

# **Mycobacterial Diseases**

## Frequency of NRAMP1 Gene Polymorphisms among Canadian First Nations Peoples Experiencing Endemic Tuberculosis

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#### Abstract

**Objectives:** The natural resistance-associated macrophage protein 1 (NRAMP1) regulates susceptibility to infectious and autoimmune diseases. NRAMP1 gene polymorphisms have been implicated in susceptibility to tuberculosis. The frequency of NRAMP1 gene polymorphisms was therefore evaluated in three Manitoba First Nations sub-groups (Dene, Cree, and Saulteaux) with differential but high rates of tuberculosis (636/100,000, 496/100,000, and 0/100,000 respectively).

**Methods:** Venous blood samples were collected from 281 study participants from three First Nations sub-groups (Dene (N=108), Cree (N=41), Saulteaux (N=49)), and a non-indigenous Canadian-born (European-descent) group (N=83). Genomic DNA was extracted and four single nucleotide polymorphisms in the NRAMP1 gene (5' (GT)n, -274 (C/T), Intron 4 (469+14 G/C), D543N (G/A)) were genotyped using restriction fragment length polymorphism. NRAMP1 SNP allele frequencies were counted and compared between studied sub-groups.

**Results:** The Dene sub-group had significantly different allele frequencies of NRAMP1 (5'(GT)n, -274 (C/T), Intron 4(G/C), D543N (G/A)) compared to the European-descent group. The NRAMP1 allele frequencies at D543N (G/A) and Intron 4(G/C) differed significantly between the Cree and the European-descent group while the allele frequencies of the Saulteaux were not significantly different from the European-descent group. Two sub-groups (Dene and Cree) had higher frequency of NRAMP1 D543N (A) allele, which is associated with tuberculosis in other populations.

**Conclusion:** High, but differential rates of tuberculosis among the First Nation sub-groups in Manitoba are related to social determinants of health (i.e. poverty, racism, inadequate housing) but other potential risk factors such as gene polymorphisms associated with tuberculosis have only recently come under investigation. NRAMP1 allele frequencies were found to be different comparing the Dene, Cree and Saulteaux and their role in tuberculosis susceptibility/resistance needs further investigation.

**Keywords:** NRAMP1 gene polymorphisms; Canadian First Nation; Aboriginal; Tuberculosis

#### Introduction

Tuberculosis is a major global health concern not only in developing countries, but also among sub-populations in industrialized countries. In the Canadian province of Manitoba, the 2012 incidence of tuberculosis was 0.8 per 100,000 in the non-indigenous Canadian-born population<sup>1</sup>, compared to 66/100,000 among the First Nation<sup>2</sup> population [1]. First Nations in Manitoba are comprised of five distinct language and cultural sub-groups three of which are included in this study Dene, Cree and Saulteaux [2]. The Dene First Nation experienced an average annual tuberculosis incidence of 636/100,000 between 1994 and 2004 and they had the highest average annual tuberculosis incidence from 1975-1994 (496/100,000) [3]. The Cree and Saulteaux on reserve had lower tuberculosis rates (291/100,000 and 0/100,000) during this same period [4]. The determinants of

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First Nations, Inuit, and Metis are the three groups that comprise Aboriginals in Canada. First Nations have Status according to the revised Canadian Indian Act (1985).

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tuberculosis incidence in many Canadian First Nations populations include socioeconomic and political factors including poverty, racism, unequal access to health care, crowded and inadequate housing, and food insecurity, and biological factors such as host/pathogen genetics and co-morbidities [5,6]. There has been little work investigating the role that gene polymorphisms may have as risk factors for tuberculosis in these groups.

Among host genetic factors, gene polymorphisms in the natural resistance-associated macrophage protein 1 (NRAMP1) (also called SLC11A1 (solute carrier family 11A member 1)) have been associated with tuberculosis in Africa, Japan, Korea, and in North American indigenous groups [7-15]. Direct in vivo evidence for the complex interaction between the human host and Mycobacterium tuberculosis (Mtb) in terms of iron competition during infection and disease outcomes is limited, and *in vitro* studies and murine models introduce contradictory evidence [7,8,16,17]. NRAMP1 is a non-heme iron factor, localized in macrophage phagosomal membrane, known to transport divalent cations (putatively iron) across the phagosomal membrane, and known to confer resistance to intraphagosomal pathogens such as Mycobacterium tuberculosis (Mtb). NRAMP1 transports iron across the phagosomal membrane, thereby altering the Fe<sup>2+</sup> concentration into a bacterium containing phagosome [18-20]. The range of pleiotropic effects that NRAMP1 has on macrophage function includes the increased expression of pro-inflammatory cytokines (interleukin-1 $\beta$  and tumor necrosis factor- $\alpha$ ), the production of pro-inflammatory effectors molecules, and regulation of the adaptive immune response [21-23]. The immune regulatory capabilities of NRAMP1 make the gene a strong candidate for influencing both infectious diseases and autoimmune conditions [24-28].



**Figure 1:** Location of NRAMP1 polymorphisms analysed in this study. Exons are shown as black boxes and their respective numbers corresponding to the length (kb) of the gene. The grey boxes indicate the 3' and 5' untranslated regions and the introns. Below each polymorphism is the reference single nucleotide polymorphism (rs#) identification number.

Four NRAMP1 SNPs (5'(GT)n, 274 (C/T), Intron 4, D543N) have been studied globally and associations between the gene variants, tuberculosis and autoimmune diseases have had mixed results, confirmed in some studies but not in others [7,8,10,11,14,15,28-31] (Figure 1). In an analysis of a Canadian Aboriginal family, gene polymorphisms in NRAMP1 were associated with the rate of progression from infection to disease [8]. As a part of a larger study on tuberculosis in First Nations in Manitoba we therefore investigated the frequency of NRAMP1 alleles in three First Nations sub-groups in Manitoba that have differential rates of tuberculosis.

#### Study design and research methodology

Study participants and ethics: This study is a part of a larger research project on risk factors for tuberculosis in Manitoba First Nations sub-groups. The Dene participants are Athapaskan speakers and reside in a remote northern community in Manitoba that is accessible only by air or winter ice-roads.

The Algonquian speaking Cree participants are located in central Manitoba (approximately 700 km south of the Dene) in a community that is accessible all year-round. The Saulteaux participants are also part of the Algonquian language family but reside in south-western part of Manitoba (approximately 775 km from the Cree community) in a largely rural community.

Community consultation determined that convenience sampling (volunteers responding to advertisement), rather than random sampling, was the only methodology considered acceptable by First Nations community members for this research. Canadian Aboriginal research principles of ownership, control, access and possession (OCAP) were followed [32]. The University of Manitoba Health Research Ethics Board (H2005-106) and the communities Chief and Council approved the study. Study participants were 18 years of age or older, were able to provide written informed consent and selfidentified. First Nation participants were recruited in one of three geographically separate First Nation Reserves in Manitoba and individuals self-identified as Dene, Cree or Saulteaux. First and second-degree relatives of already enrolled participants were excluded. The non-indigenous Canadian-born (European-descent) individuals were enrolled at the University of Manitoba.

SNP analyses: DNA was extracted from whole blood by absorption onto QIAamp silica-gel following QIAGEN protease digestion (Qiagen, Mississauga, Canada). After column elution the purity and concentration of extracted DNA was determined by UV spectroscopy (BioRad, Mississauga, Canada). The following three NRAMP1 polymorphisms were typed by PCR amplification: a single-nucleotide change in Intron 4 (469+14G/C) (Intron 4) (GenBank rs3731865); a non-conservative single-base substitution at codon 543 in exon 15 that changes aspartic acid to asparagine (D543N) (GenBank rs17235409); a single nucleotide change in exon 3 (-274 C/T) (GenBank rs2276631). Restriction fragment length polymorphism with positive controls was used detect genotypes for -274 C/T, Intron 4 G/C, and D543N G/A following published protocols [33]. Each PCR reaction contained preoptimized sequence specific primers, 100 ng of genomic DNA, and 0.25U Taq polymerase (PE Biosystems, Mississauga, Canada). Following the initial denaturation steps, samples were subjected to an initial 9 rounds of PCR consisting of 96°C for 10 sec, 63°C for 60 sec followed by 20 rounds of PCR consisting of 96°C for 10 sec, 56°C for 30 sec and 72°C for 30 sec. To visualize the PCR products 10  $\mu l$  of the amplified reaction was run in a 2.5% agarose gel containing 0.5% ethidium bromide at 150 V for 5 minutes. DNA bands were then visualized with UV light on a transilluminator and photographed for subsequent analysis. A repeat in the promoter region of NRAMP1 (5'GTn) (GenBank rs34448891) was sequenced using the standard protocols with the ABI Big Dye Terminator Sequencing V.3.1 kit<sup>®</sup> to detect the major 5'(GT)n alleles-Allele 2 (t(GT)5ac(GT)5ac(CT)10g) or Allele 3 (t(GT)5ac(GT)5ac(CT)9g) in samples where the DNA yield was sufficient.

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	MAF		Genotypes				
D543N (1703) rs17235409	A	G	G/G	G/A	A/A	HWE (P)	Pa
Dene	0.2837	0.7163	0.5145	0.4078	0.0777	0.01	<0.0001
Cree	0.1667	0.8333	0.7714	0.2286	0	0.4	0.005
Saulteaux	0.0306	0.9694	0.9388	0.0612	0	0.8	0.7
European-descent	0.0225	0.9775	0.9551	0.0449	0	0.8	-
Intron 4 (469+14G/C) rs3731865	С	G	G/G	G/C	C/C	HWE (P)	P <sup>a</sup>
Dene	0.1048	0.8952	0.8585	0.1415	0.0000	0.3	<0.0001
Cree	0.0952	0.9048	0.8286	0.1143	0.0571	0.01	0.03
Saulteaux	0.2347	0.7653	0.551	0.4286	0.0204	0.05	0.3
European-descent	0.2528	0.7472	0.5618	0.3708	0.0674	0.8	-
274 (C/T) rs2276631	т	С	C/C	С/Т	Т/Т	HWE (P)	Pa
Dene	0.1238	0.8762	0.8817	0.086	0.0323	0.3	0.01
Cree	0.119	0.881	0.7714	0.1714	0.0572	0.07	0.16
Saulteaux	0.2449	0.7551	0.5833	0.3333	0.0834	0.4	0.9
European-descent	0.2611	0.7389	0.0899	0.3371	0.573	0.2	-
5'(GT)nrs34448891	A2	A3	A3/A3	A3/A2	A2/A2	HWE (P)	Pa
Dene	0.1733	0.8267	0.8095	0.1904	0	0.2	0.02
Cree	0.2375	0.7625	0.5897	0.359	0.0513	0.06	0.68
European-descent	0.2614	0.7386	0.5682	0.3409	0.0909	0.2	-

**Table 1:** NRAMP1 allele and genotype frequencies for three First Nations and a European-descent cohort. MAF: Minor allele frequency; HWE:

 Hardy Weinberg Equilibrium; P<sup>a</sup>: Reference European-descent.

**Statistical analysis:** Genetic analyses were performed using GENEPOP (version 1.2) Inc [34]. Hardy-Weinberg Equilibrium (HWE) was assessed for the genotype distribution of each NRAMP1 restriction sites using Fishers Exact test. NRAMP1 genotype frequencies were compared using the Fisher Exact two-tailed test; p<0.05 was reported as significant.

## Results

Two hundred and eighty-one participants were enrolled into the study (Dene N=108, 52 males/56 females, mean age=43 years; Cree N=41, 19 males/ 22 females, mean age=41 years; Saulteaux N=49, 25 males/ 24 females, mean age=42 years; European-descent group N=83, 31 males/52 females, mean age=52 years). NRAMP1 SNPs (Table 1) at Intron 4 (Cree and Saulteaux) and D543N (Dene) were not in HWE (p<0.05), indicating that these genotype frequencies deviate from equilibrium and may be under selective pressure (genetic drift).

Compared to European-descent participants, Dene First Nation had significantly different allele frequencies at four NRAMP1 loci (5' (GT)n (p=0.02), -274(C/T) (p=0.01), Intron 4 (G/C) (p<0.001), and D543N (G/A) (p<0.001)) (Table 1). Cree participants were significantly different at 2 loci (Intron 4 (G/C) (p=0.03), D543N (G/A) (p=0.005)), and Saulteaux were not significantly different. Differences were also

found in the allele frequencies between the First Nations subgroups. The Dene had significantly different frequencies of NRAMP1 SNPs than the Saulteaux at all four loci and one loci was significantly different from the Cree subgroup D543N (G/A) (p<0.001).

## Discussion

This is the first report of differences in allele frequencies of the NRAMP1 gene between First Nation sub-groups that have differential rates of tuberculosis. Compared to the participants of Europeandescent, the Dene and Cree significantly differ in NRAMP1 allele frequencies at the D543N and Intron 4. The Dene also had allele frequencies at the NRAMP1 5' (GT)n and -274 (C/T) loci that were significantly different from the European-descent group. The Saulteaux were not significantly different from the European-descent sub-group. Differences in allele frequencies between European-descent groups, Dene, Cree, and Saulteaux were found in previous studies of immune regulatory genes (i.e. cytokine promoters) [5,35]. This analysis found that the Dene maintained higher frequencies of alleles (D543N (A) and -274 (C)) that have been associated with tuberculosis in other populations [11,29]. In a meta-analysis of this NRAMP1 gene polymorphism carriage of the D543N A allele, which has a frequency of 28.3% in the Dene cohort, was significantly associated with TB (OR, 1.24: 95% CI 1.04-1.49) in African and Asian populations but not Western European [7,9,14,15]. NRAMP1 D543N (G/A) (Asparagine/ Aspartic acid) codon substitution of the negatively charged aspartic acid to an uncharged asparagine residue may affect the function of the protein or be linked to NRAMP1 genes and limit the macrophage response to Mtb [16,20,24]. The A allele is rare (but not absent) in western and central European populations and in the Europeandescent group (<5%) in this study [36-38]. In studies where the A allele occurs, the variant genotypes G/A and A/A or the A allele were of significantly higher frequency in tuberculosis patient groups compared to controls in Indonesian, Cambodian and African groups [7,29,39,40]. The D543N A allele was found in 22% of Indonesian controls but only in 2-15% of other Asian populations [9,38,39]. The occurrence of D543N A/A homozygotes in the Dene but not the Cree, Saulteaux, or European-descent cohorts could indicate selective pressures or it could be a function of the small population.

The Dene but not the Cree or Saulteaux, had significantly higher frequencies of the NRAMP1 -274 C allele and C/C homozygotes compared to the European-descent groups. The NRAMP1 -274 (C/T) allele frequencies for the European-descent group were similar to those of western and northern Europe and for North American Caucasians [41]. In a study by Malik [11] the common C allele of the -274 (C/T) SNP was strongly associated with pediatric tuberculosis particularly in males. A conclusion has been made that this allele promotes rapid progression from infection to disease in primarily American, and Asian populations [11,16,42]. Further studies provided direct evidence that the -274 C/C carriers had reduced NRAMP1 function as compared to those with the C/T genotype and that the C allele may be linked with low innate macrophage function [13]. In other studies a moderate linkage disequilibrium between the 5' (GT)n and -274 (C/T) was found but no association to tuberculosis in a Polish group [36].

While the Dene have significantly different allele frequencies at NRAMP1 5' (GT)n and Intron 4 compared to the European-descent, they have higher frequencies of the alleles that may be protective against infectious diseases [43,44]. The Dene had a higher frequency of Allele 3 of the NRAMP1 5' (GT)n compared to the European-descent group and an increased percent of A3/A3 homozygotes. NRAMP1 5' (GT)n polymorphic alleles been shown to be associated with autoimmune and infectious diseases and Allele 3 has been associated with protection from infectious diseases and tuberculosis [7,14,16,45]. In a recent meta-analysis of NRAMP1 alleles and disease Allele 2 was associated with increased susceptibility to tuberculosis and autoimmune conditions [7,27,31,46,47]. Of the nine NRAMP1 5' (GT)n known to-date alleles 2 and 3 predominate and have differential frequencies in developing countries and western populations potentially reflecting differential responses to selective pressure [48,49]. The four most common alleles (Allele 1 (GT)5AC(GT)11G; Allele 2 (GT)5AC(GT [18])10G; Allele 3 (GT)5AC(GT)9G; and Allele 4 (GT)9G) have known functional differences [16,48]. Allele 3, the most common promoter allele drives high NRAMP1 expression and has a variable frequency of 0.65-0.85, depending upon geography and ethnicity [50]. Allele 2 drives low expression of NRAMP1 expression and occurs at a frequency of 0.10-0.30 [50]. The expression however can be altered in response to cytokine and other exogenous stimuli [49]. In the absence of any stimuli alleles 1, 2 and 4 were poor NRAMP1 promoters whereas Allele 3 drives high expression. INF stimulates similar levels of enhancement for all four alleles. In contrast, LPS has no effect on NRAMP1 expression Alleles 1 and 4, reduced expression in Allele 2, and enhance Allele 3 expression [18,49].

The Dene and Cree sub-groups had significantly lower frequency of the Intron 4 risk allele (C) and the genotype (G/C) compared to the European-descent, Saulteaux, and Cree. The Intron 4 C allele carriage (CC+GC) was associated with increased risk of tuberculosis as compared to GG genotype (OR, 1.23; 95% CI, 1.04-1.44) as summarized in recent meta-analyses [31,51]. The frequency of the Intron 4 G allele in the Dene (90%) and Cree (89%) was similar to that found in Asians (89%) but higher than that of an African (64%) group and a small Inuit (72%) group [43]. The allele and genotype frequencies for the European-descent and Saulteaux were similar to those for Caucasian and African groups [43]. The association of this allele with tuberculosis was significant in African and Asian but not in Western European subgroups [7,15,38,52]. Soborg et al. found a highly significant correlation between the C allele and the presence of microscopy-positive tuberculosis (p=0.004) [43]. In some populations (i.e. Indonesia) the Intron 4 polymorphism is rare [39].

The higher frequency in the Dene sub-group of the purported protective NRAMP1 Allele 3 compared to the Cree and a Europeandescent group, and the low frequency of the purported Intron 4 risk allele, is inconsistent with the rates of tuberculosis in these sub-groups. Reasons for this discrepancy include that NRAMP1 5' (GT)n and Intron 4 polymorphisms are important loci but may be linked to other functional loci. Other complex interactions (social, economic, nutritional status, other co-infections) play a role in the tuberculosis susceptibility and/or resistance. These risk factors and others are not controlled for in this study.

The Manitoba Dene First Nation sub-group have significant differences in the frequencies of NRAMP1 alleles compared to a Cree, Saulteaux, and a European-descent. The Dene had higher frequencies of NRAMP1 alleles that have been associated with tuberculosis in other populations (-274 C/T and D534N G/A) and this sub-group has experienced the highest rates of tuberculosis. In contrast, the allele frequencies of the 4 NRAMP1 polymorphisms were not significantly different between the Saulteaux and European-descent sub-groups. These groups have similar and low rates of tuberculosis. In populations with high rates tuberculosis, nutritional deficiencies of vitamin D, zinc, protein, vitamin A, and iron may be an important determinant of the incidence of tuberculosis [53]. Iron has a crucial role in mediating the host-pathogen interactions, as pathogens utilize iron to influence establishment of infection, propagate pathogenesis and sometimes overcome host defences. Even though it has been shown that iron metabolism can contribute to advancing the pathology and increase mortality in infectious disease, mechanisms in the context of Mtb infection has not been completely delineated [20]. Limitations in the study are the small, potentially non-random, samples, which may make these findings not generalizable to the larger First Nations groups. The occurrence of HWE deviation in the D543N and the Intron 4 allele frequencies for the Dene and Cree may support the supposition that these groups are subject to differential selective pressures but genotyping errors, small sample size, or disease associations can also cause HWE deviation [35]. Clinical data on the participants were not collected but individuals within each sub-group were members of First Nation Reserves undergoing tuberculosis surveillance. [4].

## Conclusions

The identification of gene polymorphisms that have immune regulatory capabilities cannot fully rationalize the differential rates of tuberculosis between First Nations sub-groups, and non-Aboriginal groups. However, the investigation of gene polymorphisms that may play a role in the control of MTB in macrophages (i.e. NRAMP1) may contribute to a more fulsome and balanced understanding of the risk factors for tuberculosis. The populations in this study are rarely included in the analyses of genetic risk factors for tuberculosis for reasons including lack of trust of researchers, community priorities other than research, and a historical lack of sharing of research findings. The findings from this study suggest that genetic risk factors for tuberculosis may play a role in susceptibility and/or resistance in Canadian First Nations and should be further investigated at the subgroup level along with social and environmental determinants of health.

A more detailed understanding of the pathways of the innate immune response to Mtb among Canadian First Nations may assist in the development of preventive (i.e. vaccine) and therapeutic modalities to be implemented in tandem with efforts to address the non-biologic determinants of this disease. This study is a part of longstanding research collaboration with First Nations peoples, investigating the social, environmental, and biological factors influencing the high rates of TB in their communities. Although the social and environmental factors are of overwhelming importance in the causation of tuberculosis in this population, and are amenable to intervention with supporting political will, greater understanding of the biologic determinants may also allow for the development and application of effective medical strategies.

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