Table 2. *NRAMP1* expression gene in the active TB, latent TB, and healthy groups.

Target	Biological group	Control	Expression	Expression 95% CI low	Expression 95% CI high	*p*-value
GAPDH	Latent TB					
GAPDH	Healthy	C				
GAPDH	TB					
NRAMP1	Latent TB		0.24970	0.07341	0.84928	0.117868
NRAMP1	Healthy	C	1.00000	0.29127	3.43322	
NRAMP1	TB		0.21752	0.12723	0.37188	0.025601

the results of our study, where 18 of 30 (60%) household contacts were positive for IGRA, while the rest were negative for IGRA. People who were negative for IGRA can be considered a healthy individual because, despite the history of contact with active pulmonary TB patients, the IGRA results remained negative. It may be due to the fact that the intensity of the exposure was low, or the number of germs that penetrate the body was below the threshold for sensitivity to the applied IGRA method.

A previous study showed that there was a lack or absence of *NRAMP1* expression in macrophages or Langerhans' giant cells found in granulomatous lesions of in Taiwanese indigenous relative to Han subjects, and there was a positive correlation of *NRAMP1* gene polymorphism to tuberculosis susceptibility in Taiwanese indigenous people (Hsu *et al*., 2006). Gallant *et al*. (2007) also suggested that, in pediatric TB patients with homozygous CC genotype NRAMP 1–274, lower *NRAMP1* activity was found in monocyte-derived macrophages with elevated risk allele than in heterozygous CT genotype patients. Lack of *NRAMP1* protein expression occurred in mouse strains prone to *Mycobacterium bovis* BCG infection (Vidal *et al*., 1996). However, in bovine TB, *NRAMP1* was high in peripheral blood mononuclear cells; alveolar macrophages (obtained by bronchioalveolar lavage) and Holsten Friesian cattle lymphatic node granulomas with self-proven bovine TB were highly expressed (Estrada-Chávez *et al*., 2001; Pereira-Suárez *et al*., 2006). Human and mouse *NRAMP1* proteins share 88% identity and 93% overall sequence similarity, with the predicted N-linked glycosylation signals being precisely conserved in the fourth extracellular loop of both proteins (Vidal *et al*., 1996).

In our study, we found that the *NRAMP1* expression gene was downregulated in TB compared to latent TB and healthy individuals. Also, the *NRAMP1* protein level in TB was lower than those with latent TB and healthy participants. The decreased *NRAMP1* action may contribute to increased bacterial availability of iron, fostering the replication of mycobacteria in macrophages. *NRAMP1* pumps iron, essential to living from the macrophages via intracellular bacteria like Mtb. Since iron is also essential for the cell to produce reactive oxygen and intermediate nitrogen, loss of intramacrophage iron availability for bacterium and simultaneous weakening of antimicrobial activities of the Fe^{2+} ion transport role of the *NRAMP1* protein could thus facilitate infection by Mtb and tuberculosis development (Fernández-Mestre *et al*., 2015). Many studies have investigated the correlation between *NRAMP* gene polymorphisms and tuberculosis (Fernández-Mestre *et al*., 2015; Meilang *et al*., 2012; Stagas *et al*., 2011), and the stability and efficiency of mRNA protein translation of these polymorphisms can impact.

CONCLUSION

NRAMP1 expression is downregulated and protein level was lower in active TB than latent TB and healthy individuals. Our results demonstrated that *NRAMP1* could contribute to the pathogenesis of TB. This study confirms that *NRAMP1* gene expression correlated with the TB infection.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICAL APPROVALS

This research had ethical approval by the Research Ethics Commission of the Faculty of Medicine, Universitas Hasanuddin, Makassar, South Sulawesi, Indonesia (No. 1125/UN4.6.4.5.31/PP36/2020).

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