

Network: Infection and Inflammation: from Pathogen-induced Signatures to Therapeutic Target Genes**Project: Association and Linkage Approaches to Identify Human Genetic Variants Contributing to Natural Protection against Pulmonary Tuberculosis**

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Introduction

Genetic association studies in tuberculosis (TB) were, until now, limited to analyses of single candidate-genes (1). A more comprehensive approach might be to dissect the analysis of complex genetic traits and to study distinct functional systems involved in the pathogenesis of TB.

With regard to the protective immune response to *Mycobacterium tuberculosis*, interferon (IFN)- γ signalling may be considered a highly relevant functional system (2), as supported by evidence from studies of humans and animal models and highlighted by studies of children susceptible to atypical mycobacterial disease (3). Accordingly, we use a large case-control group for a detailed analysis of the signal transduction pathway of the IFN- γ receptor (IFNGR).

The goal of the project is to investigate the IFNGR signalling pathway with a large matched case-control sample characterized in NGFN1. The aim is to confirm the relevance of the pathway by showing influences of variants of its components on the occurrence of TB. In addition, the influence of variants of single candidate genes, as arising from scientific evidence, shall be investigated.

Project Status**Study group**

Study subjects were recruited in Ghana, West Africa, between September 2001 and July 2004. Patients were enrolled at Korle Bu Teaching Hospital, Accra, Komfo Anokye Teaching Hospital, Kumasi, 15 additional hospitals or polyclinics in Accra and Kumasi, and at the district hospitals of Obuasi, Agona, Mampong, Agogo, Konongo and Nkawie (Ashanti Region), Nkawkaw and Atibie (Eastern Region) and in Assin Fosu and Dunkwa (Central Region).



Fig 1: TB patient in Ghana.

In a first step, a total of 3128 patients with smear-positive pulmonary TB were recruited. Symptoms including cough, hemoptysis, shortness of breath, chest pain, night sweats, fever and weight loss were documented. Clinical and laboratory assessments included a physical examination, chest X-ray, two sputum smears stained by the modified Ziehl-Neelsen staining technique, a culture of *M. tuberculosis* in Löwenstein-Jensen medium and HIV-1 and -2 testing.



Fig 2: Chest X-ray of a tuberculosis patient with extensive bronchopneumonic shadowing.

All cases were treated in the framework of the Directly Observed Treatment Short-Course Strategy organized by the National Tuberculosis Programme of Ghana. 1039 cases were excluded for HIV positivity, lost to follow-up, refusal after enrolment, evidence of alcoholism or drug addiction, diabetes, and other reasons, mostly inappropriateness for matching with controls for sex and age and incomplete documentation. The final group of cases available for further analyses comprised 2089 individuals.

For the recruitment of control groups, mobile teams enrolled 1715 unrelated personal contacts of cases and 2283 community members from neighbouring houses of cases and at public assemblies. Characterization of participants included a medical history, clinical examination, chest X-ray and a tuberculin skin test.

1629 controls were excluded for the following reasons: signs of actual or previous TB on the X-ray film, lost to follow-up, refusal after enrolment, ambiguous medical histories with, inappropriateness for matching with cases for sex and age. The final control group consisted of 1234 unrelated personal contacts and 1135 community controls. The study participants belonged to the ethnic groups of Akan, Ga-Adangbe, Ewe and various ethnic groups of northern Ghana including Mole, Dagbane, Gurma, and Grusi.

In addition to the case-control group, DNA samples from 250 cases and from both parents (trios) are available for family-based association studies (transmission disequilibrium tests).

Identification of SNPs

The characterization of the IFNGR signalling pathway includes, in a first step, the identification of single nucleotide polymorphisms (SNPs) occurring with 10% and 5% frequency at 99% and 90% power, respectively. To that aim, DNA re-sequencing of the genes encoding IFNGR1, IFNGR2, interleukin (IL)12A, IL12B, IL12RB1, IL12RB2, IL23A, IL23R, IL27A, IL27B, IL27RA, IL27RB, Janus kinase (Jak) 1, Jak2, T-box transcription factor (T-bet), interferon

regulatory factor (IRF) 1, suppressor of cytokine signalling (SOCS) 1, signal transducer and activator of transcription (STAT) 1, STAT4 and protein-tyrosine kinase (TYK) 2 is currently performed in each 23 cases and PPD-positive and negative control individuals. Sequencing all exons (average 10) and ~1000 base pairs of the 5' regions of the genes in 69 individuals requires 15,000 sequencing reactions.

In the next step, an average of 10 SNPs per gene will be selected for high-throughput SNP typing, resulting in > 0.8 Mio. genotypes to be assessed. SNPs selected for further genotyping are variants that are relevant to function (non-synonymous mutations, promoter SNPs within binding sites for transcription factors, SNPs within splice consensus sequences), candidate markers (presumed association with a distinct phenotype, SNPs suggesting differences in frequencies between cases and controls), markers defining haplotypes, and SNPs with a frequency of >10% in one of the three subgroups.

Re-sequencing of 14 genes has meanwhile been completed and a total of 134 known and 111 novel variants has been identified until now. Among the novel alleles are 23 promoter, 45 intronic, 10 nonsynonymous, 16 synonymous and 17 variants of untranslated exonic regions. So far, one hundred-fifty SNPs fulfill the criteria required to be included in genotyping, and 94 of these variants are currently being typed at the Genotyping Center, Kiel.

Statistical analyses

Stratifications for variants of other pathway components and mycobacterial variants according to the results obtained in NGFN1 (4,5) will be included in the analyses. Therefore, futility tests will not be applied, and all candidates will be analysed in the entire sample. Trios will be subjected to transmission disequilibrium tests to confirm associations obtained in the case-control group.



Fig 3: Characterizations of tuberculosis contacts included physical examinations, chest X-ray and PPD testing. Photography Mika Väisänen (www.mika-photography.de)

Candidate genes

In addition to each one SNP of the candidate genes typed in the frame of NGFN1 (IL1a, IL1b, purinergic receptor P2X (P2RX7), solute carrier family (SLC) 11A1 (formerly NRAMP1), mannose binding lectin (MBL), vitamin D receptor (VDR)), further SNPs of these genes are currently typed and additional candidate genes are included. The latter comprise cytotoxic T lymphocyte-associated (CTLA) 4, IL10, tumor necrosis factor (TNF) α , dendritic cell-specific ICAM3-grabbing nonintegrin (DC-SIGN), thymocyte antigen CD1d, IL4, IL4R, arachidonate 5-lipoxygenase (ALOX5), secreted phosphoprotein (SPP) 1 and nuclear body protein SP110.

Two genes that recently have been shown to be involved in the phenotype of *M. tuberculosis* infection in mice have attracted our interest. The mouse gene *Ipr1* is crucially involved in the pathogenesis of TB (6). Consequently the human homologue of *Ipr1*, the gene encoding the nuclear body protein SP110, is considered a candidate. Furthermore, the observation that 5-lipoxygenase (5-lo)-dependent lipoxins regulate the IL-12 production and lungs from *M. tuberculosis*-infected 5-lo-/- animals have increased IL-12, IFN-gamma, and NO synthase 2 mRNA levels compared with wild-type mice makes the human homologue arachidonate 5-lipoxygenase (ALOX5) a candidate gene (7). Consequently, variants of these and other candidates arising from scientific evidence are included in our genetic analyses.

Outlook

The large sample collected is now available for refined analyses of the influence of genetic variants involved in the IFNGR pathway and of additional candidate gene variants on the outcome of *M. tuberculosis* infection and to confirm associations with transmission disequilibrium tests. Clinical, bacteriological and demographic data will allow for stratifications according to ethnicity, clinical details such as radiological details and PPD reactivity, mycobacterial genotypes and others. Thereby, analyses will not only address the role of individual components in protection against TB but will also investigate the ability of a genetic epidemiological approach to trace entire regulatory systems, the use of stratifications for component variants on the sensitivity to identify the influence of others, and the contribution of pathway heterogeneity to the complexity of common disease.

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