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Research Report

3'UTR Polymorphism of *NRAMP1* Gene and Susceptibility to Lung Tuberculosis among Patients and Nurses in Surabaya, Indonesia

Rahayu Anggraini
Division of Tuberculosis, Institute of Tropical Disease, Airlangga University

ABSTRACT

The objectives of this study was to evaluate a potential role for natural-resistance-associated macrophage protein 1 (NRAMP1) gene in the human homologue using four single base pair polymorphisms (D543N, 3'UTR, INT4, 274C/T) for susceptibility to tuberculosis infection in Surabaya, Indonesia. The study population were 69 lung tuberculosis patients and 43 healthy nurses were genotyped with the polymerase chain reaction (PCR) and the product amplified from their genomic DNA were subjected to restriction enzyme digestion (RFLP) and were analysed using agarose gel electrophoresis. Results of this study showed only the homozygous TGTG deletion allele at the 3'untranslated region (3'UTR) of the NRAMP1 gene i.e. the TGTGdel/del genotype was more frequently found in lung tuberculosis patients (20/69=29%) compared to that found in nurses (2/43=4.7%). The Odds ratios (ORs) were 8.37 (95% confidence interval [CI], 1.85 to 37.94; p=0.002). This finding shows that polymorphism 3'UTR of NRAMP1 gene increased the risk of lung tuberculosis in Surabaya, Indonesia.

Key words: *NRAMP1 gene, pulmonary tuberculosis, Surabaya*

INTRODUCTION

Despite the availability of a number of anti-mycobacterial drug, lung tuberculosis is still difficult to eradicate. In 1990 the incidence of tuberculosis (TB) world-wide approximately 1.7 billion or about one third of the world population at that time. The incidence of TB in 1997, is about 8 million and mortality amount 3–4 million cases per annual. Therefore WHO started that TB is one of the more important infectious disease to notice and to tackle.[1]

In Indonesia, TB re-appears as a prominent cause of death. The result of family health survey held in 2005, showed that TB is the third leading cause of death after cardiovascular and respiratory tract disease in all age and is the first leading cause of death in infectious disease.[2,3]

Lung TB arises when TB bacilli present in air droplets enter the respiratory tract, finally reaching lung alveoli. Upon which it will evoke an inflammatory response causing in accumulation of macrophage and neutrophyl. These phagocyte cells will than migrate to regional lymph nodes crossing swelling of these lymph nodes, thus forming the so called primary complex. The TB bacilli in lung tissue and lymph node were engulfed by macrophages. If the host immune response is adequate, TB bacilli will be killed by macrophage causing healing of the primary complex. How ever when the host immune response not adequate, TB bacilli survive and multiply within macrophage which upon leaving these cells, finally enter lymph and blood circulation, spreading to other organs.[4]

The *NRAMP1* (natural resistance associated macrophage protein-1) gene is expected in macrophages and also in blood cells (Peripheral Blood Mononuclear Cells/ PBMC). The gene encodes a protein that function as a divalent ion channel including Fe++ ions. Fe++ ions are known to inhibit growth and ultimately kill M. tuberculosis. When a mutation in the *NRAMP1* gene results in a non functional NRAMP1 protein or the protein is rapidly degraded, then the inhibition action of the NRAMP1 protein is lost or lessened and the mycobacteria will multiply freely in macrophages.[5–7]

Based on that above facts it can be concluded that in certain cases susceptibility to lung tuberculosis is under genetic control. That certain genetic mutation in *NRAMP1* gene leads to susceptibility been reported by several

authors.[8–17] The present study is the first known study done in Indonesia involving the influence of *NRAMP1* gene polymorphism in acquiring susceptibility to lung tuberculosis involving the following genotype D543N, 3'UTR, INT4, and 274C/T polymorphism which have been previously investigated by other author outside Indonesia.

The D543N polymorphism (G/G, G/A, A/A genotype), 3'UTR polymorphism (TGTG+/+, TGTG+/del, TGTG del/del genotype), INT INT4 polymorphism (G/G, G/C, C/C genotype) and 274C/T polymorphism (C/C, C/T, T/T genotype) has been found to show a significantly different distribution frequencies between tuberculosis patients and healthy subjects (nurses), thus showing differences in susceptibility between these two groups toward acquiring tuberculosis.[8–17] Field experience have been shown that nurses serving lung tuberculosis patients in hospital wards for years often do not acquire the disease. Likewise, only 10% of people infected by M. tuberculosis actually acquire the disease.[11,13]

MATERIALS AND METHODS

Participant

Subjects participating in this study are 69 lung tuberculosis patients (38 male and 31 female) treated in Dr. Soetomo General Hospital and Karang Tembok Hospital at Surabaya, they had normal blood and serum analysis (Hb, Creatinine, BUN, Glucose, and HIV), and abnormal X-Ray and positive culture and acid-fast bacilli in sputum and 43 healthy nurses (18 male and 25 female) with normal blood and serum analysis (Hemoglobin, Creatinine, BUN, Glucose, and HIV) as well as normal X-Ray and negative cultur and acid-fast bacilli in sputum, but positive PPD. Patient ranged in age between 18 and 50 years, with average between 25 and 45 years. Ethical clearance and informed consent approval for the study was obtained from the Dr. Sutomo General Hospitals at Surabaya.

DNA Preparation

PBMC Isolations, 2 ml whole blood were obtained by vena puncture using EDTA as anticoagulant (Whole blood EDTA) is then centrifuged at 3000 rpm for 30 minutes. The resulting buffycoats containing PBMC is then pipette and stored frozen for further use. DNA is isolated using High Pure PCR Preparation Kit (Roche, Applied Science; Mannheim, Germany). The isolated DNA is then stored at 4^{o} C for immediate use.

NRAMP1 Genotyping

NRAMP1 genotyping method was performed according to the Ceillier method. PCR-RFLP analysis, to distinguish the different genotypes of each *NRAMP1* gene (D543N, 3'UTR, INT4, and 274C/T), PCR-RFLP (Polymerase chain reaction-restriction fragment length polymorphism) analysis were done. The principle of this procedure is as follow: using a pair of specific primers, part of the *NRAMP1* gene is amplified by PCR and the amplified fragment is then cut by a specific restriction enzyme so that either the normal or mutated allele is cut.

PCR (Polymerase chain Reaction) using Fast Start PCR Master (Roche, Applied Science; Mannheim, Germany) was done using a thermal cycler (model 9600; Parkin Elmer; Branchburg, NJ) as follows: addition of 5 µl DNA solution, primers, and other reagents needed for DNA fragment amplification, activation of Thermal cycler 95^{o}C for 5 minutes, denaturation 95^{o} C for 30 seconds, annealing 60^{o} C for 30 seconds, extension 72^{o} C for 3 minutes, steps 3,4,5 repeated for 35 cycles and final extension 72^{o} C for 7 minutes. The temperature of the thermal cycler is than chilled to 4^{o} C and the resulting amplified DNA fragment is stored 4^{o} C. The amplified DNA fragments (often called "amplicon") is than identified by agarose gels electrophoresis, visualized by adding ethidium bromide to the gel. If the procedure is success full a single green color bands will appear when expressed to UV radiation.

D543N polymorphism, forward primer: 5'– GCA TCT CCC CAA TTC ATG GT-3'and reverse primer: 5'- AAC TGT CCC ACT CTA TCC TG -3'. Restriction enzyme Ava*II* (Roche, Applied Science; Mannheim, Germany) with normal allele (G): 3 fragments: 126bp, 79bp, and 39bp and mutant allele (A): only 1 fragments: 244bp. Numbers of bands seen after agarose gels electrophoresis, genotype G/G: 126bp, 79bp and 39bp (3 bands), genotype G/A: 244bp, 126bp, 79bp and 39bp (4 bands), and genotype A/A: 244bp (1 bands).

3'UTR polymorphism, the primer same as used for D543N mutation, forward primer: 5'– GCA TCT CCC CAA TTC ATG GT-3' and reverse primer: 5'- AAC TGT CCC ACT CTA TCC TG -3'. Restriction enzyme: Fok*I* (Roche, Applied Science; Mannheim, Germany) with normal allele (TGTG+): 2 fragments: 211bp, and 33bp and mutant allele (TGTG del): only 1 fragments: 244bp. Numbers of bands seen after agarose gels electrophoresis, genotype TGTG+/+: 211bp and 33bp (2 bands), genotype TGTG+/del: 244 bp, 211bp, and 33bp (3 bands), and genotype TGTG del/del : 244bp (1 bands).

INT4 polymorphism, forward primer: 5'- TCT CTG GCT GAA GGC CTC TCC-3' and reverse primer: 5'-GAG GCT CAA ACT GAT AGC ACA- 3'. Restriction enzyme Apa*I* (Roche, Applied Science; Mannheim, Germany) with normal allele (G): 1 fragments: 624bp and mutant allele (C): 2 fragments: 455bp and 169bp. Numbers of bands seen after agarose gels electrophoresis, genotype G/G : 624bp (1 bands), genotype G/C : 624bp, 455bp, and 169 bp (3 bands), and genotype C/C : 455bp and 169bp (2 bands).

274C/T polymorphism, forward primer: 5'- TGC CAC CAT CCC TAT ACC CAG-3' and reverse primer: 5'-TCT CGA AAG TGT CCC ACT CAG- 3'. Restriction enzyme Mnl*I* (Roche, Applied Science; Mannheim, Germany) with normal allele (C): 4 fragments: 102bp, 65bp, 37bp, and 12bp and mutant allele (T): 3 fragments: 167bp, 37bp, and 12bp. Numbers of bands seen after agarose gels electrophoresis, genotype C/C : 102bp, 65bp, 37bp,

and 12bp (4 bands), genotype C/T : 167bp, 102bp, 65bp, 37bp, and 12bp (5 bands), and genotype T/T : 167bp, 37bp, and 12bp (3 bands).

Statistical Analysis

For each polymorphism, allele and genotype frequency differences in each group were examined using Pearson's Chi-Square or Fisher exact test. Odds ratios (ORs) and 95% confidence intervals (CI) were calculated to quantitatively assess the degree of association between these polymorphism from patient and nurses.

RESULTS

From an amplification genomic DNA with PCR were analyzed by electrophoresis on 2% agarose gel showed in figure1 the D543N and 3'UTR polymorphism *NRAMP1* gene have 244 base pair and INT4 polymorphism have 624 base pair.

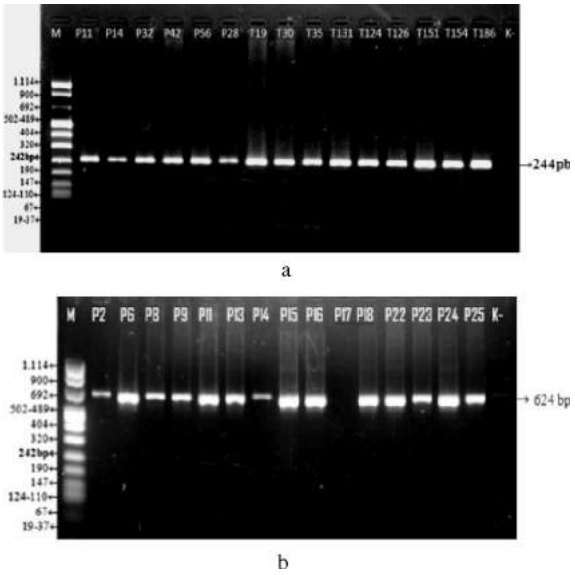

Figure 1. PCR result from genomic DNA amplification, D543N and 3'UTR (a) polymorphism *NRAMP1* gene with 244 base pair and INT4 (b) polymorphism *NRAMP1* gene with 624 base pair.

From an amplification genomic DNA 274C/T polymorphism with PCR were analyzed by electrophoresis on 2% agarose gel showed have 216 base pair can showed in figure 2.

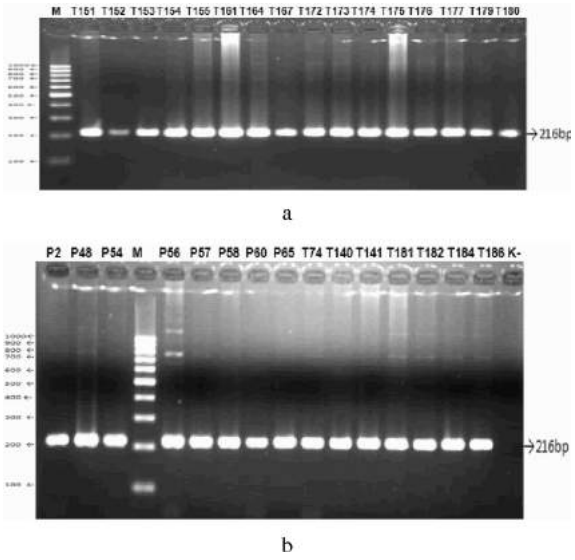

Figure 2. PCR result from genomic DNA 274C/T polymorphism *NRAMP1* gene amplification with 216 base pair.

The PCR-RFLP of D543N polymorphism from lung TB patients and nurses G/G genotype showed 3 bands of 126bp, 79bp and 39bp, genotype G/A showed 4 bands of 244bp, 126bp, 79bp, 39bp and genotype A/A showed 1 band of 244bp who show in Figure 3.

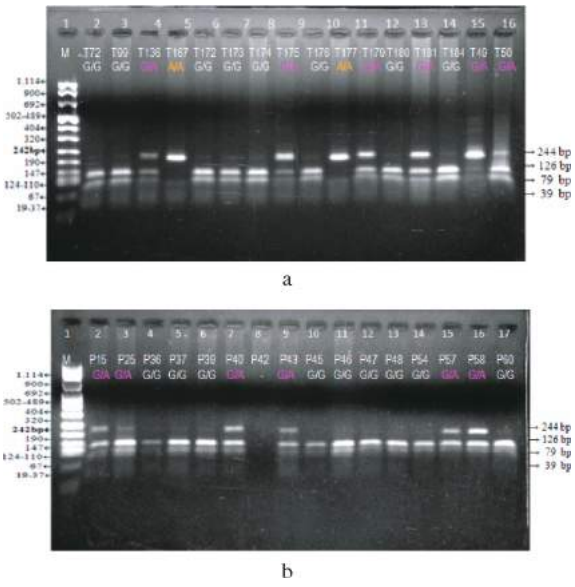

Figure 3. The PCR-RFLP of D543N polymorphism from lung TB patients (a) and nurses (b). Lane1: marker, and showed 1 band was genotype A/A, 2 bands were genotype G/G and 3 bands were genotype G/A.

The PCR-RFLP of 3'UTR polymorphism from lung TB patients and nurses. TGTG +/+ genotype showed 2band of 211bp and 33bp, TGTG +/del genotype showed 3 bands of 244bp and 211bp and 33bp and TGTG del/del genotype showed 1 band: 244bp who show in Figure 4.

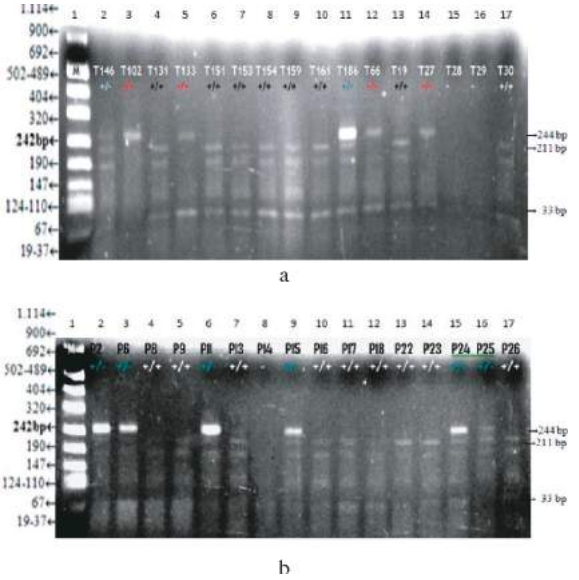

Figure 4. The PCR-RFLP genotyping polymorphism 3'UTR from lung TB patients (a) and nurses (b). Lane 1 (a, b): marker; showed 1 band was genotype TGTG del/del, 2 bands were TGTG +/+; 3 bands were TGTG +/del.

The PCR-RFLP of INT4 polymorphism from lung TB patients and nurses. G/G genotype showed 1 band of 624bp, G/C genotype showed 3 bands: 624bp, 455bp, and 169bp who show in Figure 5.

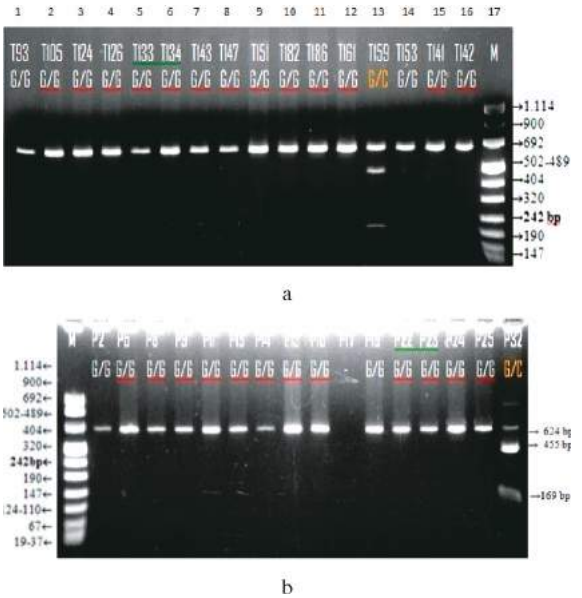

Figure 5. The PCR-RFLP genotyping polymorphism INT 4 from lung TB patients (a) and nurses (b). Lane 17 (upper) and lane 1 (lower): marker; showed 1 band was G/G and showed 3 bands were G/C genotype.

The PCR-RFLP of 274C/T polymorphism from lung TB patients and nurses. T/T genotype showed 3 band of 167bp, 37bp and 12bp, C/C genotype showed 4 band of 102bp, 65bp, 37bp and 12bp, and G/C genotype showed 5 bands: 167bp, 102bp, 65bp, 37bp and 12bp who show in Figure 6.

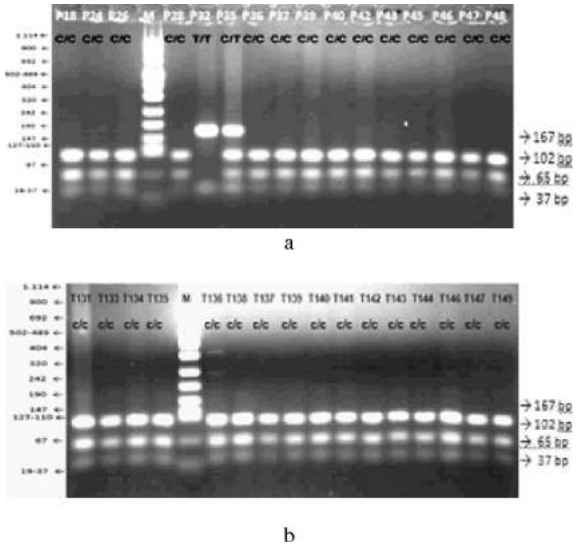

Figure 6. The PCR-RFLP genotyping polymorphism 274C/T from (a) nurses and lung TB patients (b). Lane 4 (upper) and lane 5 (lower): marker; C/C genotype were showed 3 band not 4 bands, C/T genotype were showed 4 bands not 5 bands, and T/T genotype were showed 2 bands not 3 bands, because 12bp band was disappear.

The frequencies of the D543N, 3'UTR, INT4, and 274C/T polymorphism *NRAMP1* gene mutation in lung tuberculosis patients and healthy nurses were compared using Fisher Exact test for frequencies D543N because certain cell is zero, and Chi-square statistical method for D543N, 3'UTR, INT4, and 274C/T polymorphism *NRAMP1* gene see table 1.

The above table revealed that only allele TGTG del genotype TGTG del/del of 3'UTR polymorphism showed a frequencies were statistically different (p=0.002), that was pointed to susceptibility of tuberculosis infection.

DISCUSSION

Our study shows that allelic frequencies in the D543N, INT4, and 274C/T showed no allelic association was identified different between the *NRAMP1* alleles and tuberculosis susceptibility, except the 3'UTR of *NRAMP1* differ between tuberculosis patients and healthy nurses in Surabaya, it was suggesting that *NRAMP1* gene could be associated with susceptibility to tuberculosis. We assume that genetic variants 3'UTR polymorphisms locating in *NRAMP1* are responsible for the allelic difference in our study.

Table1. The frequency of D543N, 3'UTR, INT4, and 274C/T polymorphism *NRAMP1* gene in lung TB patients and healthy nurses group

Polymorphism	Genotype	Lung TB patients (n=69)	Healthy nurses (n=43)	Risk Estimate (95% Confidence Interval [CI])	*p* value
D543N	G/G	35 (50.70%)	28 (65.10%)	0.551(0.252-1.209)	0.171
	G/A	28 (40,60%)	15 (34.90%)	1.275(0.579-2.809)	0.558
	A/A	6 (8.7%)	0 (0%)	1.095(1.018-1.178)	0.080
3'UTR	TGTG+/+	35 (50.70%)	28 (65.10%)	0.551(0.252-1.209)	0.135
	TGTG+/del	14 (20,30%)	13 (30.20%)	0.587(0.245-1.411)	0.232
	TGTGdel/del	**20 (29.0%)**	**2 (4,7%)**	**8.367(1.846-37.937)**	**0.002**
INT4	G/G	67 (97,10%)	40 (93,00%)	2.513(0.402-15.687)	0.370
	G/C	2 (2.9%)	3 (7.00%)	0.398(0.064-2.485)	0.370
	C/C	0 (0%)	0 (0%)		
274C/T	C/C	68 (98,60%)	40 (93,00%)	5,100(0.513-50.697)	0.296
	C/T	1 (1,40%)	2 (4.70%)	0.301(0.026-3.430)	0.557
	T/T	0 (0%)	1 (2.30%)	0.977(0.933-1.023)	0.384

It is not yet clearly whether the 3'UTR polymorphism directly affect *NRAMP1* function, or whether another functional polymorphism exists in the *NRAMP1* gene. Further studies are necessary to answer this question. The 3'UTR polymorphism allele associated with susceptibility to tuberculosis has been reported to be very uncommon in Caucasians, but is present in many cases in West African.[9,11] as well as in Surabaya. These observations may explain in part why African Americans and Asians have greater susceptibility to tuberculosis than Caucasians.

The *Nramp1* gene has been identified as a critical factor for host defense against some mycobacterial species among inbred mouse strains.[18] The protein encoded by the *Nramp1* gene is exclusively expressed in the macrophage/monocyte, and it is assembled onto the subcellular membrane of the lysosome / endosome and phagolysosome.[19] It is likely to restrict mycobacterial replication by influencing the trans-membrane transportation of divalent cations, which are essential for the survival of mycobacteria.[20,21]

In human *Mycobacterium tuberculosis* is found within phagosomes present in macrophages, because the bacteria is phagocyte by macrophage. When phagosome fuse with late endosome, the bacteria will then be present in late endosome. Late endosome contains a Fe^{2+} (divalent) ion channel encoded by the *NRAMP1* gene. If the NRAMP1 protein is functional, then Fe^{2+} ions will enter late endosome. Late endosome contain H_2O_2 (hydrogen peroxidase) which upon reacting with Fe^{2+} ions will then give rise to the highly reactive hydroxyl radical (OH*) through a chemical reaction called the Fenton Reaction:

$$Fe^{2+} + H_2O_2 \rightarrow OH^* + OH^- + Fe^{3+}$$

The highly reactive OH* radical will kill the mycobacteria by causing membrane demage.[15] In human with a mutated *NRAMP1* gene giving rise to a non functional NRAMP1 protein, the above reaction will not be killed and still survive within macrophage.

Several case-control study[9,10,12,20,22] have indicated that polymorphism of the human *NRAMP1* gene (ie, D543N, 3'UTR, and 274C/T polymorphism) modify the susceptibility of the host to tuberculosis, with the group affected including Africans and Asians. In addition, one interesting point is the fact that the proportion of TGTG del/del genotype in Surabaya patients is higher than that in the healthy nurses, whereas the proportion of TGTG del/del in a West African control group was slightly higher than that of West African patients, as reported in the study of Bellamy et al. The different proportions of TGTG del/del in Surabaya and West Africans in the case-control study seem to indicate that the 3'UTR polymorphism might not be the direct cause of susceptibility, but rather that another functional polymorphism exists in *NRAMP1*. The polymorphism might be strongly linked to the genotypes.

The question is why the 3'UTR mutation can result in susceptibility to tuberculosis? It was found that the mutant TGTG del allele produce a normal NRAMP1 protein, but in a much lower amount compared to the normal TGTG+ allele.

The majority of eukaryote mRNA posses a poly-A tail about 250 nucleotide long.[23,34] including the *NRAMP1* mRNA. When eukaryote mRNA enters cytosol, the poly-A tail is gradually degraded by cytosolic nuclease. Cytosol contains poly-A polymerase (PAP) which can results the poly-A tail, but it can not outcompete. The destruction due to the cytosol nuclease that the net effect is the gradually, but inescapably shortening of the poly-A tail with time.

When the poly-A tail is completely destroyed then the cytosol nuclears will eventually degrade the coding sequenes of the now tailless mRNA and protein production will stop. The TGTG del allele produce a much shorter. Shorter poly-A tail so that its mRNA will be degraded much eailier and resulting in a lower amount of NRAMP1 protein produced as compared to the TGTG+ allele with its longer poly-A tail.

Why the TGTG del allele produce a much shorter tail is not enterely clear. Apparently the 3'UTR region contain certain sequences needed to produce a normal length poly-A tail. These sequences is an AATAA sequence followed 23–24 nucleotides down stream with a GT rich region. A

loss of a TGTG sequence within the GT rich region may possibly desturb the formation of a normal poly-A tail resulting in a much shorter poly-A tail.[23,24]

This cross sectional study showed that homozygotes at TGTG del/del genotypes of 3'UTR polymorphism in *NRAMP1* gene were observed at significantly higher frequencies in patients with lung disease than in healthy nurses. This is the first study to indicate a possible genetic risk factor associated with isolated lung disease. Additional studies with patients from diverse ethnic backgrounds will be required to further investigate the relationships underlying these preliminary findings.

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