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-1R 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 leukocytes, 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 1                     | Primer 2                     | PCR condition  | Restriction enzyme |
|-------|-------|------------------------------|------------------------------|----------------|-------------------|
| TLR10 | rs4129009 | 5’CCAGAATGAGTGGTGCCATT 3’    | 5’GTATGTTGTCACCCCACTCC 3’    | 56°C, 35 cycles | VspI              |
|       |       | 5’GGCAAGAATGAGTGGTGCCATT 3’ | 5’GTATGTTGTCACCCCACTCC 3’    | 56°C, 35 cycles | SspI              |
|       | rs11096955 | 5’GGTAAGGTCATTGTGTTGACT 3’   | 5’GGTAAGGTCATTGTGTTGACT 3’   | 54.2°C, 35 cycles | Hinf I            |
|       |       | 5’ATGGGACTTGCTCCAAACCA 3’    | 5’ATGGGACTTGCTCCAAACCA 3’    | 59°C, 35 cycles | Tag I             |
|       |       | 5’GATTGGAATTGGAAGGAATGT 3’   | 5’GATTGGAATTGGAAGGAATGT 3’   | 52,5°C, 40 cycles | Msel              |
|       |       | 5’AGCTGAGCTGCCGCCATC 3’      | 5’AGCTGAGCTGCCGCCATC 3’      | 59°C, 35 cycles | MspI              |
|       |       | 5’CTGAGATCGCTAAATGGTTCCA 3’  | 5’CTGAGATCGCTAAATGGTTCCA 3’  | 54.2°C, 30 cycles | XbaI              |
|       |       | 5’CATCATTAGATGGAGG TTC 3’    | 5’CATCATTAGATGGAGG TTC 3’    | 59°C, 35 cycles | MseI              |
|       |       | 5’GATGAGGAAATTGAAGGATCT 3’   | 5’GATGAGGAAATTGAAGGATCT 3’   | 59°C, 35 cycles | BstNI             |
| TLR1  | rs5743551 | 5’GGCAGGGCAGTAAGGGAAG AT 3’  | 5’CTGCCCTGAATCCAAAAAGA 3’    | 56.5°C, 40 cycles | DpnII             |
|       |       | 5’GGCAGGGCAGTAAGGGAAG AT 3’  | 5’CTGCCCTGAATCCAAAAAGA 3’    | 56°C, 35 cycles | AvaII             |
|       |       | 5’AGCTGAGCTGCCGCCATC 3’      | 5’AGCTGAGCTGCCGCCATC 3’      | 59°C, 35 cycles | Rsal              |
|       |       | 5’CTGAGATCGCTAAATGGTTCCA 3’  | 5’CTGAGATCGCTAAATGGTTCCA 3’  | 59°C, 35 cycles | MssI              |
|       |       | 5’CATCATTAGATGGAGG TTC 3’    | 5’CATCATTAGATGGAGG TTC 3’    | 59°C, 35 cycles | MspI              |
|       |       | 5’GATGAGGAAATTGAAGGATCT 3’   | 5’GATGAGGAAATTGAAGGATCT 3’   | 59°C, 35 cycles | XbaI              |
| TLR6  | rs5743810 | 5’GCAAAAACCCTTCACCTTGT 3’    | 5’AGACTCTGACCAGGCATTTCC 3’   | 56°C, 35 cycles | AvaII             |
|       |       | 5’AGACTCTGACCAGGCATTTCC 3’   | 5’AGACTCTGACCAGGCATTTCC 3’   | 56°C, 35 cycles | AvaII             |
|       |       | 5’CATGAGACCTGTGCACAAGAGGA 3’ | 5’CATGAGACCTGTGCACAAGAGGA 3’ | 55°C, 35 cycles | Rsal              |
|       |       | 5’GCCAAGAATGAGTGGTGCCATT 3’ | 5’GCCAAGAATGAGTGGTGCCATT 3’ | 59°C, 35 cycles | Rsal              |
| TLR2  | rs3804099 | 5’GAAAACCTTAGGAGGAAAACATC 3’ | 5’CTGACGTGCTCTACCCG 3’       | 61°C, 35 cycles | Tal(I/MaeII)       |
|       |       | 5’GAAAACCTTAGGAGGAAAACATC 3’ | 5’CTGACGTGCTCTACCCG 3’       | 55°C, 35 cycles | Ddel              |
|       |       | 5’GATGAGGAAATTGAAGGATCT 3’   | 5’GATGAGGAAATTGAAGGATCT 3’   | 59°C, 35 cycles | Rsal              |
|       |       | 5’AGCTGAGCTGCCGCCATC 3’      | 5’AGCTGAGCTGCCGCCATC 3’      | 60°C, 35 cycles | TaqI              |
| TLR3  | rs3775291 | 5’GGAGGAAATTGAAGGATCT 3’     | 5’GGAGGAAATTGAAGGATCT 3’     | 59,1°C, 35 cycles | MboII             |
|       |       | 5’GGAGGAAATTGAAGGATCT 3’     | 5’GGAGGAAATTGAAGGATCT 3’     | 59,1°C, 35 cycles | MboII             |
|       |       | 5’GGAGGAAATTGAAGGATCT 3’     | 5’GGAGGAAATTGAAGGATCT 3’     | 56°C, 35 cycles | MboII             |
| TLR5  | rs5744168 | 5’GATGAGCTACATGTTGTTGACGA 3’ | 5’GATGAGCTACATGTTGTTGACGA 3’ | 65°C–55°C in 56°C–55°C steps, 55°C | Ddel |
|       |       | 5’AGACTCTGACCAGGCATTTCC 3’   | 5’AGACTCTGACCAGGCATTTCC 3’   | 65°C–55°C in 56°C–55°C steps, 55°C | Ddel |
| TLR9  | rs187084 | 5’GACATCTGACCAGGGGAAATA 3’   | 5’GACATCTGACCAGGGGAAATA 3’   | 59°C, 35 cycles | BstNI             |
|       |       | 5’GATGAGGAAATTGAAGGATCT 3’   | 5’GATGAGGAAATTGAAGGATCT 3’   | 65°C–55°C in 56°C–55°C steps, 55°C | BstNI |
|       |       | 5’GACATCTGACCAGGGGAAATA 3’   | 5’GACATCTGACCAGGGGAAATA 3’   | 65°C–55°C in 56°C–55°C steps, 55°C | BstNI |

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
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

| 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    | 0.000        | 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 | 4   | 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 then, 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 gnososomal 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

| Haplotyp e | Controls | RSV |
|------------|----------|-----|
| 111111111111 | 0.264 | 0.186 |
| 111111111112 | 0.011 | 0.021 |
| 111111111121 | 0.301 | 0.327 |
| 111111111211 | 0.016 | 0.010 |
| 111111112112 | 0.018 | 0.004 |
| 111111112121 | 0.004 | 0.014 |
| 111122211111 | 0.063 | 0.067 |
| 111222211211 | 0.049 | 0.037 |
| 111222211111 | 0.022 | 0.031 |
| 112111111111 | 0.000 | 0.015 |
| 112111111211 | 0.000 | 0.011 |
| 211222212121 | 0.011 | 0.020 |
| 211222212221 | 0.011 | 0.012 |
| 211222212222 | 0.113 | 0.128 |
| 222222222222 | 0.043 | 0.023 |

(\( p = 0.013 \)). 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 up-regulated – 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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