TBX21 gene variants increase childhood asthma risk in combination with *HLX1* variants

Kathrin Suttner, MSc,[a] Philip Rosenstiel, MD,[b] Martin Depner, MSc,[c] Michaela Schedel, PhD,[a] Leonardo A. Pinto, MD,[c] Andreas Ruether, PhD,[b] Jerzy Adamski, PhD,[d] Norman Klopp, PhD,[e] Thomas Illig, PhD,[e] Christian Vogelberg, MD,[f] Stefan Schreiber, MD,[b] Erika von Mutius, MD,[c] and Michael Kabesch, MD[a] *Hannover, Kiel, Munich, Neuherberg, and Dresden, Germany*

Background: The T cell–specific T-box transcription factor (TBX21) plays a crucial role in the regulation of the immune system because this factor induces the differentiation of T_H1 and blocks T_H2 commitment together with the homeobox transcription factor HLX1.
Objective: The role of genetic variants in *TBX21* alone and in combination with *HLX1* polymorphisms was investigated in the development of T_H2-associated atopy and asthma.
Methods: The *TBX21* gene was resequenced in 37 adult volunteers. Polymorphisms identified were genotyped in a cross-sectional (N = 3099) and nested asthma case-control population (N = 1872) using mainly matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Effects of promoter polymorphisms on *TBX21* gene expression were studied by reporter gene assays. Furthermore, the impact of combinations of *TBX21* and *HLX1* polymorphisms on the development of asthma was assessed by using a risk score model. Statistical analyses were performed by using SAS/Genetics.
Results: Forty-three polymorphisms were identified in the *TBX21* gene. Considering a minor allele frequency of at least 10%, single nucleotide polymorphisms were assigned to 7 linkage disequilibrium blocks. Three tagging single nucleotide polymorphisms increased childhood asthma risk significantly (odds ratio [OR], 2.60, 95% CI, 1.34-5.03, $P = .003$; OR, 1.39, 95% CI, 1.02-1.90, $P = .039$; and OR, 1.97, 95% CI, 1.18-3.30, $P = .009$). *TBX21* promoter polymorphisms contained in 2 blocks significantly influenced *TBX21* promoter activity. In a risk score model, the combination of *TBX21* and *HLX1* polymorphisms increased the asthma risk by more than 3-fold.
Conclusions: These data suggest that *TBX21* polymorphisms contribute to the development of asthma, potentially by altering *TBX21* promoter activity. A risk score model indicates that *TBX21* and *HLX1* polymorphisms may have synergistic effects on asthma risk. (J Allergy Clin Immunol 2009;123:1062-8.)

Key words: TBX21, *HLX1, asthma, association study, genetic analysis, functional promoter analysis, risk score model*

Asthma is the most common chronic disease in childhood, affecting as many as 30% of children worldwide.[1] Asthma risk is determined by genetic susceptibility and environmental factors.[2] A lack of T_H1 responses and a deviation toward a T_H2 reaction is observed in many patients with asthma, suggesting a profound involvement of deregulation and imbalance of the immune system in the onset of atopic disorders such as asthma.

Transcription factors play an important role in determining T-cell development. Although GATA-3 is crucial for T_H2 development,[3] the T cell–specific T-box transcription factor (TBX21) is essential for directing T cells toward T_H1 by inducing IFN-γ.[4] TBX21 is significantly less expressed in the lung tissue of patients with asthma than controls without asthma.[5] TBX21-deficient mice ($TBX21^{+/-}$ and $TBX21^{-/-}$) developed characteristic asthma symptoms such as enhanced bronchial hyperresponsiveness (BHR) and airway remodeling.[5] Further studies showed that airway remodeling and eosinophilic airway inflammation after allergen exposure were significantly reduced in TBX21-overexpressing mice compared with GATA-3–overexpressing animals.[6] However, to obtain maximal IFN-γ expression, an interaction between TBX21 and the homeobox transcription factor HLX1 (H. 20-like homeobox) is required.[7] In addition, TBX21 and HLX1 have the ability to redirect T_H2 cells into T_H1 cells.[7]

Although these functional observations indicate an important role of TBX21 in asthma and other atopic diseases, a mixed picture evolves from genetic association studies investigating the effect of *TBX21*-related single nucleotide polymorphisms (SNPs) on atopic diseases. Whereas some studies report no association with asthma,[8,9] others describe effects of different *TBX21* SNPs on asthma phenotypes.[10-12] Because the role of *TBX21* variants in asthma and atopy is not yet clear, we resequenced the *TBX21* gene to extend and verify the genetic information available for *TBX21*. In a next step, comprehensive association and interaction studies were performed, and functional analyses on selected *TBX21* SNPs in the promoter region of the gene were conducted.

From [a]the Clinic for Paediatric Pneumology and Neonatology, Hannover Medical School; [b]the Institute of Clinical Molecular Biology, University Hospital Schleswig-Holstein, Campus Kiel; [c]the University Children's Hospital, Ludwig-Maximilians-Universität, Munich; [d]the Institute of Experimental Genetics and [e]the Institute of Epidemiology, Helmholtz Zentrum Munich, Neuherberg; and [f]the University Children's Hospital Dresden.

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Reprint requests: Michael Kabesch, MD, Center for Paediatrics, Clinic for Paediatric Pneumology and Neonatology, Medical School Hannover, Carl-Neuberg-Str 1, D-30625 Hannover, Germany. E-mail: Kabesch.Michael@mh-hannover.de.

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Abbreviations used
3′UTR: 3′ flanking region
BHR: Bronchial hyperresponsiveness
HLX1: H 2.0-like homeobox
LD: Linkage disequilibrium
MAF: Minor allele frequency
OR: Odds ratio
SNP: Single nucleotide polymorphism
TBX21: T cell–specific T-box transcription factor

METHODS

Population

Between 1995 and 1996, cross-sectional studies were carried out in the German cities of Munich, Leipzig, and Dresden to assess the prevalence of asthma and allergies in white German schoolchildren age 9 to 11 years.[13,14] Informed written consent was obtained from all parents, and all study methods were approved by the local ethics committees.

All children with asthma and/or BHR were selected (n = 624) and matched with a stratified random selection of healthy, nonatopic children without asthma or BHR (n = 1248; age 9-11) at a 1:2 ratio from the same population as previously described.[15] To investigate the influence of *TBX21* tagging SNPs on other atopic phenotypes, polymorphisms were also genotyped in the original cross-sectional population from Munich and Dresden (N = 3099; Munich, n = 1159; Dresden, n = 1940). Consequently, overlaps between the case-control and the cross-sectional study populations exist (see this article's Fig E1 in the Online Repository at www.jacionline.org).[16]

Parental questionnaires for self-completion were sent through the schools to the families. Children whose parents reported a physician's diagnosis of asthma or recurrent spastic or asthmatic bronchitis were classified as having asthma. Atopic asthma was defined as the concomitant co-occurrence of asthma with a positive skin prick test result, whereas nonatopic asthma was defined as asthma without a positive skin test result. Current environmental smoke exposure was defined as any current environmental tobacco smoke exposure at the age of 9 to 11 years.

Resequencing and mutation screening

To cover 15,199 bp in and around the *TBX21* gene (exons, introns, 2297 bp upstream and 990 bp downstream of the gene), 36 overlapping fragments were designed. Gene fragments of interest were amplified by PCR with specific primers (see this article's Table E1 in the Online Repository at www.jacionline.org). Primers were designed using the NetPrimer software and obtained from Metabion GmbH (Planegg-Martinsried, Germany). PCR was carried out on standard cyclers (Eppendorf GmbH, Eppendorf, Germany), in a total volume of 50 μL with 60 ng genomic DNA, 0.5 μmol/L of each amplification primer, 0.2 mmol/L of each deoxynucleotide triphosphate, and 0.8 U of Taq DNA Polymerase. PCR fragments were sequenced in at least 37 unrelated randomly selected adult volunteers by using ABI 310 and ABI 3730 sequencers (Applied Biosystems, Lincoln, Calif).

Genotyping

Genomic DNA was extracted from whole blood by a standard salting out method.[17] To minimize the use of genomic DNA, a modified primer extension preamplification[18] or alternatively the GenomiPhi procedure (Amersham Biosciences, Freiburg, Germany) was applied for random DNA preamplification. DNA samples were genotyped by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Sequenom Inc, San Diego, Calif),[19] except for G-999A, for which a restriction-endonuclease–based assay was used, and A12406C, for which a solid-phase oligonucleotide ligation assay was performed (Variom Biotechnology AG, Berlin, Germany). SNPs A4704T, T7729C, and A8385T were genotyped by using the TaqMan MGB bialleleic discrimination system (Applied Biosystems) as previously described.[20] PCR assays and associated extension reactions were designed by using the SpectroDESIGNER software (Sequenom Inc). All amplification and extension reaction conditions have been previously described,[21] and specific primers are given in this article's Tables E2 and E3 in the Online Repository at www.jacionline.org. Primer extension products were loaded onto a 384-elements chip with a nanoliter pipetting system (SpectroCHIP, SpectroJet; Sequenom Inc) and analyzed by a MassARRAY mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). The resulting mass spectra were analyzed for peak identification by using the SpectroTYPER RT 2.0 software (Sequenom Inc). For genotyping quality control, Hardy-Weinberg calculations were performed to ensure that each marker was within the expected allelic population equilibrium.

Luciferase assay

Jurkat cells were seeded in a 96-well plate at a density of 8×10^4 per well. The next day, cells were transfected with 35 ng of the plasmids expressing a *luciferase* gene under the control of the *TBX21* promoter containing either the risk or the nonrisk allele at position T-1993C (block 2) or the combination of risk alleles at positions T-1514C and G-999A (both block 1) together with 15 ng pRL-TK *Renilla* reporter plasmid (Promega, Madison, Wis) for normalization of transfection efficiency and cell viability. Plasmids always contained wild-type/nonrisk alleles at those polymorphic positions that were not tested. Xtreme Gene Q was used as transfection reagent according to the manufacturer's protocol (Roche, Mannheim, Germany). Eight hours after transfection, medium was exchanged by medium containing 50 ng/mL ionomycin or pure medium. After 18 hours of incubation, cells were washed in PBS and lysed in 1× passive lysis buffer (Promega). A dual luciferase assay was performed by using a dual luciferase reporter assay system (Promega) and a Genios Pro luminometer (Tecan, Crailsheim, Germany).

Bioinformatics and statistical analysis

Linkage disequilibrium (LD) patterns were assessed by Haploview,[22] and a threshold of $r^2 \geq 0.8$ was used to build LD blocks (also denoted as "bins"). Deviations from Hardy-Weinberg equilibrium were tested by using the χ^2 test, with expected frequencies derived from allele frequencies.

χ^2 Tests and logistic regression models were used to test for associations between dichotomous traits and single SNPs. We tested a recessive model and used a conservative Bonferroni correction for all tagging SNPs in every phenotype to adjust for multiple testing.

A risk score was established for *TBX21* and previously described *HLX1* variants[23] showing associations with asthma in the single SNP analyses to account for possible gene-by-gene interaction effects. Risk alleles were determined and given a risk value of either 1 (presence of risk in dominant or recessive model) or 0 (absence of genetic risk variant). Risk values were calculated and combinations were compared to the reference population with a risk score of 0. Risk score effects were assessed by using logistic regression in the cross-sectional study population. All calculations were carried out by using the SAS/Genetics software package (version 9.13) (SAS Institute Inc, Cary, NC).

RESULTS

Mutation screening and polymorphism identification in the *TBX21* gene

Resequencing of at least 37 adult volunteers led to the identification of 43 polymorphisms in the *TBX21* gene with minor allele frequencies (MAFs) of at least 3% (see this article's Table E4 in the Online Repository at www.jacionline.org). Thirteen polymorphisms (C533G, G2011A, T2473A, G2761A, C3075T, G3078A, T4716A, C5287T, A6618G, T6902C, G8760A, T10386C, delT12564) were previously not described in public SNP databases (dbSNP) and were submitted to dbSNP. Three polymorphisms were found in the promoter region, 1 in the 5′ untranslated region, 31 in the intronic regions, and 5 in the 3′