Confirmation of an association between single nucleotide polymorphisms in the VDR gene with Respiratory Syncytial virus related disease in South African children

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Running title

SNPs in South African children with RSV

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Respiratory syncytial virus is a leading cause of lower respiratory tract infection in infants. Disease severity has been linked to host immune responses and polymorphisms in genes associated with innate immunity. A large scale genetics study of single nucleotide polymorphisms (SNPs) in children in the Netherlands identified SNPs in the Vitamin D receptor (VDR) and JUN genes which have a strong association with an increased risk of developing bronchiolitis following the first RSV infection. The Toll-like receptor 4 (TLR4) gene has two SNPs which have been associated previously with RSV disease severity in various populations. The aim of this study was to determine if these SNPs may be associated with RSV disease in African children in South Africa.

RSV patient (n=296) and control (n=113) groups were established (median ages: 3 and 3.5 months) and DNA extracted from the collected specimens. Real-time polymerase chain reaction using hydrolysis probes was used to screen for SNPs in the VDR (Thr1Meth; rs10735810), TLR4 (Asp299Gly; rs4986790 and Thr399Ile; rs4986791) and JUN (c.750G/A; rs11688) genes.

Carriers of the VDR (Thr1Meth) SNP minor T allele were more prone to RSV disease than individuals in the control group. The TLR4 (Asp299Gly), TLR4 (Thr399Ile) and JUN (c.750G/A) SNPs showed no significant association with RSV disease.

It is concluded that children carrying the minor T allele of the VDR (Thr1Meth) SNP may be predisposed to RSV disease, as this SNP was identified as a risk factor for severe RSV disease in South African children, confirming the findings in the Netherlands.

Key words

genotype, allele, disease susceptibility, heterozygous, homozygous

INTRODUCTION

Human respiratory syncytial virus (RSV), a member of the family Paramyxoviridae, which occurs commonly in infants and young children, is the foremost viral respiratory pathogen and a major cause of lower respiratory tract infection worldwide (van Drunen Little-van den Hurk, 2007; Amanatidou et al, 2009). In industrial countries, RSV is the most common cause of the hospitalization of infants in their first year of life, resulting in clinical diseases such as bronchiolitis and pneumonia (Amanatidou et al, 2009; Mejías et al, 2005). Children who are at a higher risk of infection include infants who are premature, and those who suffer from bronchopulmonary dysplasia, congenital heart

defects, congenital or acquired immunodeficiency and children with Down's Syndrome (Ogra, 2004; Bloemers et al, 2007).

RSV disease susceptibility has been associated with host genetic factors, specifically in genes associated with the innate immune response (Amanatidou et al, 2009; Janssen et al, 2007). A large genetic study involving analysis of 347 SNPs in 470 children hospitalized for RSV bronchiolitis, identified single nucleotide polymorphisms (SNPs) located in the vitamin D receptor (VDR Thr1Met; rs10735810) and JUN (c.750G/A; rs11688) genes to have the strongest association to RSV infection, at both an allele and genotype level (Janssen et al 2007). VDR has been associated with illness such as tuberculosis, and the JUN gene is involved in pro-inflammatory cytokine production (Wilkinson et al, 2000; Adcock and Caramori, 2001). In general, SNPs in genes involved in innate immunity displayed the strongest associations in this study (Janssen et al, 2007). A strong association has also been found between the Asp299Gly and Thr399Ile polymorphisms of Toll-like receptor 4 (TLR4), which is also important in innate immunity, and RSV disease in high risk infants and young children (Babb et al, 2007; Awomoyi et al, 2007). However this could not be replicated in other studies (Janssen et al, 2007) suggesting that this association may only be present in certain populations. A previous study showed that the TLR4 genotype distribution is different in Africa and that this has consequences for TLR4 function (Ferwerda et al, 2007). Therefore, the effect of TLR4 SNPs could be different in the African setting.

The frequency of these SNPs associated with severe lower respiratory tract infection in

the African setting has not yet been determined. Identification of SNPs associated with RSV disease in the South African population may help identify general mechanisms that are important for development of severe RSV disease, and treatment targets for disease in Southern African children. The aim of the present study was to replicate previous studies on the association between genetic polymorphisms and risk of RSV disease in European and North American studies, in black South African children. SNPs in the VDR (Thr1Met), TLR4 (Asp299Gly), TLR4 (Thr399Ile) and JUN (c.750G/A) genes were screened for in South African children with RSV disease compared to a healthy control group, and the influence of these genotypes was correlated to the pathogenesis of RSV infection.

MATERIALS AND METHODS

Study design

Specimens

This was a blinded study, comprised of a RSV patient group and a control group, each consisting of specimens from individuals who were treated anonymously. For the patient group, nasopharyngeal aspirates collected over five consecutive years (2006- 2010) were included in the study. The nasopharyngeal aspirates were centrifuged and separated immediately following collection, and the cell pellets and supernatants were stored at -70 °C. For the control group, buccal swabs were collected from the infants under the consent and supervision of the parent or guardian.

Patient group

The patient group consisted of 296 infants, less than 2 years of age (median age, 3 months) who were hospitalized and showed signs of acute respiratory infection. The patients were all positive for RSV infection, based on immunofluorescence (IF), rapid testing or an "in-house" multiplex real-time PCR assay (Lassauniére et al, 2010), and no other co-infections were observed. All of the children included in this study were of the black South African population group.

Specimens were submitted to the Department of Medical Virology, University of Pretoria/NHLS Tshwane Academic Division, which serves three public sector hospitals in the Pretoria region (Kalafong Secondary Hospital, Steve Biko Academic Hospital and 1-Military Hospital), for diagnosis of respiratory viral infection. These specimens were obtained from patients seeking medical attention at outpatients or who were admitted to hospital or to the intensive care unit (ICU) due to acute lower respiratory infections. Ethical approval was obtained from the University of Pretoria Ethics committee to enter routine diagnostic specimens in an anonymous database. By removing all identifiers from the patients, the investigators waived the need for informed consent.

Control group

The control group included 113 healthy infants receiving immunizations from a local maternity clinic. All of the children were of the black South African population group, and were less than 2 years of age (median age, 3.5 months). Written informed consent was obtained from the parents/guardians of all patients

This study was cleared by the ethics committee, University of Pretoria (protocol no. 2006/25) as well as the ethics committee of the Netcare hospital group, South Africa.

DNA extraction and identification of SNPs

Genomic DNA was extracted from the NPA cell pellets and from the buccal swabs using the QIAmp® DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

Real time polymerase chain reaction (PCR), using the Lightcycler® 2.0 carousel-based system and the Lightcycler® 480 96-well plate-based system (Roche Diagnostics, Mannheim, Germany), was used to screen for SNPs in the VDR gene (NCBI SNP reference rs10735810) and the JUN gene (NCBI SNP reference rs11688). The 7500 Fast Real-Time PCR System (Applied Biosciences, Warrington, UK) was used to screen for SNPs in the TLR4 gene, at the Asp299Gly (NCBI SNP reference rs4986790 and Thr399Ile (NCBI SNP reference rs4986791) positions.

The primer and probe sequences are shown in Table I. Minor groove binder – non-fluorescent quencher (MGB-NFQ) hydrolysis probes (Applied Biosystems, Warrington, UK), labelled with the fluorogenic dyes VIC and 6-FAM, were used to distinguish between homozygous and heterozygous SNPS in each gene.

Cycling conditions for both VDR and JUN were 95°C for 15 min, followed by 45 cycles of

95°C for 0 sec, 62°C for 1 min and then 40°C for 30 sec using the QuantiTect™ Probe PCR Kit (Qiagen, Valencia, CA, USA). Cycling conditions for TLR4 Asp299Gly and TLR4 Thr399Ile were 95°C for 10 min, followed by 40 cycles of 92.1°C for 15 sec and 60°C for 1 min using the Taqman® Genotyping Master Mix (Applied Biosystems, Warrington, UK).

The TLR4 (Asp299Gly; rs4986790), TLR4 (Thr399Ile; rs4986791) and JUN (c.750G/A) SNPs were unable to be detected in 13, 4 and 2 of the specimens included in the RSV patient group.

Statistical analysis

The statistical difference in the genotype and allele frequency between the different sample groups was assessed by the χ^2 test using the Chi Square Statistic website http://math.hws.edu/javamath/ryan/ChiSquare.html. Probability testing to determine p-values was also conducted using this website. Odds ratios and confidence intervals were calculated using the Simple Interactive Statistical Analysis software available at http://www.quantitativeskills.com/sisa/statistics/twoby2Old.htm.

RESULTS

Vitamin D receptor (VDR)

After investigating the VDR (Thr1Met; rs10735810) C/T SNP, it was observed that a higher percentage of RSV-positive patients (37%) possessed the heterozygous (CT) genotype compared to the control group (21%; Table II), and a higher percentage of

controls (76%) were found to present the homozygous genotype for the C allele (CC) compared to the patient group (60%). These data were statistically significant (p=0.008; χ^2=9.63). In accordance with this the allele frequencies of the VDR (Thr1Met) minor T allele occurs at a higher frequency in the patient group (22%) compared to the control group (13%; Table III), while the C allele is more prevalent in the control group (87%) than in the patient group (78%). These data were statistically significant, and indicate that the minor T allele is associated with RSV-disease (p=0.006; χ^2=7.58).

The frequency at which the C allele of the VDR (Thr1Met; rs10735810) SNP occurred in the South African population was higher (Table III) than the frequency at which this allele occurred in the European (Utah residents with ancestry from northern and western Europe (CEU)) and Asian (Han Chinese in Beijing, China (CHB) and Japanese in Tokyo, Japan (JPT)) populations studied in the International HapMap project (International HapMap Consortium, 2003).

Toll-like receptor 4 (TLR4)

TLR4 (Asp299Gly)

The frequency of the GG genotype for the TLR4 (Asp299Gly; rs4986790) SNP was so low (Table II) that associations could only be analysed at the allele level. In both the patient and control groups, the frequency at which the G allele occurred was low (Table III). Although not a significant association, the TLR4 (Asp299Gly) wild-type A allele occurred at a marginally higher frequency in the patient group compared to the control group (p=0.052; χ^2=3.79).

The allele frequency distribution of the TLR4 (Asp299Gly; rs4986790) SNP in the South African population was similar to that found in the European population. This SNP does not occur in the Asian population (International HapMap Consortium, 2003; Ferwerda at al, 2007).

TLR4 (Thr399Ile)

The TLR4 (Thr399Ile) SNP is not present in the South African population group, as only the CC genotype was prevalent in both the patient (99%) and control (100%; Table II) groups. The difference in the frequency of the C allele between the patient (99%) and control (100%; Table III) groups was negligible. This SNP is prevalent in the European population, but is absent from the Asian population (Ferwerda et al, 2007).

JUN

For JUN (c.750G/A), no significant difference in genotype distribution was found between the patient and control groups (Table II). Similarly, at the allele level, no difference in allele frequency was observed between the patient and control groups (Table III). The frequency at which the A allele occurs in the South African population is higher than the frequency of this allele in the European (CEU) and Asian (CHB and JPT) populations. The frequency of the heterozygous genotype is also present at a higher frequency in the South African population compared to European (CEU) and Asian (CHB and JPT) population groups (International HapMap Consortium, 2003).

None of the allele distributions for the VDR (Thr1Met), JUN (c.750A/G), TLR4

(Asp299Gly) and TLR4 (Thr399Ile) SNPs, for both the patient group and the negative control group, were different from those expected according to Hardy-Weinberg calculations. The odds ratios were below 1 for the genotypes and alleles which occurred at a lower frequency in the patient group compared to the control group, indicating that those genotypes and alleles are less likely to occur in the patient group than in the control group. The odds ratios were higher than 1 for the genotypes and alleles which occurred at a higher frequency in the patient group compared to the control group, indicating that those genotypes and alleles are more likely to occur in the patient group than in the control group (Tables II and III). The p-values were adjusted for multiple testing using the Bonferroni correction, and the associations found between the patient and control groups for the VDR (Thr1Met) SNP remained significant.

DISCUSSION

There are a number of predisposing factors which may contribute toward the severity of RSV disease. None-the-less severe RSV-related illness has been diagnosed in individuals who were not considered as high risk candidates for severe RSV disease. The study of host genetic factors, specifically in genes associated with the innate immune response, may account for these observations (Amanatidou et al, 2009). Most studies of RSV genetic associations have targeted Caucasian groups, with little knowledge of African populations and no data about Southern African populations that are known to differ genetically from African American or central African population groups.

The main findings of the present study are that the VDR (Thr1Met; rs107358) SNP, which was associated previously with severe RSV disease in Caucasians, shows an association with a Southern African population, highlighting the importance of the VDR pathway in RSV disease. In addition, an association of the TLR4 (Asp299Gly; rs4986790) and TLR4 (Thr399Ile; rs4986791) SNPs with severe RSV disease in the African setting was not found, even though it was postulated that functional effects of TLR4 SNPs would be much more pronounced in this setting because of the difference in genetic variation in TLR4 in African populations as compared to Caucasians. The contribution of TLR4 SNPs to RSV disease therefore remains elusive.

Vitamin D has important immunomodulatory effects; therefore varying levels of VDR may influence the mechanism of VDR in immune-mediated diseases (Poon et al, 2004). Increased levels of vitamin D, caused by the conversion of inactive vitamin D to the active form by airway epithelial cells, may result in the activation of vitamin D-responsive genes. These genes lead to the production of proteins which are important for innate immunity (Hansdottir et al, 2008). Interference with production of these proteins may influence susceptibility to RSV-disease. In the present study, the C allele was over-represented in the control group, which may have resulted in increased transcriptional activity of the VDR gene compared to the RSV patient group (Table III). In a study by Roth et al (2008), acute lower respiratory tract infection was more prevalent in infants with the VDR (Fok1, Thr1Met) gene polymorphism TT genotype compared to children with the CC genotype. This may be explained by the T to C transition in the first codon of the VDR gene, which causes the abolishment of the first translational site in the start

codon. This results in a peptide lacking the first three amino acids, which increases the transcriptional activity of VDR (Jurutka et al, 2000).

Toll-like receptor 4 (TLR4) plays an important role in regulating innate and adaptive immune responses by recognising pathogen-associated molecular patterns (PAMPs) (Netea et al, 2004; Amanatidou et al, 2009). TLR4 is also able to detect viral infection, as observed following the interaction of TLR4 with the RSV fusion (F) protein, which was shown to mediate cytokine production during innate immune response (Vaidya et al, 2003; Amanatidou et al, 2009). An association has been observed between RSV disease severity and the polymorphisms which occur in the TLR4 gene at the Asp299Gly and Thr399Ile positions, as reported in previous studies (Tal et al, 2004; Puthothu et al, 2006; Tulic et al, 2007; Awomoyi, 2007). The TLR4 (Asp299Gly) genotype distributions, observed in both the RSV patient group and in the control group, were similar to those seen by Puthothu et al (2006) where the wild-type genotype (AA) was present in the majority of the population while the mutant genotype (GG) was found in only a minute percentage of the population. The genotype distribution of the TLR4 (Asp299Gly) SNP is therefore not unique to the South African population. Awomoyi et al (2007) found that the heterozygous genotype occurred at the highest frequency in their RSV patient group; however their RSV patient group consisted of individuals who were pre-disposed to RSV infection due to the infants being premature or suffering from congenital heart defects. In their population group, which was not selected as being high risk for RSV but who presented with symptoms consistent with respiratory infection, the AA genotype was demonstrated to occur at a frequency of 95.6%, which coincides with

observed genotype frequency in the present study. Tal et al (2004) found that the wild type allele was over-represented in their control group; however their population group was of Jewish origin whereas a South African population group was used in the current study. The increased frequency of the G allele in the control group, compared to the patient group, was also observed by Puthothu et al (2006) who found a weak association between the TLR4 (Asp299Gly) SNP and severe RSV infection in a German population group.

The association between the TLR4 (Thr399Ile) SNP and RSV disease severity is a topic of debate, as some studies have found an association between the TLR4 (Thr399Ile) SNP and RSV disease severity while others have not. This SNP has been identified previously in the European population, however it is missing from African and Asian populations (Ferwerda et al, 2007). In the South African population, an association was not observed between RSV disease severity and the TLR4 (Thr399Ile) SNP, which is similar to the findings observed in the German population (Puthothu et al, 2006). In contrast to these findings, an association was observed between the TLR4 (Thr399Ile) SNP and RSV disease severity in a Jewish population (Tal et al, 2004). The association between the TLR4 (Asp299Gly) and TLR4 (Thr399Ile) SNPs and RSV disease severity therefore appears to be population-specific. The genotype distribution of the TLR4 (Thr399Ile) SNP in both the RSV patient group and in the control group was similar to that observed by Puthothu et al (2006), where the wild-type genotype (CC) was present in the majority of the population while the heterozygous (CT) and mutant (TT) genotypes were almost completely absent from the population. The genotype distribution of the

TLR4 (Thr399Ile) SNP was therefore not found to be unique to the South African population.

JUN is an oncoprotein which is involved in the induction and prevention of apoptosis, a process which suppresses oncogenic transformation and controls organogenesis and immune responses (Shaulian and Karin, 2002). JUN forms part of the transcription factor AP-1, which together with other transcription factors such as NF-κB, is involved in the regulation of many inflammatory and immune genes and mediates pro-inflammatory cytokine production. Over-expression of these genes has been linked to asthma, where an inflammatory response may occur if both AP-1 and NF-κB are activated simultaneously (Adcock and Caramori, 2001). A strong association was found between the JUN (c.750A/G) SNP and RSV disease severity in the Dutch population (Janssen et al, 2007). In contrast to this, a significant association between RSV disease severity and the JUN (c.750A/G) SNP was not found in the South African population.

In this study, an association was found between the C/T transition in the VDR (Thr1Meth) gene and RSV disease in children of African descent in South Africa, confirming the association of polymorphisms in these innate immunity genes with RSV disease identified previously in children from the Netherlands. No association was found between the TLR4 (Asp299Gly), TLR4 (Thr399Ile) and JUN (c.750A/G) SNPs and RSV disease severity. The size of the South African population which was used in this study may have contributed to lack of association between these SNPs and RSV disease severity. The association of specific SNPs with RSV disease severity in certain populations should be taken into account when identifying SNPs for therapeutic

purposes, as therapies targeting SNPs which are population-specific will not be logistically or financially viable. However the identification of SNPs associated with RSV disease severity, which are common to all population groups, will be good candidates for treatment modalities against RSV infection.

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Table I – Primer and probe sequences used to screen for SNPs in the VDR, JUN and TLR4 genes.

SNP	Primer/ Probe*	Sequence
VDR	Forward primer	5'-GGGTCAGGCAGGGAAGTG-3'
	Reverse primer	5'-TGGCCTGCTTGCTGTTCTT-3'
	Probe 1	5'-ATTGCCTCC**A**TCCCTGT-3'
	Probe 2	5'-TGCCTCC**G**TCCCTGT-3'
TLR4 (Asp299Gly)	Forward primer	5'-TGACCATTGAAGAATTCCGATTAGCA-3'
	Reverse primer	5'-ACACTCACCAGGGAAAATGAAGAA -3'
	Probe 1	5'-TACCTCGATG**A**TATTATT-3'
	Probe 2	5'-CCTCGATG**G**TATTATT-3'
TLR4 (Thr399Ile)	Forward primer	5'-CAAAGGTTGCTGTTCTCAAAGTGAT-3'
	Reverse primer	5'-GGTAATAACACCATTGAAGCTCAGATCTA-3'
	Probe 1	5'-CTTTAGGCTG**A**TTGTCC-3'
	Probe 2	5'-TTTAGGCTG**G**TTGTCC-3'
JUN	Forward primer	5'-GGTTCCTCATGCGCTTCCT-3'
	Reverse primer	5'-CCCCTGTCCCCCATCGA-3'
	Probe 1	5'-TCCGCTC**C**TGGGACT-3'
	Probe 2	5'-ATCCGCTC**T**TGGGACT-3'

* Specific allele detected by Probe 1 and Probe 2 for each SNP is indicated in bold

Table II – Frequencies and odds ratios for the different genotypes of the VDR, TLR4 and JUN genes in the RSV patient group and negative control group.

Gene	Genotype	Patient group (‡‡n = 296)	Control Group (n = 113)	SNP	χ^2	*p-value	‡OR (95% CI)
VDR	CC	177 (60%)	86 (76%)	Thr1Met	9.63	0.008	0.65 (0.552-0.768)
	CT	109 (37%)	24 (21%)				3.183 (1.895-5.345)
	TT	10 (3%)	3 (3%)			(**0.032)	1.622 (0.438-6.014)
TLR4	AA	265 (94%)	99 (88%)	Asp299Gly	Can't calculate		2.103 (1.008-4.39)
	AG	18 (6%)	14 (12%)				0.476 (0.228-0.993)
	GG	0 (0%)	0 (0%)				
TLR4	CC	288 (99%)	113 (100%)	Thr399Ile	Can't calculate		
	TC	4 (1%)	0 (0%)				
	TT	0 (0%)	0 (0%)				
JUN	GG	192 (65%)	79 (77%)	c.750G/A	1.99	0.370	0.810 (0.507-1.294)
	AG	93 (32%)	33 (32%)				1.122 (0.698-1.802)
	AA	9 (3%)	1 (1%)			(**1.48)	3.537 (0.443-28.241)

* According to χ^2 distribution of a 3 x 2 table on genotype frequencies

** Bonferroni correction-adjusted p-value

‡ OR, odds ratio; CI, confidence interval

‡‡ SNPs undetected in 13xTLR4 (Asp299Gly), 4xTLR4 (Thr399Ile) and 2xJUN in RSV patient group

Table III – Frequencies and odds ratios for the allele distributions of the VDR, TLR4 and JUN genes in the RSV patient group and negative control group.

Gene	Allele	Patient group (‡‡n = 296)	Control Group (n = 113)	SNP	χ^2	*p-value	‡OR (95% CI)
VDR	C	463 (78%)	196 (87%)	Thr1Met	7.58	0.006	0.55 (0.357-0.845)
	T	129 (22%)	30 (13%)			(**0.024)	1.820 (1.183-2.801)
TLR4	A	548 (97%)	212 (94%)	Asp299Gly	3.79	0.052	2.010 (0.982-4.115)
	G	18 (3%)	14 (6%)			(**0.208)	0.497(0.243-1.018)
TLR4	C	580 (99%)	226 (100%)	Thr399Ile	1.56	0.212	
	T	4 (1%)	0 (0%)			(**0.848)	
JUN	G	477 (81%)	191 (84.5%)	c.750G/A	1.28	0.259	0.788 (0.520-1.193)
	A	111 (19%)	35 (15.5%)			(**1.036)	1.270 (0.838-1.924)

* According to χ^2 distribution of a 2 x 2 table on allele frequencies

** Bonferroni correction-adjusted p-value

‡ OR, odds ratio; CI, confidence interval

‡‡ SNPs undetected in 13xTLR4 (Asp299Gly), 4xTLR4 (Thr399Ile) and 2xJUN in RSV patient group