Nontuberculous mycobacterial cervicofacial lymphadenitis in children from the multicenter, randomized, controlled trial in The Netherlands: Relevance of polymorphisms in candidate host immunity genes

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1. Introduction

In a prospective nationwide surveillance study of nontuberculous mycobacterial (NTM) infection in Dutch children, published in 2004, we reported an annual incidence rate of 0.77 NTM infections per 100,000 children [1]. NTM disease arose most often as localized cervicofacial lymphadenitis. NTM thrive everywhere and pose a serious threat of generalized infections in patients with a vital breach in their defense mechanism, e.g., patients on immunosuppressive medication, HIV-positive individuals and patients with mutations in the IL-12/IFN-γ pathway or in NF-κB essential modulator protein (NEMO) [2]. However, most children, teens and healthy adults remain unaffected. Therefore, the high occurrence of NTM infections in very young and apparently healthy children is puzzling. So far, there is no evidence that toddlers lack essential immunological protection against mycobacteria as compared to adults. The 2004 study suggested that direct exposure of oral mucosa to mycobacteria during eruption of teeth could be relevant to the etiology of NTM lymphadenitis [1]. We hypothesize that children prone to NTM bear single nucleotide polymorphisms (SNPs) in genes that are...
Table 1
Distribution of alleles and genotypes of two SNPs related to NTM infection in children: 3953C>T in IL1B and −592C>A in IL10.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Alleles/genotypes</th>
<th>Frequency in patients (%)</th>
<th>Frequency in controls (%)</th>
<th>( \chi^2 ) test p-value OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL1B</td>
<td>3953C&gt;T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>108 (66.7)</td>
<td>318 (74.0)</td>
<td>0.098</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>54 (33.3)</td>
<td>112 (26.0)</td>
<td>0.057</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>37 (45.7)</td>
<td>113 (52.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>34 (42.0)</td>
<td>92 (42.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>10 (12.3)</td>
<td>10 (4.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CC/CT</td>
<td>71 (87.7)</td>
<td>205 (95.3)</td>
<td>0.036 Reference 2.5 (1.2–7.2)</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>10 (12.3)</td>
<td>10 (4.7)</td>
<td></td>
</tr>
<tr>
<td>IL10</td>
<td>−592C&gt;A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>125 (79.1)</td>
<td>311 (73.7)</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>33 (20.9)</td>
<td>111 (26.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>52 (65.8)</td>
<td>113 (53.6)</td>
<td>0.098</td>
</tr>
<tr>
<td></td>
<td>AC</td>
<td>21 (26.6)</td>
<td>85 (40.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>6 (7.6)</td>
<td>13 (6.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Homozygotes</td>
<td>58 (73.4)</td>
<td>126 (59.7)</td>
<td>0.043 Reference 0.54 (0.3–0.95)</td>
</tr>
<tr>
<td></td>
<td>Heterozygotes</td>
<td>21 (26.6)</td>
<td>85 (40.3)</td>
<td></td>
</tr>
</tbody>
</table>

Relevant to mycobacterial immunity and associated with severity of periodontal disease. Accordingly, we determined 22 SNPs in a patient cohort from the 2007 CHIMED study (surgical excision versus antibiotic treatment for cervicofacial NTM lymphadenitis in children) [3]. All polymorphisms, with the exception of SNPs in TLR4 and IL1B, have been associated in previous studies either with susceptibility to or protection from tuberculosis (TB). Some of the SNPs we know are also related to periodontal disease. Furthermore, we screened for the most common mutation (818del4 in IFNGR1) that leads to Mendelian Susceptibility to Mycobacterial Disease (MSMD).

### 2. Methods

This genetic association study includes 81 Dutch children (0–18 y/o) with NTM cervicofacial lymphadenitis, referred by general and specialist health care centers nationwide ('CHIMED-cohort', previously described [3]). We excluded patients with serologic evidence of other infectious agents causing chronic lymphadenitis (i.e., active infection by cytomegalovirus, Epstein-Barr virus, Adenovirus, Bartonella species and toxoplasmosis). We included only those patients with a culture-confirmed and/or real-time PCR positive NTM infection of the lymph node, as investigated with fine-needle aspirate. All patients were PCR-negative for M. tuberculosis complex. We excluded patients immunocompromised by medication such as prednisone or anti-TNF blockers. The control group consisted of 215 randomly selected adults from the center of The Netherlands. We obtained written informed consent from all patients and/or their guardians and from all control subjects. The local Ethics Committees approved the study.

We genotyped the following 22 SNPs [gene(SNPs)]: CD209(−139G>A), IL1B(−511C>T, 3953C>T), IL8(−251A>T), IL10(−1082A>G, −592C>A), IL12B(ins/del, 1188A>C), IL12R(−2C>T, 641A>G), IL18(−137G>C, 105A>C), PTX3(143A>C, 130+9G>A, 532+326G>A), TLR4(896A>G, 1196C>T), TNF(−238G>A, −308G>A), VDR(61968T>C) and SLC11A1(1627G>A, −INT4). These SNPs were selected from the literature and databases on the web because of their link to mycobacterial immunity. We isolated genomic DNA from whole blood using standard methods. Multiplex assays were designed using Assay designer software (Sequenom). We genotyped using the MassArray platform according to manufacturers protocols (Sequenom). For quality control, we genotyped 10% of samples in duplo and observed no inconsistencies. The program CONTING v2.62 analyzed the association of the polymorphisms to localized NTM disease [4]. To screen for the 818del4 mutation (TATA in exon 6 of IFNGR1), we performed a PCR on 100 ng DNA following standard procedures and digested the DNA with Vsp1 (Fermentas) on a potential AT-TAAT site.

### 3. Results

All 81 patients were born in The Netherlands, with 11 coming from non-European extraction. More than half of the children are below the age of 4 (53%, median age 3.8 years, range 1.2–14). Three patients are known to have other serious infections, namely viral meningitis, laryngitis subglottica and mononucleosis infectiosa. We noted 2 cases of growth retardation and autism each. Sixty-nine children (85%) were affected in 1 lymph node only and exhibited no fatigue, fever or weight loss. Generalized symptoms were seen in 13 of the cases. All patients were diagnosed with NTM infection, by culture (55 cases), PCR (26 cases) or both (1 case). In most cases, the infection was caused by M. avium (55 cases, 68%) or M. haemophilum (20 cases, 25%). In the remaining individuals, we isolated M. malmseomae (3 cases), M. fortuitum (1 case) or M. chelonae (1 case). The mycobacterium could not be classified in one instance.

We did not detect a single case among these 81 children of 818del4 in IFNGR1, the most common MSMD mutation. Neither patients nor controls exhibited significant difference from Hardy–Weinberg proportions for the 22 SNPs we analyzed. The relative allele frequencies and genotype distributions for the SNPs 3953C>T in IL1B and −592C>A in IL10 reported in Table 1 show no significant correlation between NTM infection and genotypes, respectively. However, a closer look at CC/CT versus TT in +3953 (IL1B) and homozygotes versus heterozygotes in −592 (IL10) revealed significant differences between cases and controls using uncorrected p-values (\( p = 0.036 \) and 0.043 respectively). Therefore, carrying the IL1B +3953TT genotype seems to be linked to susceptibility to NTM infection (OR 2.9, 95%-CI: 1.2–7.2). In addition, IL1O −592 heterozygosity would be protective (OR 0.54, 95%-CI: 0.3–0.95). These statistics should be interpreted with caution given the small sample size. Importantly, none of the patient-control differences remained significant after Bonferroni’s correction for multiple testing (0.05/n = 0.0522 informative SNPs = 0.0023). The other 20 polymorphisms were not associated with NTM in children (data not shown).

### 4. Discussion

+3953TT in IL1B and −592 homozygosity in IL10 are significantly correlated with localized NTM cervicofacial lymphadenitis in Dutch children. All other candidate SNPs we selected were...
unrelated to NTM infection, despite their role in granulomatous inflammation and cellular immunity. This report is one of precious few studies addressing the possible link between SNPs in candidate genes and NTM infections (as opposed to tuberculosis). A role for genetic factors in susceptibility to NTM disease has been accepted ever since research uncovered mutations in the IL12/IFN-γ-signaling pathways and NEMO (MSMD) in a select group of patients suffering from generalized mycobacterial infections [2]. In our sample, we have excluded the most prevalent of those mutations, 818del4 in IFNGR1. Neither of the correlations between IL1B and IL10 with NTM infection was robust to Bonferroni’s correction. However, to reach adequate power (>0.8) with major allele frequencies such as those carried by 3953C>T and −592C>A (74% in controls and 67–79% in patients [Table 1], we would have needed an unrealistic sample size of about 2000 cases and controls. Furthermore, as in most SNP studies, associations between gene variations and disease might be swamped by confounders such as population heterogeneity and common unobserved components (linkage disequilibrium, LD). Also, one could argue that Bonferroni’s test will be overly conservative because it does not take into account the selection bias of our candidate SNPs that arises from our theoretical understanding of underlying immunological pathways.

Our case for biological plausibility of the link between the IL1B SNP 3953C>T and childhood NTM lymphadenitis is as follows. We initially genotyped the IL1B SNP 3953C>T in our study because of its supposed relationship to tuberculosis. Indeed, the SNP influences IL1B mRNA levels, which are important for host defense to mycobacteria [5]. Additionally, various reports have linked the same SNP to periodontal disease where IL1B plays a crucial role [6–9]. In particular, the cytokine facilitates leukocyte migration via adhesion molecules and regulates matrix metalloproteinases, prostaglandin E2 production and osteoclastic activity [10]. Many studies typically analyze 3953C>T jointly with −889C>T in IL1A and conclude that the composite genotype (+3953T−889T) is related to earlier incidence of advanced periodontitis in non-smokers (see overview in [8,10]). Other studies, however, reject this link or cannot separate it from other factors such as age, smoking and the presence of P.gingivalis [10]. Taken together, these considerations suggest a case for linking 3953C>T in IL1B to childhood NTM lymphadenitis via its influence on periodontal inflammation.

In our hypothesis, NTM in children likely enter the oropharyngeal mucosa via gingival damage. Such damage could be caused by teething where periodontal reactivity is potentially genetic— or periodontitis. Indeed, more than 70% of children eight years and older in the general population suffer from bacterial biofilm-induced gingivitis that could lead to prepubertal periodontitis (age of onset: 4 years and older; prevalence 0.8–27%) [11]. We conjecture that mycobacteria target dendritic cells which then transfer them to the next lymph node station. This mechanism has already been established for TB, where the cervicofacial lymph node is considered part of the primary complex. In an earlier article, we had already suggested that children could contract NTM lymphadenitis via oral-inflamed mucosa when using their mouth as an exploratory instrument, thus sampling objects that can harbor NTM [1]. Autistic children are particularly susceptible to this habit (“pica”, an abnormal appetite for inedible objects). Indeed, the incidence of autism in our admittedly small sample (2/81) exceeds that of the general population (3–6/1000) [12].

With regard to the IL10 −592C>A, the other SNP that is the focus of our study, we found an association between heterozygosity of the IL10 SNP and protection against NTM infection in young children. The −592 SNP alters IL10 cytokine production because it is located in a region of the gene with a negative regulatory function [13]. Research shows that the more frequent C-allele protects against TB [14]. This confirms the influence of the −592 SNP on mycobacterial susceptibility. We hypothesize that −592 homozygosity in IL10 alters the immunological Th1/Th2 balance in such a way that it curtails periodontal inflammation initiated by teething. If so, this homozygosity will hamper the clearance of mycobacteria and sustain infection. Consequently, NTM disease and periodontal inflammation would appear to have two common components, 3953C>T in IL1B and −592C>A in IL10.

This study brought to light new but statistically tenuous genetic associations between localized NTM disease and periodontitis in children. We believe that this statistical fragility should not deter from conducting further SNP studies explaining NTM infections in otherwise healthy children. Results may be individually weak but cumulatively meaningful. Also, there is neither evidence nor theory suggesting the existence of another etiological mechanism for NTM. Furthermore, we cannot exclude that there may be other, yet unidentified SNPs with a link to NTM disease. A targeted search for NTM-related polymorphisms in cohorts that are as large as possible remains justified.

Conflicts of interest

No conflict of interest is stated by any of the authors.

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