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ORIGINAL ARTICLE

A haplotype spanning *P2X7R*, *P2X4R* and *CAMKK2* may mark susceptibility to pulmonary non-tuberculous mycobacterial disease

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Abstract Despite widespread exposure to potentially pathogenic mycobacteria present in the soil and in domestic water supplies, it is not clear why only a small proportion of individuals contract pulmonary nontuberculous mycobacterial (NTM) infections. Here, we explore the impact of polymorphisms within three genes: P2X ligand gated ion channel 7 (*P2X7R*), P2X ligand gated ion channel 4 (*P2X4R*) and calcium/calmodulin-dependent protein kinase kinase 2 beta (*CAMKK2*) on susceptibility. Thirty single nucleotide polymorphisms (SNPs) were genotyped in NTM patients (n = 124) and healthy controls (n = 229). Weak associations were found between individual alleles in *P2X7R* and disease but were not significant in multivariate analyses adjusted to account for gender. Haplotypes spanning the three genes were derived using the fastPHASE algorithm. This yielded 27 haplotypes with frequencies >1% and accounting for 63.3% of the combined cohort. In univariate analyses, seven of these haplotypes displayed associations with NTM disease above our preliminary cut-off ($p \leq 0.20$). When these were carried forward in a logistic regression model, gender and one haplotype (SH95) were independently associated with the disease (model $p < 0.0001$; $R^2 = 0.05$). Examination of individual alleles within these haplotypes implicated *P2X7R* and *CAMKK2* in pathways affecting pulmonary NTM disease.

Keywords Non-tuberculous mycobacteria · Genetic polymorphism · Pulmonary disease · Immunogenetics

Introduction

Pulmonary disease is the most common presentation of infections with nontuberculous mycobacteria (NTM) in otherwise healthy adults. Hence, NTM are considered an emerging public health risk in Australia and around the world (Thomson 2010; Winthrop et al. 2010). Exposure through soil or water is extremely common but only a small subset of the population develops pulmonary NTM disease. Estimates in Queensland, Australia, suggest 22.1 persons per 100,000 in the population will develop the disease (Thomson 2010). Known risk factors include smoking, excessive alcohol intake, past tuberculosis and underlying structural lung disease such as COPD and

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bronchiectasis (Cassidy et al. 2009; Winthrop et al. 2010). However, these factors do not account for all cases—a notable exception being patients with the "Lady Windermere" phenotype. These individuals are lean and tall, with higher than normal incidence of thoracic abnormalities including scoliosis, *pectus excavatum* and mitral valve prolapse. The majority of cases are Caucasian (91%) and female (95%), with an average age of 60 years (Kim et al. 2008). Behavioural questionnaires found no links between pulmonary NTM disease and patients' activities, exposures or habits (Kim et al. 2008). Genetic factors should be investigated further.

The risk of pulmonary NTM disease is increased in individuals carrying certain alleles of genes encoding cytokines including IL-10, TNFα and IL-28B (Affandi et al. 2013). The critical effect of *IL10* polymorphisms was replicated in the present cohort (Halstrom et al. 2017). Other studies have linked polymorphisms in natural-resistance-associated macrophage protein 1 (*NRAMP1*) and cystic fibrosis transmembrane conductance regulator (*CFTR*) to an increased risk of NTM disease (Jang et al. 2013; Kim et al. 2008; Koh et al. 2005; Tanaka et al. 2007; Ziedalski et al. 2006). Genes of relevance showing no genetic associations with pulmonary NTM disease risk also include *VDR* and *TLR2* (Huang et al. 1998; Park et al. 2008a; Park et al. 2008b; Ryu et al. 2006). An intronic microsatellite repeat polymorphism in *TLR2* (Yim et al. 2008) has been linked with pulmonary NTM disease. Mendelian susceptibility to mycobacterial disease (MSMD) is a disease associated with bioactive polymorphisms in genes involved in IFN-γ dependent immunity (*IFNGR1*, *IFNGR2*, *STAT1*, *IL12B*, *IL12RB1*, *ISG15*, *IRF8*, *NEMO* and *CYBB*) (Al-Muhsen and Casanova 2008; Bustamante et al. 2014), but these alleles are not associated with pulmonary NTM disease.

No study yet has investigated possible associations between polymorphisms in P2X ligand gated ion channel 4 (*P2X4R*), P2X ligand gated ion channel 7 (*P2X7R*), calcium/calmodulin-dependent protein kinase kinase 2 beta (*CAMKK2*) and pulmonary NTM disease risk. Their potential for modulation of the inflammatory immune response makes them viable candidates. In particular, *P2X7R* has been associated with aggressive tuberculosis presentations; *P2X4R* and *CAMKK2* are important in macrophage function and activated killing of phagocytosed bacteria (central to mycobacterial disease pathogenesis), and all three lie in a cluster on chromosome 12 exhibiting some measure of linkage disequilibrium (Goullee et al. 2016).

The *P2X7R* gene encodes the purinergic receptor P2X7R protein, an ATP-gated ion channel which plays an important role in the innate immune response. When bound by its ligand, extracellular ATP, P2X7R enables the intracellular killing of phagocytosed bacteria (e.g. NTM) in the presence of local inflammatory stress. An important role for the P2X7R has been identified in aggressive forms of tuberculosis (Amaral et al. 2014; Ge and Chen 2016; Wu et al. 2014). A *P2X7R* knockout mouse model implicated *P2X7R* in *Mycobacterium tuberculosis* infection (Santos et al. 2013). These data make *P2X7R* a candidate for modulation of NTM disease.

P2X4R plays a similar role to *P2X7R* in immune regulation. The gene encodes the purinergic receptor P2X4R protein, a purinoreceptor activated by ATP allowing the transport of calcium ions across a membrane. *P2X4R* is generally co-expressed with *P2X7R* in intracellular lysosomal compartments of macrophages and microglia but can be rapidly trafficked to the surface membrane via the process of endolysosomal secretion (Stokes and Surprenant 2009). P2X4R appears to be involved in the process of phagocytosis, lysosomal destruction and removal of pathogens taken up by macrophages, specifically post-fusion phase exocytosis (Miklavc et al. 2011). Polymorphisms in *P2X4R* may allow NTM to exploit this system, and so persist in macrophage phagosomes via inhibition of lysosome binding.

CAMKK2 encodes the CaMKK2 protein, a calcium ion activated protein kinase involved in many different pathways, including lymphocyte activation. CaMKK2 may act downstream of P2X4R in the process of exocytosis in macrophages that have taken up NTM, with P2X4R allowing calcium ions to traverse cell membranes activating Calmodulin-dependant pathways. This function may be affected by the total or partial loss-of-function mutations leading to an increased susceptibility to NTM. *CAMKK2* is selectively expressed at higher than base levels in monocytes, macrophages and B-lymphocytes in response to lipopolysaccharide (LPS) stimulation (Racioppi et al. 2012). In knockout mice, *CAMKK2*-null macrophages displayed deficiencies in motility, phagocytosis of bacteria and synthesis of cytokines in response to the TLR4 agonist LPS (Racioppi et al. 2012). Loss of CaMKK2 function uncouples the TLR4 cascade, thereby failing to properly activate the inflammatory response. Poor macrophage function in *CAMKK2* knockout-mice establishes the potential for mutations in this gene to affect NTM disease.

Here, single nucleotide polymorphisms (SNPs) and haplotypes spanning *P2X7R*, *P2X4R* and *CAMKK2* are investigated for associations with the incidence of pulmonary NTM disease in Caucasians. Associations were corrected for the disproportionate incidence of pulmonary NTM disease in females.

Materials and methods

Patients and controls

DNA was obtained from patients attending Greenslopes Private Hospital and Prince Charles Hospital with pulmonary NTM disease between 2005 and 2014. All participants

provided a written informed consent to participate in this work, which was approved by the Greenslopes Research and Ethics Committee (Protocol 12/12) in accordance with the National Statement on Ethical Conduct in Human Research. Control samples were supplied by the Institute for Respiratory Health, the University of Western Australia, in accordance with the Royal Perth Hospital Human Research Ethics Committee. This cohort is a representative of the Caucasian Australian population from which patients were also derived.

Genotyping

DNA samples were quantified with Qubit Fluorometric Quantitation technology (Thermo Fisher Scientific, Waltham, MA, USA) and adjusted to 50 ng/uL. Genotyping was performed using TaqMan OpenArray Genotyping Plates (Life Technologies, Grand Island, NY, USA) (Goullee et al. 2016). DNA samples were diluted at 1:1 in TaqMan OpenArray Genotyping Master Mix for 50 cycles of PCR amplification. The output was viewed using OpenArray™ SNP Genotyping Analysis software, and genotypes were allocated manually. Several SNPs were excluded from further analysis, as they did not meet Hardy-Weinberg Equilibrium (HWE) or had a high percentage of no call readings (rs11065456, rs208294, rs208307, rs12299020, rs504677, rs10160951, rs2230912, rs2668252, rs11608486, rs1169719, rs11065502, rs11065503, rs11837114, rs1718120 and rs2686367).

Haplotype construction

Haplotypes were identified using the fastPHASE algorithm (Scheet and Stephens 2006). As per convention, the most frequent allele in the population is denoted 0, with the "minor" allele denoted 1. This system is used to portray haplotypes in this study. During haplotype construction, multiple possible haplotypes were generated with fastPHASE for each individual based on their genotypes. The most probable combination of haplotypes for each was selected. From these, 188 different haplotypes were identified from the combined patient and control samples (n = 353). Haplotypes with a combined frequency of 1% or less across patient and control populations were excluded from further analysis.

Statistical analyses

Statistical analyses were performed in Stata 12 (StataCorp, Collage Station, TX, USA). Univariate analyses evaluating associations between pulmonary NTM lung disease and gender, age, SNP and haplotypes were performed using two-tailed Fisher's exact or Chi^2 tests (χ^2) as appropriate. Multivariate analyses were performed using logistic regression modelling where all included factors associated weakly with NTM in univariate analysis ($p < 0.20$ cut-off chosen for this exploratory study), followed by a stepwise removal procedure to obtain the model of best fit.

Results

The demographic profile of patients with pulmonary NTM disease

Samples were available from 124 patients with pulmonary NTM disease (attending clinics at the Prince Charles and Greenslopes Private Hospitals, Queensland, Australia) and 229 healthy controls (resident in Western Australia). All participants declared Caucasian ethnicity. Seventy-three percent of the patients and 55% of the healthy controls were female. As this is a significant difference (χ^2, $p = 0.001$), gender was included in logistic regression models for SNP and haplotypes of interest.

NTM patients were older than healthy controls [median (range) 67 (25–89) vs. 44 (21–75) years respectively]. Whilst some control donors may develop pulmonary NTM lung disease later in life, the impact on the data is considered minor as pulmonary NTM disease is rare. Patients displayed infections from *Mycobacterium intracellulare* (n = 64), *Mycobacterium avium* (n = 13), *Mycobacterium abscessus* (n = 10), *Mycobacterium kansasii* (n = 3), *Mycobacterium triplex* (n = 2), *Mycobacterium xenopi* (n = 1), *Mycobacterium simiae* (n = 1), *Mycobacterium interjectum* (n = 1), *Mycobacterium shimoidei* (n = 1), *Mycobacterium terrae* (n = 1), *Mycobacterium gordonae* (n = 1) or *M. lentiflavum* (n = 1). Some individuals were infected with more than one mycobacterial species (n = 12).

Associations between SNP genotypes and pulmonary NTM disease

Of the SNPs tested, four in *P2X7R* (rs208288, rs1718125, rs1186055 and rs2857585) were weakly associated with pulmonary NTM disease ($p \leq 0.20$) in univariate χ^2 analysis. These SNP and gender met our criteria for inclusion in logistic regression modelling (Tables 1 and 3). The optimal model (model $p = 0.0004$; $R^2 = 0.027$) did not retain any SNP, only gender. No SNP from *P2X4R* or *CAMKK2* were associated with NTM in univariate analyses ($p > 0.20$).

Table 1 No SNP associated with NTM in univariate analyses

SNP[a]	Position	MA	MAF[c] Control (%)	MAF[c] NTM (%)	*p* value (χ^2)
P2X7R					
rs10849849	121586395	G	6.6	7.0	0.90
rs208288[b]	121588088	G	19.5	12.8	0.14
rs17525767	121588125	T	21.2	21.7	0.91
rs1718125[b]	121593019	T	26.2	19.5	0.16
rs1169737	121600294	T	5.3	7.4	0.43
rs1186055[b]	121600529	C	47.8	39.2	0.13
rs2857585[b]	121600802	G	14.3	9.4	0.19
rs208296	121600953	G	45.4	42.9	0.65
rs11065464	121602135	C	45.8	50.9	0.38
rs503720	121605074	G	45.5	40.0	0.33
rs1653609	121605919	C	25.4	21.4	0.46
rs2230911	121615131	G	11.7	13.7	0.59
rs1653598	121615283	T	40.0	41.3	0.54
rs3751144	121622239	T	11.2	14.4	0.39
rs3751143	121622304	C	33.9	31.7	0.67
rs12301635	121624108	G	20.0	14.6	0.21
P2X4R					
rs2686387	121648870	G	48.0	43.3	0.41
rs2303998	121655063	G	4.0	5.7	0.47
rs7298368	121659684	T	29.9	33.3	0.51
rs25643	121660787	T	31.5	27.9	0.47
rs10849860	121668254	T	28.1	30.6	0.63
CAMKK2					
rs1653587	121676232	G	12.0	13.3	0.73
rs1653588	121676666	T	11.6	11.4	0.96
rs7961979	121671261	C	27.0	24.8	0.66
rs11065504	121680460	G	44.3	46.2	0.74
rs7975295	121689101	T	20.7	21.5	0.87
rs2686344	121690548	T	46.7	40.3	0.26
rs1560568	121690587	G	19.5	22.8	0.47
rs7314454	121698785	T	19.1	16.4	0.54
rs3817190	121712077	A	40.3	35.6	0.44

MA minor allele in this cohort, *MAF* minor allele frequency

[a] SNP not in Hardy-Weinberg equilibrium were excluded. Remaining SNP are shown in chromosomal order (same as the order of appearance in the haplotypes)

[b] Four SNP carried forward into multivariable analyses

[c] Minor allele frequencies generated are based on the number of individuals carrying each allele, so patients with no calls for a particular SNP are excluded from the frequency calculations

Associations between haplotypes and pulmonary NTM disease

From the 30 SNPs analysed, 188 unique haplotypes were generated by fastPHASE. Haplotypes present in less than 1% of the cohort were excluded, leaving 27 haplotypes of interest. One haplotype (SH95) was significantly associated with pulmonary NTM disease (χ^2, $p = 0.019$) on univariate analysis (Table 2). Seven haplotypes (SH5, SH11, SH17, SH70, SH83, SH95 and SH97) displayed associations above our exploratory cut-off ($p \leq 0.20$) on univariate analysis and were included in logistic regression models with gender (Table 2). After a stepwise removal process, the final model (model $p < 0.0001$; $R^2 = 0.05$) included haplotypes SH95, SH70 and gender (Table 3). The haplotype most strongly associated with NTM disease in multivariate analysis was SH95. Associations between haplotypes and NTM disease were similar when gender was excluded as a variable (model $p = 0.0013$; $R^2 = 0.04$) (Table 3).

The results of patient radiological findings (nodular bronchiectasis, cavitary or mixed) (χ^2, $p = 0.631$) and mycobacterial isolates from the lung (fast or slow growing species) (χ^2, $p = 0.590$) were tested for an elevated co-incidence with SH95. No associations were observed.

Discussion

Exposure to potentially pathogenic mycobacteria from the environment is ubiquitous, so the mechanism behind the rare and selective distribution of infection in the population presents an apparent paradox. Genetic predisposition seems the most likely influence. Here, polymorphisms within three genes involved in the inflammatory immune response were investigated. As pulmonary NTM disease generally develops during the later years of life (average patient age 60 years (Kim et al. 2008)) in individuals without histories of bacterial infection, it is unlikely that factors increasing susceptibility reflect a primary immunodeficiency. Hence, loss-of-function mutations in any of the genes of interest would be poor candidate polymorphisms. The SNP investigated in this study are likely to have more subtle effects. Here, the clearest confounding factor was gender, with 59–95% of the reported cases being in females (Huang et al. 1998; Kim et al. 2008; Winthrop et al. 2010). Gender was included in multivariate analyses here in order to confirm an independent effect from the genes under study.

Of the seven haplotypes, meeting the $p \leq 0.20$ cut-off in univariate analyses, SH95 was most clearly associated with pulmonary NTM disease (Tables 2 and 3). None of the three SNP displaying minor alleles (rs12301635, rs7975295 and rs7314454) in this haplotype (present in *P2X7R* and *CAMKK2*) were independently associated with the disease (i.e. none met the $p < 0.20$ exploratory cut-off).

SH70 and SH83 associated weakly with risk and protection (respectively) in univariate analysis and shared several SNP alleles. The three alleles (rs10849849, rs1718125 and

Table 2 One haplotype (SH95) associated with NTM in univariate analyses

Haplotype (SH number)	Haplotype sequence[a]	Frequency[b] controls (n = 229)	Frequency NTM (n = 124)	p value (χ^2)[d]
SH6	00000000001000000100010000011	45 (19.6%)	22 (17.7%)	0.662
SH11[c]	00000000000001000000000010000	31 (13.5%)	11 (8.9%)	0.196
SH1	00000001110000000000001000000	28 (12.2%)	14 (11.3%)	0.795
SH2	00000001100000000000001000000	27 (11.8%)	13 (10.5%)	0.712
SH5[c]	00000001110000000000001000011	22 (9.6%)	18 (14.5%)	0.165
SH9	00100110011000010011000000000	11 (4.8%)	8 (6.4%)	0.512
SH27	00000000011000100000001000000	10 (4.4%)	7 (5.6%)	0.592
SH47	00000101011000010110110000010	10 (4.4%)	6 (4.8%)	0.839
SH74	00100100011000010011000000011	9 (3.9%)	7 (5.6%)	0.460
SH20	00000000010000000000001010000	8 (3.5%)	6 (4.8%)	0.536
SH83[c]	10010100001110010110000010100	11 (4.8%)	2 (1.6%)	0.151
SH8	00000000000001010110000010100	6 (2.6%)	6 (4.8%)	0.272
SH19	00000000001000010011000000000	7 (3.1%)	5 (4.0%)	0.629
SH97[c]	00000110011000100000001000000	11 (4.8%)	1 (0.8%)	0.063
SH95[c, e]	00000000000001000000000010010	3 (1.3%)	8 (6.4%)	0.019
SH84	01011100000000000000000010000	7 (3.1%)	4 (3.2%)	1.00
SH104	01010000011000111011001000111	6 (2.6%)	4 (3.2%)	0.746
SH39	00000000000001010011000000111	6 (2.6%)	3 (2.4%)	1.00
SH10	00000001110000000000000010000	3 (1.3%)	4 (3.2%)	0.247
SH71	00000000000000000000000001000011	3 (1.3%)	4 (3.2%)	0.247
SH92	00000100000000000000000001000011	6 (2.6%)	1 (0.8%)	0.429
SH70[c]	00000000001110010110000010100	2 (0.9%)	5 (4.0%)	0.055
SH17[c]	00000001100000010110000010100	2 (0.87%)	4 (3.2%)	0.190
SH34	00000001101000010110110000010	3 (1.3%)	3 (2.4%)	0.428
SH41	00000001000001000000000010111	5 (2.2%)	1 (0.80%)	0.669
SH94	00100100011000010011000000000	3 (1.3%)	3 (2.4%)	0.428
SH105	01010000011000100000000010000	5 (2.2%)	1 (0.8%)	0.669

[a] Defined by alleles of SNP in the order shown in Table 1

[b] Frequency of patients with each haplotype based on the most probable assignment for each individual

[c] Seven haplotypes carried forward into multivariable analyses

[d] Two-tailed Fishers exact tests were substituted where any categorical frequencies fell below 5

[e] Only a single haplotype reached significance in univariate analysis

rs1186055) distinguishing these haplotypes lie within *P2X7R* and do not explain the association observed at the haplotype level. We also compared SH97 (potentially protective) with the three haplotypes most closely linked with risk (SH95, SH70 and SH17). No minor alleles of SH97 were shared with either SH95 or SH17, but single minor allele was present in both SH97 and SH70, rs1653609 (*P2X7R*). As SH97 and SH70 are associated with protection and risk respectively, the impact of a minor allele at rs1653609 remains unclear.

These discrepancies between haplotypes and their contained SNP allele associations may be explained by linkage disequilibrium between the alleles identified and other as yet unidentified alleles. These may be within *P2X7R* or *CAMKK2* regions of the genome or in strong linkage disequilibrium with them. Alternatively, these alleles may require a second polymorphic allele in a pathway-related gene before a contextual loss-of-function phenotype is observed. Further work is required to characterise the relationships between these haplotypes and pulmonary NTM disease.

Whilst haplotypes trending to an association with NTM disease carried minor alleles of *P2X7R* and *CAMKK2*, there was no evidence of a similar relationship for *P2X4R* alleles. Moreover no SNP alleles investigated in *P2X4R* met the exploratory cut-off ($p < 0.20$) in univariate χ^2 analysis.

This exploratory study implicates *P2X7R* and perhaps *CAMKK2* (and their protein products P2X7R and CaMKK2) in pathways affecting pulmonary NTM disease, whilst *P2X4R* was not associated with disease protection or risk.

Table 3 Logistic regression models define gender and haplotypes as predictors of NTM disease

Variable	Odds ratio	*p* value	95% confidence interval
Model combining gender and haplotypes ($n = 353$[a], $p < 0.0001$; $R^2 = 0.05$)			
Gender[b]	0.43	0.001	0.267–0.70
SH95[b]	5.62	0.014	1.425–22.13
SH70	4.90	0.064	0.910–26.36
Model combining haplotypes ($n = 353$[a], $p = 0.0013$; $R^2 = 0.04$)			
SH97	0.18	0.104	0.023–1.420
SH95[b]	5.31	0.015	1.380–20.42
SH70	4.98	0.058	0.950–26.08
SH17	3.98	0.114	0.718–22.08

[a] Excluding samples with genotyping failures

[b] Variables achieving significant association with pulmonary NTM disease within the logistic regression models

Confirmatory studies in additional cohorts are required to confirm the results obtained. Future work would investigate *P2X7R* and *CAMKK2*, genes in linkage disequilibrium with them, and genes encoding products in the same pathways in more depth and in larger cohorts.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Informed consent Informed consent was obtained from all individual participants included in the study.

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