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Original Article

Toll-interacting protein polymorphisms in viral bronchiolitis outcomes

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Abstract

***Background*:** Toll-interacting protein is a key factor in regulating innate immunity responses via gatekeeping Toll-like receptors. Genetic variance in innate immunity has been linked with susceptibility to infections. Children with viral bronchiolitis in infancy are at increased risk of later asthma. The aim was to evaluate the role of *toll-interacting protein* gene point mutations in severity of bronchiolitis and subsequent risk of asthma.

***Methods*:** Infants less than 6 months old were recruited during hospitalization due to bronchiolitis. In all, 166 children were prospectively followed up to age of 1.5, 6, and 11 years. Clinical data on viral etiology and severity markers, and further post-bronchiolitis asthma and lung function outcomes were compared with genetic differences in two single-nucleotide point mutations rs116938768 and rs5743854 in the *toll-interacting protein* gene.

***Results*:** *Toll-interacting protein* rs116938768 or rs5743854 did not show significant associations with severity markers or viral etiology of bronchiolitis. Follow-up data on current asthma or lung function at 6 or 11 years of age after bronchiolitis were not associated with the investigated mutations.

***Conclusion*:** *Toll-interacting protein* gene point mutations in rs116938768 or rs5743854 were not involved with the clinical course of viral bronchiolitis in early infancy, and did not predict post-bronchiolitis asthma or lung function reduction by the age of 11 years.

Key words asthma, innate immunity, lung function, toll-interacting protein, viral bronchiolitis.

Bronchiolitis in infancy has long-term consequences for the later respiratory health of the child. According to long-term follow-up studies, up to 40% of children hospitalized for bronchiolitis in infancy are diagnosed with asthma by the time they reach teenage years. Parental asthma, atopic eczema of the child, or rhinovirus as a causative agent of bronchiolitis are well-known risk factors but do not fully explain why only some children with bronchiolitis develop asthma. Therefore, host genetics, especially genes regulating innate immunity, are likely to play an important role in the disease process.

Toll-like receptors (TLRs) are key molecules in detecting foreign pathogens, inducing innate immune responses and activating adaptive immunity to defend host against outside intruders. Toll-interacting protein (Tollip) is an inhibitory adaptive protein, widely expressed in the respiratory tract,[1] which dampens TLR downstream signaling and controls the subsequent inflammatory responses.[2] Several *TLR* gene polymorphisms have been associated with infectious and inflammatory diseases, including asthma and related atopic diseases, but the *Tollip* gene is less studied. As a negative regulator of TLR signaling it is, however, an interesting molecule in the TLR pathway and therefore worth of investigating, as impairment in Tollip function might lead to excessive inflammatory responses.

Tollip rs5743854 and rs116938768 are closely located in the promoter region of the gene, and rs5743854 has previously been associated with attenuated mRNA expression in monocytes and increased inflammatory response after TLR2 stimulation.[3] The carriage of *Tollip* rs5743854 wild allele has been linked with atopic eczema in one study.[4] Data on *Tollip* rs116938768 functionality are lacking, but the reason to explore it was that, due to the apparent closeness of only 10 base pairs, it was suspected to have similar properties to rs5743854. Further support for this rationale came from a study looking at another SNP in close proximity *Tollip* rs5743899, where the carriage of variant allele was associated with attenuated lung function and a lower *in vitro* anti-viral response to rhinovirus infection in asthmatic subjects.[5]

We aimed to investigate the role of two *Tollip* gene polymorphisms, rs116938768 and rs5743854, in the viral etiology and severity of bronchiolitis under six months of age, and in the post-bronchiolitis outcome at 5–7 years and 11–13 years of ages.

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Methods

This study was conducted at the Department of Paediatrics, Tampere University Hospital, Finland, and the detailed design of this study has been described previously.[6] In brief, 187 previously healthy infants were included in this study in 2001 to 2004, when they were hospitalized for bronchiolitis at less than 6 months of age. Bronchiolitis was defined as a first episode of an acute lower respiratory tract infection characterized with runny nose, cough, and diffuse wheezes or inspiratory crackles on auscultation. The first follow-up visit was arranged in 2003 to 2005 at the mean age of 18 months, and 139 children attended.[7] The second follow-up visit was arranged in 2008 to 2010 when the children were 5 to 7 years of age and 166 children attended.[8] The third follow-up visit was arranged in 2014 to 2015 when the children were 11 to 13 years of age, and 138 children attended.[9] Whole blood samples were obtained for genetic studies at the first or second follow-up visit.

The viral etiology of bronchiolitis was studied by antigen detection and polymerase chain reaction from nasopharyngeal aspirates. The viruses that were studied were respiratory syncytial virus (RSV), rhinovirus, human metapneumovirus, influenza A and B virus, parainfluenza type 1, 2 and 3 viruses, adenovirus, and human bocavirus. The severity of bronchiolitis was evaluated as the need of supplementary oxygen, length-of-stay in hospital and the need of feeding support.

At the follow-up visits, data were collected on the occurrence of atopic eczema, wheezing episodes, asthma and allergy diagnoses and the use of asthma medications since the last follow-up visit by structured questionnaires. All follow-up visits also included clinical examination and interview by a doctor. Lung function and bronchial responsiveness were evaluated by impulse oscillometry (Jaeger, Master Screen IOS, Höchberg, Germany) with exercise challenge test at the 5–7 years control visit,[10] and by flow-volume spirometry (Vmax™ Carefusion, Becton, Dickinson and Company, NJ, USA) with a bronchodilation test at the 11–13 years control visit.[11]

Current asthma was defined as the current use of inhaled corticosteroid (ICS) medication or having asthma-suggestive symptoms (repeated wheezing, or prolonged cough, or night cough for at least 4 weeks) during the last 12 months prior to the control visit with a diagnostic finding in the exercise challenge or bronchodilation test. Previous asthma at the 5–7 years control visit was defined as the previous use of ICS as continuous or intermittent maintenance medication for asthma, and if the child had previous or current asthma, the term "asthma ever" was used. Persistent asthma at 11–13 years control visit was defined as the presence of current asthma at both the 5–7 years and 11–13 years control visits. Allergic rhinitis was defined as symptoms of runny or stuffy nose or repeated sneezing outside infection during the last 12 months before the control visits. Atopic dermatitis was defined as doctor-diagnosed allergic eczema.

Genetic studies

In the present study, we determined two single nucleotide polymorphisms (SNPs) of the *Tollip* gene, rs5743854 and rs116938768, in samples from 166 children. Polymerase chain reaction-based sequencing was used for the SNP detection and the following primers were designed: (forward) 5′-TTCGGACGTGCGACCC −3 and (reverse) 5′-AACCGCGCCCCATCTTTA-3 and purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The sequencing was performed at the Institute for Molecular Medicine Finland (FIMM) laboratories, Helsinki, Finland.

Population-based data on the *Tollip* rs116938768 and rs5743854 gene polymorphisms were available from the 1000 Genomes Project for 99,[12] and from the Genome Aggregation Database for 1738 Finnish subjects (3476alleles).[13] The minor allele frequencies (MAFs) were compared between the study population and the Finnish data.

Ethics

The study was carried out in accordance with the World Medical Association's (WMA) Helsinki Declaration. We obtained informed parental consent, including the use of samples for genetic studies on bronchiolitis and asthma risk both during hospitalization and at the control visits. The protocol of the study was approved by the Ethics committee of the Tampere University Hospital district, Tampere, Finland. The personal data of the study subject were not given to the laboratories that performed the genetic studies, the Department of Medical Microbiology and Immunology, Turku, Finland, or the Institute for Molecular Medicine Finland laboratories, Helsinki, Finland.

Statistics

Statistical analyses were carried out with SPSS version 25.0 software (IBM Corp, NY, USA). The χ^2 test and Fisher's exact tests were used, as appropriate, for categorized variables and pairwise comparison of two groups with Student′s *t*-test for continuous variables.

Results

Hospitalization data

There were 166 children with genetic and clinical data available during hospitalization and 84 (50.6%) of them were boys. Respiratory syncytial virus was the causative agent in 114 (68.7%) cases, rhinovirus in 19 cases, and other viruses in 20 cases (human metapneumovirus in 6, influenza A virus in 9, parainfluenza type 3 virus in 4 and adenovirus in 1 cases). The virus was not identified in 13 samples. Fifty-seven (34.3%) children needed feeding support, 31 (18.7%) needed supplemental oxygen, and the mean length of hospital stay was 4.49 (range 0–22, SD 3.19) days.

Table 1 *Tollip* rs116938768 and rs5743854 genotypes in relation to the clinical disease severity markers in 166 children admitted due to bronchiolitis before 6 months of age

Tollip rs116938768	Genotype CC (wild) n = 150		Genotype CT (variant) n = 16		
Clinical finding	*n*	%	*n*	%	*p*-value*
Feeding support (n = 57)	52	34.7	5	31.3	1.00
Oxygen supplementation (n = 31)	29	19.3	2	12.5	0.74
RSV positive (n = 114)	103	68.7	11	68.8	1.00
Length of stay (mean, SD)	4.57 (SD 3.26)		3.69 (SD 2.14)		0.30
Tollip rs5743854	Genotype CC (wild) n = 118		Genotype CG or GG (variant) n = 48		
Clinical finding	*n*	%	*n*	%	*p*-value*
Feeding support (n = 57)	43	36.4	14	29.2	0.47
Oxygen supplementation (n = 31)	25	21.2	6	12.5	0.27
RSV positive (n = 114)	82	69.5	32	66.7	0.72
Length of stay (mean, SD)	4.64 (SD 3.05)		4.10 (SD3.5)		0.33

*Pairwise comparison wild versus variant

Table 2 *Tollip* rs116938768 and rs5743854 genotypes in relation to clinical outcomes at 5–7 years of age in 141 children admitted due to bronchiolitis before 6 months of age

Tollip rs116938768	Genotype CC (wild) n = 128		Genotype CT (variant) n = 13		
Clinical finding	*n*	%	*n*	%	*p*-value†
ICS use (n = 18)	17	13.3	1	7.7	1.00
Current asthma (n = 19)	19	14.8	0	0.0	0.22
Asthma ever (n = 38)	35	27.3	3	23.1	1.00
Allergic rhinitis (n = 40)	35	27.3	5	38.5	0.29
Atopic dermatitis (n = 42)	40	31.3	2	15.4	0.27
Tollip rs5743854	Genotype CC (wild) n = 103		Genotype CG or GG (variant) n = 38		
Clinical finding	*n*	%	*n*	%	*p*-value†
ICS use (n = 18)	12	11.7	6	15.8	0.57
Current asthma (n = 19)	13	12.6	6	15.8	0.63
Asthma ever (n = 38)	24	23.3	14	36.8	0.11
Allergic rhinitis (n = 40)	29	28.2	11	28.9	0.93
Atopic dermatitis (n = 42)	28	27.2	14	36.8	0.27

†Pairwise comparison wild versus variant

The *Tollip* rs116938768 genotype was CC (wild) in 150 (90.4%) cases and variant CT in 16 (9.6%) cases. The variant TT genotype was not detected. The *Tollip* rs5743854 genotype was CC (wild) in 118 (71.1%) cases, variant CG in 41 (24.7%) cases and variant GG in 7 (4.2%) cases. There were no significant associations between the wild and variant genotypes and the virus etiology of bronchiolitis nor the severity markers of bronchiolitis (Table 1).

Comparison with population genetic data

The minor allele frequency (MAF) of *Tollip* rs116938768 (allele T) in this study population was 0.05 and the MAF (allele G) of *Tollip* rs5743854 was 0.17. The MAFs in the bronchiolitis group did not differ from the general Finnish population according to FIN data in the 1000 Genomes project (p = 0.48 and p = 0.56, respectively), nor from the Finnish data in Genome Aggregation Database (p = 0.67 and p = 0.88, respectively).

5–7-year follow-up data

There were 141 children with clinical and genetic data available from the second follow-up visit at 5–7 years of age. In all, 18 (12.8%) of the children had continuous ICS medication for asthma and in addition, one child was diagnosed with asthma at the control visit based on asthma presumptive symptoms during the last 12 months and a positive finding in the exercise challenge test. Therefore, 19 (13.5%) of the children were regarded as having current asthma. Altogether 38 (27.0%) of the children had been diagnosed with current or previous asthma and according to the definition, had asthma ever. In addition, 42 (29.8%) children had current atopic dermatitis and 40 (28.4%) had current allergic rhinitis.

Asthma ever was somewhat more common in children with the *Tollip* rs5743854 variant CG or GG genotypes when compared together to wild CC genotype (38.8 vs. 23.3%, p = 0.11) (Table 2). Further, the *Tollip* rs5743854 variant homozygous GG genotype alone compared to other genotypes combined (CC or CG) showed a weak association with asthma ever (66.7% vs. 25.2%, p = 0.045). *Tollip* rs116938768 wild and variant genotypes did not show any significant association with asthma ever. There were no significant associations between the *Tollip* rs5743854 or rs116938768 wild and variant genotypes and ICS use, current asthma or presence of atopic dermatitis or allergic rhinitis (Table 2).

Lung-function data were available from 100 cases with genetic data available. Neither *Tollip* rs5743854 or *Tollip* rs116938768 showed any significant difference between wild and variant genotypes in baseline lung function by IOS (data not shown). *Tollip* rs5743854 variant CG or GG genotypes as combined (n = 75) showed on average less airways reactivity (increase in Rrs5 +3.4% vs. +10.4%, p = 0.04) in response to exercise challenge compared to children with wild CC genotype (n = 25). *Tollip* rs116938768 did not show any significant differences between wild and variant genotypes in the exercise challenge test (data not shown).

11–13-year follow-up data

There were 125 children with clinical and genetic data available from the third follow-up visit at 11–13 years of age. In

Table 3 *Tollip* rs116938768 and rs5743854 genotypes in relation to clinical outcomes at 11–13 years of age in 125 children admitted due to bronchiolitis before 6 months of age

Tollip rs116938768	Genotype CC (wild) n = 115		Genotype CT (variant) n = 10		
Clinical finding	*n*	%	*n*	%	*p*-value[†]
ICS use (n = 11)	9	7.8	2	20.0	0.21
Current asthma (n = 15)	13	11.3	2	20.0	0.34
Persistent asthma (n = 9)	8	7.0	1	10.0	0.54
Atopic dermatitis (n = 31)	30	26.1	1	10.0	0.45
Allergic rhinitis (n = 19)	18	15.7	1	10.0	0.63
Tollip rs5743854	Genotype CC (wild) n = 92		Genotype CG or GG (variant) n = 33		
Clinical finding	*n*	%	*N*	%	*p*-value[†]
ICS use (n = 11)	8	8.7	3	9.1	1.00
Current asthma (n = 15)	10	10.9	5	15.2	0.54
Persistent asthma (n = 9)	6	6.5	3	9.1	0.70
Atopic dermatitis (n = 31)	22	23.9	9	27.3	0.82
Allergic rhinitis (n = 19)	14	15.2	5	15.2	1.00

[†]Pairwise comparison wild versus variant

all, 11 (8.9%) of the children had continuous ICS medication for asthma and four children were diagnosed with asthma at the control visit based on asthma presumptive symptoms during the last 12 months and a positive finding in spirometry. Therefore, 15 (12.0%) of the children were regarded as having current asthma. Nine children (7.2%) had had current asthma at both the 5–7 years and 11–13 years control visits and were regarded as having persistent asthma. In addition, 31 (24.8%) children had current atopic dermatitis and 19 (15.2%) had current doctor-diagnosed allergic rhinitis. There were no significant associations between the *Tollip* rs5743854 or rs116938768 wild and variant genotypes and ICS use, current asthma, persistent asthma or presence of atopic dermatitis or allergic rhinitis (Table 3).

Lung function by spirometry and bronchodilation tests were available covering 84 cases with genetic data. There were no significant differences between *Tollip* rs5743854 or *Tollip* rs116938768 wild and variant genotypes in baseline spirometry or bronchial reversibility to bronchodilation (data not shown).

Discussion

This long-term follow-up study did not find any significant associations between *Tollip* rs5743854 or rs116938768 polymorphisms and bronchiolitis in infancy or post-bronchiolitis outcome at 5–7 or 11–13 years of age.

Early-life contacts with microbes and subsequent activation of innate immunity via TLRs are thought to be important for the normal maturation of adaptive immunity. Lack of microbial contacts or impaired function or defect in downstream signaling of TLRs may lead to sustained Th2-cytokine milieu that is characteristic for asthmatic airways. Tollip is a negative regulator of TLR-induced inflammatory responses, and a mutation in the encoding gene may therefore attenuate its anti-inflammatory function and lead to excessive inflammatory responses.

Tollip gene polymorphisms have previously been associated e.g. with the susceptibility to sepsis[14] and tuberculosis.[15] In a mouse study, Tollip deficiency was associated with enhanced lung eosinophilia.[16] Only one previous study has investigated the connection between asthma and *Tollip* gene polymorphisms being located in close proximity to the now studied SNPs. *Tollip* rs5743899 in adult asthmatic subjects with variant genotype was associated with attenuated lung function indicated by lower FEV1/FVC-ratio when compared to subjects with wild genotype. In addition, this variant genotype was also associated with lower anti-viral response to rhinovirus infection *in vitro*.[5]

In the present study, we found that *Tollip* rs5743854 variant genotypes showed a trend with having had an asthma diagnosis ever by 5–7 years of age, but this was no longer present at the 11–13 years visit. Further, the *Tollip* rs5743854 variant homozygous GG genotype did show a weak association with asthma ever at 5–7 years of age, when analyzed as a subgroup against other genotypes. *Tollip* rs5743854 variation was associated with decreased *Tollip* messenger ribonucleic acid expression in monocytes of infants vaccinated with BCG only if the variant genotype was homozygous.[3]

The group defined as "asthma ever" consisted of 19 children with current asthma and 19 children with previous asthmatic symptoms before 5–7 years of age. In subgroup analyses, cases with rs5743854 variant genotypes, now analyzed as combined, showed less reactivity in response to exercise challenge compared to wild genotype at preschool age. This suggests that *Tollip* rs5743854 variant genotypes may be associated with transient asthmatic symptoms after bronchiolitis, remitting by school-age and showing less airway reactivity in response to exercise.

This is interesting in that the majority of the study population presented RSV as a causative agent of bronchiolitis. Respiratory syncytial virus infections before the age of 3 years have been associated with non-atopic asthma phenotype characterized by wheezing up to age of 11 years and lower lung function at age 13 years.[17] In the current study, baseline lung function testing did not show any significant associations with the investigated *Tollip* SNPs at 5–7 or 11–13 years of age. In addition, the investigated SNPs in this study were not associated with the viral etiology or the severity outcomes of bronchiolitis in infancy. Thus, as the *Tollip* rs5743854 variant genotype may be associated with transient wheezing symptoms, evidence for persistent post-bronchiolitis asthma or lower lung function was not found at the age of 11–13 years.

The main strengths of our study are the prospective design with carefully collected data during more than 10 years of follow-up time, and the depth of phenotyping clinical outcomes. The study population is of Finnish origin and therefore

highly homogeneous, which is a clear benefit in genetic studies enabling reliable comparisons to population-based databases. We also acknowledge a few limitations in our study. First, the sample size is relatively small for genetic studies, which may have led to false negative results. Second, we did not study the functionality of the *Tollip* SNPs. *Tollip* rs5743854 is located in the non-coding promoter region of the gene and has been shown to be functional in previous studies.[3] Data on the functionality of *Tollip* rs116938768, however, are lacking. Third, in terms of outcomes, such as post-bronchiolitis asthma, the results are applicable mainly for children, who have experienced moderate-to-severe bronchiolitis. Instead, in terms of bronchiolitis susceptibility, the negative results are applicable to population, but only to the Finnish population, as bronchiolitis cases were compared with two sets of Finnish population data.

The negative results of this study suggest that the investigated polymorphisms in the *Tollip* gene are not involved in the severity or viral etiology of bronchiolitis nor in the development of post-bronchiolitis asthma. However, other SNPs in the *Tollip* gene are still worthy of investigation in the future as Tollip clearly plays an important role in modulating inflammatory responses induced by TLRs.

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Disclosure

The authors declare no conflict of interest.

Author contributions

S.T. was responsible for analyses and wrote the first draft, J.T. participated in the analyses and writing, K.N. and M.K., attended to the design of the study and writing, Q.H. was responsible for the genetic analysis and participated in the writing, E.L. was responsible for the final paper. All authors read and approved the final manuscript.

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