

## Original article

Putative association of a *TLR9* promoter polymorphism with atopic eczema

**Background:** Toll-like receptors (TLR) play a pivotal role in the induction of first-line defense mechanisms of the innate immune system and trigger adaptive immune responses to microbial pathogens. Genetic variations in innate immunity genes have been reported to be associated with a range of inflammatory disorders. Deficiencies on the level of immunity receptors such as pathogen-recognition receptors are suspected to affect the maturation of our immune system and to avail thereby the high prevalence of atopic diseases and susceptibility of atopic patients to microbial infections.

**Aims of the study:** We evaluated *TLR9* as susceptibility gene for atopic eczema (AE).

**Methods:** Analyses of four tag single-nucleotide polymorphisms in two panels of families containing a total of 483 parent-affected offspring trios as well as a cohort of 274 unrelated adult AE cases and 252 hypernormal population-based controls have been performed.

**Results:** In both family cohorts, polymorphism C-1237T, which is located within the promoter region of the *TLR9* gene, was significantly associated with AE, in particular the intrinsic subtype of AE. No associations were seen in the case-control cohort. Luciferase reporter gene assays revealed significantly higher promoter activity of the TT allelic variant at this single nucleotide polymorphism site.

**Conclusion:** These observations suggest that the *TLR9* promoter polymorphism C-1237T might affect AE susceptibility in particular in patients with the intrinsic variant of AE.

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Key words: atopic eczema; immunoglobulin E; innate immunity; polymorphisms; toll-like receptor.

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The aetiology of atopic diseases such as asthma, allergic rhino-conjunctivitis and atopic eczema (AE) is complex and multifactorial, involving both genetic and environmental components (1). As possible cause for the increase in prevalence of allergic diseases seen in Western countries over the past decades, it has been suggested that an

altered stimulation of innate immune responses during childhood contributes to a deregulated immune response because of a reduction of regulatory T lymphocyte activities and missing immune deviation from allergen-specific immune responses of the Th2 type to protective immune responses of the Th1 type (2). This, in turn, might lead to the manifestation of atopic diseases in genetically susceptible hosts (3, 4). As a complex sequence of events, recognition of microbial components involves innate and acquired immunity mechanisms (5). To discriminate between diverse pathogen associated molecular patterns (PAMPs), our innate immune system uses a variety of pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) or intracellular nucleotide-binding oligomerization domain (NOD) proteins (6).

**Abbreviations:** AE, atopic eczema; CTR, controls; DC, dendritic cells; IgE, immunoglobulin E; LD, linkage disequilibrium; MAF, minor allele frequency; NOD, nucleotide-binding oligomerization domain; PAMP, pathogen associated molecular patterns; PBMC, peripheral blood mononuclear cells; pDC, plasmacytoid dendritic cells; PRR, pattern recognition receptors; SNP, single nucleotide polymorphism; TDT, transmission disequilibrium test; TLR, toll-like receptor.

TLRs are known to function as mediators between innate and adaptive immunity via induction of dendritic cell (DC) maturation and activation of naive T cells primarily skewing the adaptive immune response towards Th1 (7). So far, 10 different human TLRs which recognize distinct pathogen-associated molecular pattern found in bacteria, viruses, fungi, helminths and protozoa have been identified. TLR9 is mainly expressed by plasmacytoid dendritic cells (pDC) and B cells and recognizes unmethylated CpG motifs present in bacterial DNA (8) and intracellular viral antigens (9). Activation of TLR9 in pDC induces Th1 cytokines such as interferon- $\alpha$  or interleukin-12 (IL-12) and Th1-biased immune response (10). In murine B cells, TLR9 activation up-regulates the expression of the transcription factor T-bet and inhibits IgG1 and IgE class switching (11). Genetic variations in innate immunity genes have been reported to be associated with a range of inflammatory disorders, including both Th2-driven atopic and Th1-dominated autoimmune diseases (12). Associations between a polymorphism of the gene encoding TLR2 and asthma (13) as well as another *TLR2* single nucleotide polymorphism (SNP) with severe AE (14) and a *TLR4* polymorphism with asthma (15) have been reported. So far, only limited data on the role of *TLR9* polymorphisms for atopic diseases are available (16) (17,18), and none of the previous studies investigated a putative association of *TLR9* polymorphisms with AE. However, genetic variations in innate immunity receptor genes such as *TLR9* might be of particular importance in patients with AE, as these patients are highly susceptible to bacterial and viral infections, which frequently impair the course of their disease. About 80–90% of adult AE patients suffer from so called extrinsic AE with concomitant sensitizations, allergic rhinitis or asthma. Conversely, 10–20% of adult AE patients with clinically and histologically indistinguishable eczematous skin lesions lack sensitizations and other clinical manifestations of the atopic triad and suffer from 'intrinsic AE'. Despite the clinical similarity, extrinsic AE patients exhibit a higher tissue eosinophilia enhanced lesional cytokine expression, including IL-5, -13, -1 $\beta$  and CCL11 and higher surface expression of the high-affinity receptor for IgE (Fc $\epsilon$ RI) on epidermal DC compared with intrinsic eczema patients (19). The *TLR9* gene maps to chromosome 3p21.3 and contains two exons with exon 2 being the major coding exon (20) (Fig. 1). To evaluate the candidacy of *TLR9* as AE susceptibility gene, we

investigated *TLR9* SNPs in two independent panels of German families recruited through children with AE and an independent adult case-control cohort for AE.

## Material and methods

### Study population

Families were recruited from the Dermatology Clinics at the Technical University Munich and the University of Bonn, through a child or children with active AE. A physician-administered questionnaire was completed for each individual.

The questionnaire apart from demographic data included the basis allergy questions of the European Community Respiratory Health Survey on respiratory health (21). In addition, all probands were interviewed in a standardized manner to report on symptoms of atopic diseases, basic family data and parental history of atopic diseases. In all subjects, total as well as specific IgE antibodies against common environmental allergens (grass, birch pollen, cat and house dust mite) were measured with the help of an enzyme-immunoassay (CAP-FEIA, Pharmacia, Uppsala, Sweden and Immulite, DPC Biemann, Bad Nauheim, Germany). Atopic eczema was diagnosed on the basis of a skin examination by experienced senior dermatologists using the UK diagnostic criteria for AE (22). Specific sensitization was defined to be present if at least one of the specific IgE antibodies was positive (RAST class $\geq$ 1, corresponding to  $\geq$ 0.35 KU/l). Extrinsic AE was defined as AE with total IgE levels  $\geq$ 150 KU/l and/or specific sensitizations. The severity of eczema was assessed by using the SCORAD scoring system (23).

Initially, DNA samples from a panel of 281 parent-offspring trios of German origin were examined. For the purpose of replication, a second independent eczema panel comprising of 202 families was investigated. In addition, an independent collection of 274 unrelated adult AE cases and 252 population-based controls as described elsewhere (24) was analysed. All study methods followed the Declaration of Helsinki protocols, were approved by the Ethics Committee of the 'Bayerische Landesärztekammer' Munich and the Ethics Committee of the University of Bonn and a written informed consent was obtained from all participants prior to the beginning of the study.

### Genetic analyses

Genomic DNA was extracted from leucocytes with a commercial DNA isolation kit (Gentra Systems, Minneapolis, MN, USA) according to the manufacturer's recommendation and as described previously (25).

Single nucleotide polymorphism information for this paper was retrieved from the Innate Immunity PGA, NHLBI Program in Genomic Applications, URL: <http://innateimmunity.net/data/homology> June 2005. Assays for SNP genotyping were developed for all four SNPs of the *TLR9* gene. Genotyping analyses were carried out by using the MASSARRAY system (Sequenom, San Diego, CA, USA). Briefly, genomic DNAs were amplified by polymerase chain reaction (PCR) using HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany). Genotyping assays were carried out by using 5 ng of genomic DNA. Polymerase chain reaction primers were used at 167 nM final concentrations for a PCR volume of 6  $\mu$ l. The PCR condition was 95°C for 15 min for hot start, followed by denaturing at 95°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 1 min for 45 cycles, and finally incubation at 72°C for 10 min. Polymerase chain reaction products were first treated with shrimp alkaline phosphatase (SAP, Amersham, Freiburg, Germany) for 20 min at 37°C to

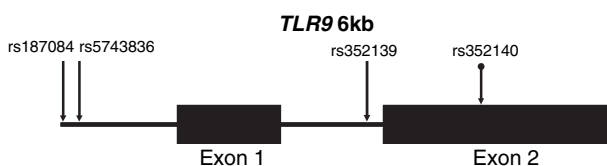


Figure 1. Overview of the gene structure and location of the four *TLR9* single nucleotide polymorphism (SNPs).

Table 1. *TLR9* htSNPs, dbSNP accession numbers, localization, genotype frequencies (combined family panels) and genotyping primers

| Single nucleotide polymorphism (SNP) ID | Location   | Genotype | Frequency (%) |           | Direction | Polymerase chain CR primer     | Extension primer    |
|-----------------------------------------|------------|----------|---------------|-----------|-----------|--------------------------------|---------------------|
|                                         |            |          | Parents       | Offspring |           |                                |                     |
| rs187084 (C-1486T)                      | 5'flanking | CC       | 19.3          | 17.7      | fwd       | ACGTTGGATGTTACTATGTGCTGGGCACTG | GCTGGAATGTCAGCTTCTT |
|                                         |            | CT       | 47.1          | 52.6      | rev       | ACGTTGGATGTATCCCCTGCTGGAATGTC  |                     |
|                                         |            | TT       | 33.6          | 29.7      |           |                                |                     |
| rs5743836 (C-1237T)                     | 5'flanking | CC       | 2.2           | 1.4       | fwd       | ACGTTGGATGAGCAGAGACATAATGGAGGC | GCTGTTCCCTCTGCCTG   |
|                                         |            | CT       | 25.2          | 21.6      | rev       | ACGTTGGATGTTGGGATGTGCTGTTCCCTC |                     |
|                                         |            | TT       | 72.6          | 77.0      |           |                                |                     |
| rs352139 (G1174A)                       | Intron 1   | AA       | 19.9          | 21.0      | fwd       | ACGTTGGATGTGGGAGGGCTGTGTGAGTG  | AAGTGGAGTGGGTGGAGGT |
|                                         |            | AG       | 44.0          | 46.1      | rev       | ACGTTGGATGGAAGATGCTAGAAGATGCC  |                     |
|                                         |            | GG       | 36.1          | 32.9      |           |                                |                     |
| rs352140 (G2848A)                       | Exon 2     | CC       | 18.5          | 18.5      | fwd       | ACGTTGGATGTCTACCACGAGCACTCATTC | TCCAGGGCCTCCAGTCG   |
|                                         |            | CT       | 48.1          | 50.0      | rev       | ACGTTGGATGCAAAGGGCTGGCTGTTGTAG |                     |
|                                         |            | TT       | 33.4          | 31.5      |           |                                |                     |

remove excess dNTPs and afterwards for 10 min at 85°C to inactivate SAP. Thermo Sequenase (Amersham, Freiburg, Germany) was used for the base extension reactions. Extension primers were used at a final concentration of 5.4  $\mu$ M in 10  $\mu$ l reactions (Table 1). The base extension reaction condition was 94°C for 2 min, followed by 94°C for 5 s, 52°C for 5 s, and 72°C for 5 s for 40 cycles. All reactions (PCR amplification, base extension) were carried out in a Tetrad PCR thermal cycler (MJ Research, Waltham, MA, USA). The final base extension products were treated with SpectroCLEAN resin (Sequenom, San Diego, CA, USA) to remove salts in the reaction buffer. This step was carried out with a Multimek 96-channel autopipette (Beckman Coulter, Fullerton, CA, USA), and 16  $\mu$ l of resin-water suspension was added into each base extension reaction, making the total volume 26  $\mu$ l. After a quick centrifugation (644 g, 3 min) in an Eppendorf Centrifuge 5810, 10 nl of reaction solution was dispensed onto a 384 format SpectroCHIP (Sequenom) prespotted with a matrix of 3-hydroxypicolinic acid by using a SpectroPoint nanodispenser (Sequenom). A modified Bruker Biflex matrix-assisted laser desorption ionization-time-of-flight mass spectrometer (Sequenom) was used for data acquisitions from the SpectroCHIP. Genotyping calls were made in real time with MASSARRAY RT software (Sequenom). Potential-binding sites for transcription factors were identified on forward and backward strands of genomic DNA using the MATINSPECTOR programme (release 7.0; Genomatix, Munich, Germany).

#### Luciferase reporter gene assay

Two *TLR9* promoter sequences (–839 to +240) including polymorphism (C-1237T) were separately cloned from CC or TT allelic variant patient genomic DNA. Two *firefly* luciferase reporter vectors (pGL2-basic-*TLR9*/TT and pGL2-basic-*TLR9*/CC) were constructed by inserting the promoter sequences into the pGL2-Basic vector at Mlu I and Xho I sites (Promega, Madison, WI, USA). Ninety per cent confluent HEK 293 cells (CRL-1537, ATCC, LGC Promochem, UK) in 24-well tissue culture plates were transiently transfected with 1  $\mu$ g of one of reporter constructs or pGL-Basic and 0.5  $\mu$ g of the thymidine kinase-driven *Renilla* plasmid (pTK-RL) as internal control of transfection efficiency by using lipofectamine<sup>TM</sup> 2000 (Invitrogen, Carlsbad, CA, USA). After 24 h, cells were lysed and dual-luciferase measurement was performed by utilizing the Dual-Luciferase Reporter Assays System (Promega, Madison, WI, USA). All plasmid constructs were verified by sequencing to exclude cloning errors (MWG-BIOTECH AG, Martinsried, Germany). Seven independent experiments were

performed and duplicate wells were done for each sample at every time.

#### Statistics

For the family-based association analysis, descriptive statistics for quantitative and qualitative values was calculated with SAS version 9.1 (SAS Institute, Cary, NC, USA) and are given by mean  $\pm$  SD, and relative frequencies or absolute numbers, respectively. The PEDCHECK program (26) was used to exclude Mendelian inconsistencies in allele assignment in each family. Genotyping success rates, Hardy–Weinberg equilibrium and minor allele frequencies were calculated for any SNP considered with HAPLOVIEW version 3.2 (<http://www.broad.mit.edu>). Linkage disequilibrium (LD) was assessed with procedure ldmax in the software package GOLD (<http://www.well.ox.ac.uk/asthma/GOLD>). Haplotype frequencies were estimated from SNP genotype data using the EM algorithm implemented in HAPLOVIEW. To examine the association of SNPs and haplotypes with the binary traits AE, total serum IgE and allergic sensitization, the classical transmission disequilibrium test (TDT) was used, which is implemented in HAPLOVIEW for both single-marker and multimarker association testing. Transmitted and untransmitted allele frequencies as well as contingency tables for TDT analyses were calculated by using SAS version 9.1. *P*-values for TDT analyses were obtained from the conditional exact McNemar test using R version 2.0.1 (R Foundation for Statistical Computing, Vienna, Austria). Power calculations were carried out according to McNemar's test of equality of paired proportions with two-sided significance level of 0.05. Results from functional analyses were evaluated with the Wilcoxon or Mann–Whitney *U*-tests using SPSS 12.0 for Windows (SPSS, Chicago, IL, USA). Results are given in mean percentage  $\pm$  SD. Any *P*-values given are two-sided, subject to a significance level of 0.05 and not adjusted for multiple comparisons, because the comparisons do not represent completely independent tests, and replication of results was achieved in two independent cohorts.

Results in functional analyses were expressed as mean  $\pm$  SEM and compared using a *Tukey's post hoc* test.

#### Results

##### Association analysis

Table 2 gives an overview on clinical characteristics of the two family panels investigated. Panel 1 contains 281

Table 2. Phenotype characteristics of all individuals (parents and affected) included in analyses. Relative proportions are calculated without considering missing values

|                                                  | Panel1 ( <i>n</i> = 281 families) |                      | Panel2 ( <i>n</i> = 202 families) |                     |
|--------------------------------------------------|-----------------------------------|----------------------|-----------------------------------|---------------------|
|                                                  | Parents                           | Offspring            | Parents                           | Offspring           |
| Male gender                                      | 50.0                              | 42.0                 | 50.0                              | 37.62               |
| Mean ( $\pm$ SD) age                             | 54.62 ( $\pm$ 9.05)               | 19.32 ( $\pm$ 11.32) | 55.08 ( $\pm$ 9.56)               | 25.07 ( $\pm$ 8.65) |
| Atopic eczema (AE) [%]                           | 14.41                             | 100                  | 4.46                              | 100                 |
| Extrinsic AE [%]                                 | 8.01                              | 82.31                | 3.47                              | 84.65               |
| Intrinsic AE [%]                                 | 3.56                              | 15.30                | 1.0                               | 15.35               |
| Allergic sensitization [%]                       | 40.21                             | 69.04                | 45.79                             | 82.67               |
| Mean ( $\pm$ SD) log-transformed total serum IgE | 3.27 ( $\pm$ 2.15)                | 4.71 ( $\pm$ 2.63)   | 3.66 ( $\pm$ 1.78)                | 5.45 ( $\pm$ 1.90)  |

Table 3. Association analysis of *TLR9* single nucleotide polymorphism (SNPs) in the trio cohort

| Polymorphism             |                   | MAF (%) | Panel 1 ( <i>n</i> = 281 families) |              |                               |              | Panel 2 ( <i>n</i> = 202 families) |              |                               |              | Combined panel ( <i>n</i> = 483 families) |              |                               |              |
|--------------------------|-------------------|---------|------------------------------------|--------------|-------------------------------|--------------|------------------------------------|--------------|-------------------------------|--------------|-------------------------------------------|--------------|-------------------------------|--------------|
|                          |                   |         | AE ( <i>n</i> = 281)               |              | Intrinsic AE ( <i>n</i> = 43) |              | AE ( <i>n</i> = 202)               |              | Intrinsic AE ( <i>n</i> = 31) |              | AE ( <i>n</i> = 483)                      |              | Intrinsic AE ( <i>n</i> = 74) |              |
|                          |                   |         | T : U                              | <i>P</i>     | T : U                         | <i>P</i>     | T : U                              | <i>P</i>     | T : U                         | <i>P</i>     | T : U                                     | <i>P</i>     | T : U                         | <i>P</i>     |
| rs187084 (C-1486T)       | T $\rightarrow$ C | 0.421   | 129 : 116                          | 0.406        | 17 : 16                       | 0.862        | 91 : 86                            | 0.707        | 13 : 12                       | 0.842        | 220 : 202                                 | 0.382        | 30 : 28                       | 0.793        |
| rs5743836 (C-1237T)      | T $\rightarrow$ C | 0.153   | 84 : 57                            | <b>0.023</b> | 21 : 8                        | <b>0.016</b> | 55 : 36                            | <b>0.046</b> | 13 : 4                        | <b>0.029</b> | 139 : 93                                  | <b>0.003</b> | 34 : 12                       | <b>0.001</b> |
| rs352139 (G1174A)        | G $\rightarrow$ A | 0.427   | 99 : 99                            | 1.000        | 13 : 10*                      | 0.532        | 93 : 90                            | 0.825        | 18 : 13*                      | 0.369        | 192 : 189                                 | 0.878        | 31 : 23*                      | 0.276        |
| rs352140 (G2848A)        | T $\rightarrow$ C | 0.430   | 133 : 129                          | 0.805        | 21 : 17                       | 0.516        | 96 : 95                            | 0.942        | 18 : 12                       | 0.273        | 229 : 224                                 | 0.814        | 39 : 29                       | 0.225        |
| C-G (rs5743836-rs352139) |                   | 0.156   | 61 : 87                            | <b>0.034</b> | 10 : 22                       | <b>0.029</b> | 36 : 52                            | <b>0.090</b> | 4 : 13                        | <b>0.029</b> | 97 : 139                                  | <b>0.007</b> | 14 : 35                       | <b>0.002</b> |

Alleles are coded as major allele  $\rightarrow$  minor allele. T : U ratio refers to the transmission–untransmission ratio of the underlined allele from heterozygote parents to affected offsprings except for \* (opposite allele). MAF, minor allele frequency in combined family panel, dbSNP rs, reference marker.

families and panel 2 202 families. The case–control cohort has been described recently (27). Briefly, it consists of 274 unrelated adult AE cases (average age  $35.9 \pm 10.8$  years; 61.2% female and 38.8% male) and 252 hypernormal population-based controls (average age  $39.4 \pm 16.1$  years; 61.5% female and 38.5% male).

The location of the genotyped *TLR9* SNPs and their frequencies are provided in Table 3 and Fig. 1. Analysis of Mendelian inheritance within the families revealed no major errors. None of the genotyped markers showed a significant deviation from Hardy–Weinberg equilibrium. Results of single locus association analysis are summarized in Table 3. In both family, panels significant associations were seen for the single nucleotide polymorphism C-1237T (rs5743836) with AE ( $P = 0.023$ ,  $P = 0.046$ ) and intrinsic AE ( $P = 0.016$ ,  $P = 0.029$ ) using the TDT (Table 2). Associations between the C-1237T T allele and AE ( $P = 0.003$ ) and intrinsic AE ( $P = 0.001$ ) were also seen in the combined family panels. None of the other SNPs genotyped in this study showed any significant association results with AE. The resulting sample size of  $n = 483$  families has more than 80% power to detect an odds ratio of 1.5 between transmitted and untransmitted alleles when the observed proportion of discordant pairs in the TDT is 0.18 and the two-sided significance level is 5%. Analyzing the sequence variation introduced by the

Table 4. Associations of the *TLR9* SNP in the case-control cohort. Frequencies of the different SNP in the case ( $n = 274$ ) and control cohort ( $n = 252$ ) are shown

| Single nucleotide polymorphism (SNP) ID | Genotype  | Frequency (%) |          |
|-----------------------------------------|-----------|---------------|----------|
|                                         |           | Cases         | Controls |
| rs187084 (C-1486T)                      | CC        | 17.88         | 21.03    |
|                                         | CT        | 51.09         | 51.19    |
|                                         | TT        | 30.29         | 25.79    |
| rs5743836 (C-1237T)                     | CC        | 2.55          | 1.19     |
|                                         | CT        | 20.07         | 19.44    |
|                                         | TT        | 76.28         | 76.59    |
| rs352139 (G1174A)                       | AA        | 20.07         | 16.27    |
|                                         | AG        | 48.54         | 49.21    |
|                                         | GG        | 31.02         | 32.54    |
| rs352140 (G2848A)                       | CC        | 21.53         | 16.67    |
|                                         | CT        | 47.81         | 49.21    |
|                                         | TT        | 30.29         | 32.14    |
| rs5743836-rs352139                      | Diplotype |               |          |
|                                         | TG        | 43            | 47.2     |
|                                         | TA        | 44.3          | 41.6     |
|                                         | CG        | 12.5          | 11.1     |

polymorphism, we found that a potential-binding site for nuclear factor- $\kappa$ B (NF- $\kappa$ B) might be created by the T/C exchange (28).

The *TLR9* SNPs selected were in strong LD ( $D'$ ) with each other indicating a single LD block (Fig. 2). Two

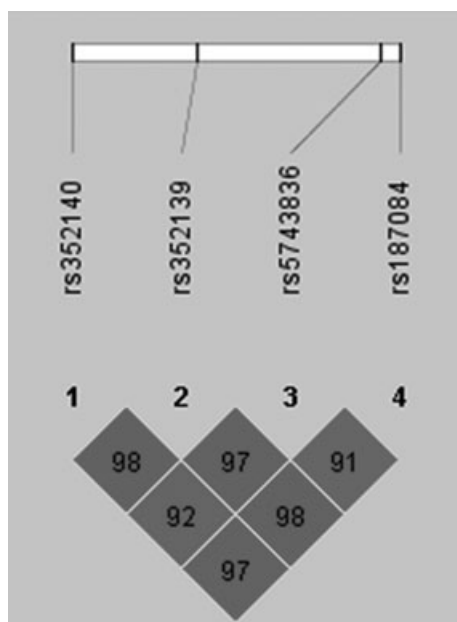


Figure 2. Linkage disequilibrium plot of the tSNPs in the *TLR9* gene in the combined family panels based on the measure of  $D'$ . The  $D'$  values for any two tSNPs are presented in the box representing their intersection.

SNPs (C-1237T and G1174A) were sufficient to distinguish the common *TLR9* haplotypes. In the combined analysis, haplotype C-G showed significant transmission distortion for AE ( $P = 0.034$ ,  $P = 0.006$ ) and intrinsic AE ( $P = 0.029$ ,  $P = 0.002$ ) (data not shown). This corresponds to results from single analyses where presence of a T allele at C-1237T was associated with increased risks. In contrast to the family cohorts, no significant associations were seen in the case-control cohort (Table 4).

#### Higher promoter activity of the TT allelic variant at C-1237T

To clarify whether *TLR9* promoter polymorphism C-1237T affects TLR promoter activity, TT allele and CC allele promoter sequences were cloned to *firefly* luciferase reporter vectors to construct pGL2-basic-TLR9/TT and pGL2-basic-TLR9/CC. The *TLR9* promoters' activities were monitored by dual-luciferase assays. As a result, the TT allelic variant sequence showed a significantly higher promoter activity than the CC allelic variant sequence (Fig. 3).

#### Discussion

*TLR9* plays a pivotal role in the induction of first-line defense mechanisms of the innate immune system and triggers effective adaptive immune responses to different bacterial and viral pathogens. On the functional level, the concept of a counter-regulation of innate immune

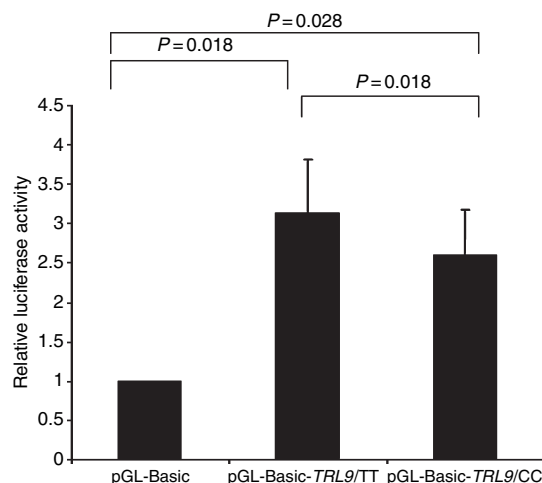


Figure 3. Higher promoter activity of the TT allelic variant at C-1237T. HEK 293 cells were transiently transfected with a reporter construct containing *TLR9* promoter polymorphism sequence fused to the *firefly* luciferase gene. A thymidine kinase-driven *ranilla* plasmid was co-transfected for monitoring transfection efficiency. After 24 h, *firefly* luciferase activity in cellular extracts of these cells was determined and normalized to *ranilla* luciferase activity and expressed as fold induction to control vector (pGL-Basic) defined as 1.0. The mean values of seven independent transfection experiments are shown, SEM is given.

responses implemented by *TLR9* and IgE-mediated adaptive immune responses conciliated via IgE-receptors has been introduced in a recent study (29) and implies a direct link between *TLR9* and atopic disorders. Further on, it has been speculated that polymorphisms in the *TLR9* gene might influence the functional capability of *TLR9* to elicit effective defense mechanism against microbial pathogens in a subgroup of patients with atopic diseases (1,12,17,18), who are characterized by a high susceptibility to microbial infections. In the present study, we evaluated four common *TLR9* SNPs for association with AE and related phenotypes in two panels of well characterized families. We observed a significant overtransmission of the common T-allele of the C-1237T promoter polymorphism in both family panels. Along with this, the two locus haplotype G-C showed transmission distortion for AE and intrinsic AE. However, an additional attempt to replicate these findings in an adult case-control cohort did not show any associations. This lack of replication might be due to the higher age of AE cases in this cohort and reflect the complex interactions between multiple population-specific genetic and environmental factors, but it also illustrates the need for further validation in independent cohorts. In addition, we cannot exclude that polymorphism C-1237T is only in LD with the causal variant(s). However, since it is located in the putative promoter

region of *TLR9*, and analysis of variant sequences predicted creation of a potential transcription factor binding site for c-rel/NFkB by the T/C transition implying a potential influence on the transcriptional regulation (28), it seems to be a good candidate for a functional SNP. This has been further supported by our functional studies, which revealed a higher transcriptional activity of the TT allelic variant at C-1237T.

Different associations of polymorphisms in candidate gene regions for AE in patients with the extrinsic and intrinsic variant of the disease have been already shown in previous studies and might point to genetic modifications which are supposedly of critical immunologic importance in patients with pure eczema, in which IgE-mediated mechanisms are not in the foreground and other factors, such as microbial influences, might represent important trigger factors of the recurrent flare ups of the eczematous skin lesions (30,31). One might speculate whether modifications in gene regions involved in the regulation of the IgE synthesis and B-cell network in intrinsic AE might protect these patients to some degree from the production of excessive IgE levels after concomitant challenge by allergens and microbial antigens. In this context, the advantage of simultaneous TLR9 activation with allergens and protective effect on Th2 immune responses has been already shown in a murine model and first clinical trials of TLR9-based immunotherapies (32).

It remains to be elucidated whether the capacity of B cells of intrinsic AE patients with the TT allele in the *TLR9* gene region to proliferate and perform a class switch after TLR9 stimulation differs and contributes to the lower IgE serum levels in this subgroup of patients (11,33,34). In a recent study, a higher incidence of eczema herpeticum in patients with extrinsic AE (35) could be demonstrated. In the light of the observations made in the current study, it seems tempting to speculate whether a genetically determined increased *TLR9* promoter activity in patients with intrinsic AE causes stronger immune responses after TLR9 activation by viruses and thereby confers protective effects against viral infections in these patients. However, neither differences in TLR9 protein expression of pDC nor in functional IFN $\alpha$ /IFN $\beta$  production after TLR9 stimulation related to the allelic variations at the SNP sites could be observed in this study (data not shown) nor in previous studies (18). An increased promoter activity of *TLR9* of skin cells together with repetitive challenges by bacterial DNA

might on the other hand represent an alternative way for the development of eczema in intrinsic AE patients independently from IgE-mediated mechanisms, as it was recently shown in a murine model (36). Clearly further studies are needed to clarify the role of the *TLR9* promoter polymorphisms in these putative interactions.

So far, three studies have investigated the role of *TLR9* polymorphisms in atopic diseases. In an exploratory case-control study for various phenotypes, an association of the C allele at C-1237T with asthma has been shown, thus directed opposite to the one we observed for AE in the present study (12). This study could not be confirmed in a second small-sized association study in Japanese children (17). Recently, another study (18) did not detect relevant associations between *TLR9* SNPs and atopy. However, a rather small number of healthy subjects was investigated, and the phenotype 'atopy' was defined by biochemical markers (positive SX1 test, elevated total IgE > 100 kU/l) or subject history of rather global atopy-related symptoms only. None of these studies evaluated the phenotype AE so far. In the present study, all *TLR9* SNPs were investigated in two panels of extensively phenotyped families with good power to detect associations with AE. With 86.5% in AE cases and 87.8% in intrinsic AE cases, the frequency of the associated common T allele of C-1237T was slightly higher than observed in other studies (12) (18). Although our transmission studies and functional data suggest a role for *TLR9* concerning the balance between Th1 and Th2 immune responses and IgE/IgG production in AE patients, the lack of replication in an additional case-control cohort implies that further investigations in independent cohorts are necessary to validate our findings.

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