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Polymorphisms of toll like receptors in the genetics of severe RSV associated diseases

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Abstract. Toll like receptors (TLRs) are an essential part of the innate immune response. So far, ten different TLRs were identified in humans. They recognize a wide range of microbial and viral pathogens. Infection by respiratory syncytial virus (RSV) is still a major health problem, about 2% of all children are hospitalised due to RSV bronchiolitis during their first 2 years of live. TLR4 has already been described in association with RSV associated diseases by us and others.
Thus we were interested whether other TLRs are also involved in the genetics of severe RSV infection. We genotyped 19 polymorphisms in the autosomal TLRs, these are TLR1, 2, 3, 5, 6, 9 and 10. Association analyses by the Armitage's Trend test revealed weak association of one TLR9 promoter polymorphism with RSV infection ($p = 0.013$). In addition, association was found with TLR10 haplotypes ($p = 0.024$).
We conclude from our data – that – although we can not rule out a minor involvement of *TLR9* polymorphism and *TLR10* haplotypes – TLRs other than TLR4 do not play a major role in the genetics of severe RSV associated diseases. Future studies should focus on additional genes of the innate immune response.

Keywords: Toll like receptor (TLR), polymorphisms, association, RSV

1. Introduction

Immunity in vertebrates is categorised into innate and adaptive immunity. Innate immunity is the ancient form of host defence against pathogens and exhibits important functions in activation and formation of the adaptive immunity through the induction of cytokines, chemokines and other co-stimulatory molecules. As a consequence, T and B cells are activated in host individuals and a highly diverse repertoire of T and B cell receptors is generated, followed by clonal selection and expansion of pathogen specific receptors. The immunological memory of these cells offers advantage and at the same times some limitation (four to seven days delayed immune response [1]).

In contrast to specific receptors in adaptive immune defence, the innate immune system recognizes a non-clonal pattern of molecules by so called pattern recognition receptors (PRRs). They bind conserved molecular structures as parts of pathogens (pathogen-associated molecular patterns, or PAMPs). The group of PRRs is large and diverse. They can be secreted or expressed on the cell surface or in intracellular compartments. One of the most important groups of pattern recognition receptors is the toll like receptor (TLR) family.

So far, ten different TLRs have been identified in humans. The genes for *TLR1, 2, 3, 4, 5, 6, 9* and 10 are located on autosomes, whereas *TLR7* and *TLR8* are encoded on the X-chromosome. Recently TLR11 has been identified in mice as an important defence mechanism against bacterial infection [2], but it was not yet found in humans.

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TLRs are evolutionarily conserved proteins with an extra cellular domain of leucine-rich repeats and an intracellular Toll/IL-IR domain [3]. They play an important role in the innate immune recognition of microbes, mycobacteria, virus particles and fungi. They are also important for the above mentioned interplay between the innate and adaptive immune response [4,5].

TLR signalling regulates the expression of many genes. Thereby a significant redundancy and collaboration is detectable between different TLRs. For example, TLR2 recognizes PAMPs from gram positive bacteria; simultaneously it interacts with TLR6 or TLR1 to mediate responses to diverse other microbial pathogens [6]. TLRs can also detect viral PAMPs: Double stranded RNA from viral genomes is detected by TLR3 [7] and F protein of respiratory syncytial virus (RSV) is detected by TLR4 [8].

The clinical course of RSV infection is highly variable, and genetic variations in genes of the immune response might be important in the determination whether a child suffers from a banal rhinitis or severe bronchiolitis after exposure to RSV. Interestingly, PBMCs of children expressing two amino acid variants in TLR4 show an impaired cellular response upon *in vitro* RSV infection [9]. Thus these variants might increase the susceptibility of these children to more severe RSV associated diseases.

Due to the interaction between different members of the TLR family, their interplay between the innate and adaptive immune responses and on the basis of genetic association between *TLR4* and severe RSV bronchiolitis in our own population as well as in other studies [10–12], we were interested whether other *TLRs* are also associated with severe RSV associated diseases. Therefore we investigated 19 single nucleotide polymorphisms (SNPs) within the autosomal *TLRs* in a population of children affected by severe RSV infection and controls.

2. Materials and methods

2.1. Subjects

Population of infants with severe RSV infection The population was recruited at the Centre for Paediatrics and Adolescent Medicine, Freiburg, Germany and at the Community Children's Hospital of Freiburg, St. Josef's Hospital. Infants were eligible for the study when hospitalized due to RSV infection between September 1998 and March 2005. Infection with RSV was confirmed by antigen test and/or RSV-specific PCR [13]. According to the case definition, children had symptoms of bronchiolitis, such as wheezing and tachypnea and needed either supplementary oxygen and/or gavage feeding and/or intravenous fluids. Children with congenital heart defects, immune deficiency, or chromosomal aberrations were excluded. DNA samples were collected either by blood-taking or buccal smears with sterile cotton sticks. In total 156 children were included.

2.2. Control population

Two hundred and seventy randomly chosen probands were used as controls (aged 19 to 40 years). They originate from the same area in the South-Western part of Germany. No medical history was taken and no medical testing was performed on controls.

2.3. Genotyping

DNA was extracted from peripheral blood leucocytes, or buccal smears following standard protocols and column purified (DNA midikit, Qiagen, Germany). Genotyping was performed by restriction fragment length polymorphism (RFLP). Table 1 gives an overview of the primers, PCR conditions and the restriction enzymes, which were used for genotyping. After digestion, all PCR products were resolved on Agarose gels and stained with Ethidium bromide.

2.4. Sequencing

For each polymorphism three controls (homozygous wild type, heterozygous and homozygous mutation) were sequenced by the dideoxy chain termination method [14] using the Big Dye Terminator cycle sequencing kit on an ABI 310 sequencer (Applied Biosystems). All following studies included these reference individuals.

2.5. Statistical analysis

For each SNP a separate association analysis based on the case-control design was performed. Hereby we used the Armitage's trend test. This test takes into account the individuals' genotypes rather than just the alleles, following the guidelines given by Sasieni [15], as implemented in the program Finetti (Thomas F. Wienker, unpublished data; http://ihg.gsf.de/cgi-bin/hw/hwa1.pl and http://ihg.gsf.

Table 1
Experimental conditions for genotyping of the SNPs by Restriction Fragment Length Polymorphisms (RFLP). * indicates primers in which a mutation was incorporated for RFLP analyses. The mutated nucleotide is shown underscored and in bold

Gene	SNP	Primer	PCR condition	Restriction enzyme
TLR10	rs4129009	5'CCAGAATGAGTGGTGCCATT 3' 5'GTATGTGGTCCCCAACTTCCC 3'	56°C, 35 cycles	VspI
	rs11466657	5'GGCCAGAAACTGTGGTCAAT 3' 5'TGAATCTGACCATCCAACCA 3'	56°C, 35 cycles	SspI
	rs11096955*	5'GGTAAGGCTTATCTTGACCACA3' 5'GACGAGTTGTTTAAAAG **G**ACT 3'	54.2°C, 35 cycles	Hifn I
	rs11096956*	5'ATGCCACATGCTTTTCTC 3' 5'AGTTTCTGGCCATGAGCAAT 3'	59°C, 35 cycles	Tag I
	rs11096957	5'AGAAGGTAGCCTGCCCATC 3' 5'TCGGATCTGAAAGTGTTCCA 3'	59°C, 35 cycles	Hin III
	rs10856839*	5'CATCATTCATATGAGGAAT**T** 3' 5'AAGTCTGCGGGAACCTTTCT 3'	52,5°C, 40 cycles	MseI
	rs4274855*	5'TCCTGACTTACCTCAACACC 3' 5'AAGTCTGCGGGAACCTTTCT 3'	59°C, 35 cycles	MspI
	rs11466645	5'GATGAGGAAATTGAAGGATCT 3' 5'CACTGCTACTTCCCCAGTGC 3'	54.2°C, 30 cycles	XbaI
TLR1	rs5743551*	5'GGCAGGGCAGTAAGGGAAG**A**T 3' 5'CTGCCCTGAATCCAAAAAGA 3'	56,5°C, 40 cycles	DpnII
TLR6	rs5743810	5'GCAAAAACCCTTCACCTTGT 3' 5'AGACTCTGACCAGGCATTTCC 3'	56°C, 35 cycles	AvaII
	rs5743794*	5'CATTGAGACTTGCTACAAAG**G**TA 3' 5'TTCCAGCACCATTTGTTGAA 3'	59°C, 35 cycles	RsaI
TLR2	rs3804099	5'CAAAACCCTAGGGGAAACATC 3' 5'CTCGCAGTTCCAAACATTCC 3'	61°C, 35 cycles	TaiI(MaeII)
TLR3	rs3775291*	5'GTGGCTAAAATGTTTGGAGCA 3' 5'TGAGATTTTATTCTTGGTTAGG**C**TGA 3'	55°C, 35 cycles	DdeI
	rs3775290	5'CTTGCTCATTCTCCCTTACACA 3' 5'TTACGAAGAGGCTGGAATGG 3'	60°C, 35 cycles	TaqI
	rs3775296	5'CCGTTTGATGTATGACTTGT 3' 5'GCTGCAGTCAGCAACTTCAT 3'	59,1°C, 35 cycles	MboII
TLR5	rs5744168	5'GGTAGCCTACATTGATTTGC 3' 5'GAGAATCTGGAGATGAGGTACCCG 3'	65°–55°C in −0.5°C steps, 55°C 20 cycles	DdeI
TLR9	rs187084	5'TATTCCCCTGCTGGAATGTC 3' 5'CTCCCAGCAGCAACAATTC 3'	65°–55°C in −0.5°C steps, 55°C 20 cycles	MseI
	rs5743836	5'GACATTCCAGCAGGGGAATA 3' 5'GTCCACAGATGGCCAACAAG 3'	59°C, 35 cycles	BstNI
	rs352140	5'GGACACTCCCAGCTCTGAAG 3' 5'CACTTGGCTGTGGATGTTGT 3'	65°–55°C in −0.5°C steps, 55°C 30 cycles	Bsh12361

de/linkage/download/finetti.zip). The program Finetti was also used to calculate the Hardy Weinberg equilibrium (HWE) for each polymorphism in both populations. A significant deviation from HWE could be a hint to population admixture or genotyping error [16]. In addition, we performed haplotype frequency estimations and association analyses with FAMHAP [17].

2.6. Approval

The collection of blood and the experimental procedures were approved by the Ethics Committee of the University of Freiburg. A statement of informed consent was signed by all participants, or in the case of children, signed by their parents.

3. Results

3.1. Genotyping of TLRs polymorphisms

In this study we included all *TLRs* located on autosomal chromosomes, except *TLR4* which we have already investigated in a previous study. In Table 2 the SNPs under investigation are listed for each gene. In addition the distributions of genotypes (homozygous for wild-type, heterozygous and homozygous for the mutation) as well as the allelic frequency in both populations are given.

Table 3 summarizes some additional information about the polymorphisms like the chromosomal localisations, the corresponding nucleotide or amino acid exchange, respectively and the p values of the Hardy

Table 2
Listed are the polymorphisms under investigation and their genotype distribution as well as the allelic frequency in both study populations

Gene	SNP	Genotype distribution Controls	Allelic frequency Controls	Genotype distribution RSV	Allelic frequency RSV
TLR10	rs4129009	169; 83; 18	0.780	91; 54; 9	0.766
	rs11466657	241; 24; 3	0.944	137; 13; 1	0.950
	rs11096955	112; 105; 51	0.614	67; 58; 29	0.623
	rs11096956	152; 102; 16	0.752	86; 53; 14	0.735
	rs11096957	109; 114; 46	0.617	60, 67; 27	0.607
	rs10856839	182; 72; 10	0.826	114; 44; 2	0.855
	rs4274855	171; 86; 13	0.793	96; 54; 11	0.764
	rs11466645	169; 86; 14	0.788	98; 41; 11	0.790
TLR1	rs5743551	143; 102; 25	0.719	76; 59; 18	0.690
TLR6	rs5743810	105; 120; 43	0.616	72; 65; 17	0.679
	rs5743794	160; 93; 13	0.776	90; 52; 9	0.768
TLR2	rs3804099	94; 126; 47	0.588	52; 67; 32	0.566
TLR3	rs3775291	139; 109; 22	0.717	74; 66; 14	0.695
	rs3775290	124; 107; 31	0.677	73; 75; 6	0.718
	rs3775296	169; 86; 15	0.785	101; 50; 2	0.824
TLR5	rs5744168	222; 36; 0	0.930	132; 14; 1	0.946
TLR9	rs187084	92; 124; 45	0.590	43; 56; 13	0.634
	rs5743836	204; 57; 6	0.871	98; 49; 5	0.806
	rs352140	81; 130; 57	0.455	44; 79; 28	0.447

Weinberg equilibrium (HWE). Two SNPs out of 19 investigated polymorphisms deviated from HWE in the control population; three in the population of RSV affected children. After correction for multiple testing no departure from HWE stayed statistically significant.

3.2. Association studies

The p values for association with severe RSV associated diseases obtained by the Armitage's Trend test are also listed in Table 3. Association was observed between severe RSV infection and rs5743836 in *TLR9* ($p = 0.013$). However, this did not remain statistically significant after correcting for multiple testing.

3.3. Haplotype analysis

Polymorphisms which are located in close proximity to one another in the genome tend to remain on the same allele during meiosis and mitosis. Thus they are not inherited independently from each other. This is known as linkage disequilibrium. Combining SNPs which are inherited together on the same allele – so called haplotypes – might reveal interaction of these SNPS.

Thus we subdivided the polymorphisms for haplotype analysis in three groups according to their position on the chromosomes: The first group consisted of SNPs within *TLR1, TLR6* and *TLR10*, the next group included SNPs from *TLR2* and *TLR3* and the last group comprised the SNPs from *TLR9*. Because we analysed only one SNP in *TLR5*, we were not able to conduct a haplotype analysis of this gene.

Combining the eight SNPs in *TLR10* a p-value of 0.024 was found for association of haplotypes with severe RSV infection. Including the *TLR1* and *TLR6* SNPs (all three genes are located within 58 kb on chromosome 4) the p-value for haplotype association was $p = 0.091$. The haplotype distribution is listed in Table 4. Out of the 2048 theoretically possible haplotypes only 15 haplotypes exist in our populations (with a frequency of at least 0.01 in either population). Thus extensive linkage disequilibrium exists between the SNPs of these three genes. The haplotype bearing the wild type allele for all polymorphisms was markedly overrepresented in the control population in comparison to the population of infants affected with severe RSV infection (frequency of 0.264 in controls compared to 0.186 in RSV population).

The other haplotypes showed no association with severe RSV associated diseases ($p = 0.758$ for *TLR2/TLR3* and $p = 0.257$ for *TLR9*, respectively).

4. Discussion

So far only a few studies investigated the genetic background of severe RSV associated diseases in infants. In these studies, predominantly genes involved in inflammatory processes and the immune system have

Table 3
Shown is the location of the investigated polymorphisms on the chromosomes (Chr) and their position within the genes. The results of Hardy Weinberg Equilibrium (HWE) as calculated by Finetti are listed as well as the p-value for association with severe RSV associated disease as given by the Armitage's trend test

Gene	Chr	SNP	Position	HWE controls	HWE RSV	p-value for association
TLR10	4	rs4129009	I775V	0.083	0.781	0.604
		rs11466657	I473T	0.040	0.306	0.715
		rs11096955	I369L	0.005	0.014	0.800
		rs11096956	P344P	0.838	0.291	0.573
		rs11096957	N241H	0.090	0.275	0.785
		rs10856839	G-25GT	0.396	0.529	0.359
		rs4274855	C-260T	0.501	0.250	0.399
		rs11466645	A-992T	0.483	0.031	0.951
TLR1	4	rs5743551	C-7202T	0.278	0.219	0.391
TLR6	4	rs5743810	S249P	0.378	0.687	0.074
		rs5743794	C-1633T	0.685	0.913	0.789
TLR2	4	rs3804099	N199N	0.671	0.235	0.550
TLR3	4	rs3775291	L412F	0.922	0.896	0.501
		rs3775290	F459F	0.289	0.012	0.221
		rs3775296	A-7C	0.359	0.168	0.181
TLR5	1	rs5744168	R392*	0.620	0.350	0.385
TLR9	3	rs187084	A-2871G	0.772	0.413	0.260
		rs5743836	A-2622G	0.411	0.710	0.013
		rs352140	P488P	0.719	0.474	0.818

been identified as susceptibility genes. The impact of genes of the innate immune response was further strengthened by a recent study: Janssen et al. analysed more than two hundred candidate genes for RSV bronchiolitis. For their analysis, they chose genes involved in airway mucosal responses, innate immunity, chemotaxis, adaptive immunity, and allergic asthma. Interestingly, association was predominantly reported with genes of the innate immune system like VDR, JUN, IFNA5 and NOS2 [18].

TLRs are an essential component of innate immunity. Studies using human and murine *in vitro* models illustrated the importance of TLR4 in the response to RSV [8,9,19]).

Furthermore, a few genetic association studies have already investigated the role of polymorphisms in different *TLRs* in the context of severe RSV infections. Association of two amino acid variants in TLR4 with RSV bronchiolitis was first reported by Gal *et al* four years ago [11]. Since than, two additional studies – including one in our own RSV population – have confirmed these results [10,12]. However, others failed to do so [18,20]. Interestingly, a functional impact of these variants has also been demonstrated: The response to inhalative LPS is changed in humans bearing these variants [21]. Finally, the above mentioned association study by Janssen et al. [18] included all *TLRs*. They found weak association with only one gene, i.e. *TLR8*.

The human *TLRs* have different chromosomal localisations and genomic structures: *TLR1*,*TLR6* and *TLR10* are located within only 58 kb on chromosome 4p14, whereas *TLR2* and *TLR3* map closely to each other on 4q32. The other autosomal TLRs are located separately, *TLR4* on 9q33-35, *TLR5* on 1q33.3 and *TLR9* on 3p21.3. Finally, *TLR7* and *TLR8* are in near proximity on Xp22.2.

We focused our study on all autosomal *TLRs* except *TLR4* which we have already analysed in a previous study. We did not include *TLR7* and *TLR8* in our association study as both genes are located on the X chromosome. So far there is no evidence that the disposition to severe RSV infection is inherited X-chromosomal. More important, our population of RSV affected children is so far too small for the examination of gonosomal genes.

We chose SNPs most likely to be important in the genetics of RSV infections, that is amino acid variants, SNPs which were already demonstrated to have functional consequences, or so called tag SNPs. A tag SNP is in high linkage disequilibrium with other polymorphisms. By genotyping a tag SNP these polymorphisms are also covered and consecutively more information about a gene is gathered in association analyses. None of the chosen SNPs was reported in association with severe RSV infection before.

The promoter polymorphism rs5743836 in *TLR9* shows weak association with severe RSV infection

Table 4
Haplotypes distribution in both populations combining polymorphisms within *TLR10*, *TLR1* and *TLR6*. Listed are haplotypes with a frequency of at least 0.01 in either population (1 = wildtype allele and 2 = mutant allele). The polymorphisms are arranged according to Table 1

Haplotype	Controls	RSV
1 1 1 1 1 1 1 1 1 1 1	0.264	0.186
1 1 1 1 1 1 1 1 1 1 2	0.011	0.021
1 1 1 1 1 1 1 1 1 2 1	0.301	0.327
1 1 1 1 1 1 1 1 2 1 1	0.016	0.010
1 1 1 1 1 1 1 1 2 1 2	0.018	0.004
1 1 1 1 1 1 1 2 1 2 1	0.004	0.014
1 1 2 1 2 2 1 1 1 1 1	0.063	0.067
1 1 2 1 2 2 1 1 2 1 1	0.049	0.037
1 1 2 2 2 2 1 1 1 1 1	0.022	0.031
1 2 1 1 1 1 1 1 1 1 1	0.000	0.015
1 2 1 1 1 1 1 1 1 2 1	0.000	0.011
2 1 2 2 2 1 2 1 2 1 2	0.011	0.020
2 1 2 2 2 1 2 2 2 1 1	0.011	0.012
2 1 2 2 2 1 2 2 2 1 2	0.113	0.128
2 2 2 2 2 1 2 2 2 1 2	0.043	0.022

($p = 0.013$). *TLR9* was already found in association with different diseases, for example with bronchial asthma [22]. Previously it has been demonstrated that RSV inhibits the production of interferon gamma in human plasmacytoid dendritic cells by signalling through TLR9 [23]. Consecutively the TH2 response is upregulated – a feature which is characteristically seen in severe RSV associated diseases. Thus an involvement of *TLR9* in the genetics of the disease seems reasonable. However, as we studied 19 SNPs in seven different genes the association does not stay significant after correcting for multiple testing. Also the haplotypes consisting out of three *TLR9* SNPs showed no association. Thus genetic variation in *TLR9* has – if at all – only minor effects on the development of severe RSV infections in our population.

In addition, haplotypes combining SNPs of *TLR10* showed a weak, but significant association with RSV infection as calculated by FAMHAP ($p = 0.024$). This statistical program automatically corrects for multiple testing by performing multiple permutations, so this p-value is realistic. However, we were not able to identify one single polymorphism in *TLR10* responsible for the observed effect. This is in contrast to a previous study describing association of individual SNPs within *TLR10* with bronchial asthma. Including polymorphisms of *TLR1* and *TLR6* into haplotype analyses the p-value did not stay significant ($p = 0.091$). Taking into account the results in our RSV as well as previous in our asthma population [24] we assume the following: As all three genes are located in close distance on the same chromosome and extensive linkage disequilibrium exists between the polymorphisms, their might be no single variant but only a combination of several polymorphisms responsible for association with the diseases. However, clearly functional studies are needed to clarify this issue.

Taken together, we can not exclude that *TLR9* and *TLR10* have some minor effects on the development of severe RSV infections in infants. However, this effect might be small and not critical for the individual child.

Thus we conclude from our data that *toll like receptors* – with the exception of *TLR4* – do not play a major role in the genetic predisposition to severe RSV associated diseases. Future association studies should focus on other genes of the innate and adaptive immune response.

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