Polymorphism in a lincRNA Associates with a Doubled Risk of Pneumococcal Bacteremia in Kenyan Children

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Bacteremia (bacterial bloodstream infection) is a major cause of illness and death in sub-Saharan Africa but little is known about the role of human genetics in susceptibility. We conducted a genome-wide association study of bacteremia susceptibility in more than 5,000 Kenyan children as part of the Wellcome Trust Case Control Consortium 2 (WTCCC2). Both the blood-culture-proven bacteremia case subjects and healthy infants as controls were recruited from Kilifi, on the east coast of Kenya. *Streptococcus pneumoniae* is the most common cause of bacteremia in Kilifi and was thus the focus of this study. We identified an association between polymorphisms in a long intergenic non-coding RNA (lincRNA) gene (AC011288.2) and pneumococcal bacteremia and replicated the results in the same population (p combined = 1.69 × 10⁻⁴; OR = 2.47, 95% CI = 1.84–3.31). The susceptibility allele is African specific, derived rather than ancestral, and occurs at low frequency (2.7% in control subjects and 6.4% in case subjects). Our further studies showed AC011288.2 expression only in neutrophils, a cell type that is known to play a major role in pneumococcal clearance. Identification of this novel association will further focus research on the role of lincRNAs in human infectious disease.

Introduction

Bacteremia is a common pathway in the progression to death from severe pneumonia, meningitis, and sepsis, which together account for an estimated 3 million deaths each year globally in children under the age of 5 years. Even in developed countries the mortality rate from bacteremia remains unacceptably high.1–3 The leading bacterial...
cause of death in young children worldwide is *Streptococcus pneumoniae* (pneumococcus), and 14.5 million episodes of serious pneumococcal disease occur in young children annually. A key question is why only a proportion of individuals develop invasive disease despite widespread exposure and asymptomatic carriage of bacteria. Host genetic factors play an important role in explaining inter-individual variation in susceptibility to different infectious diseases. However, the relevant genes for bacteremia susceptibility remain largely unknown.

To identify genetic correlates of bacteremia susceptibility, we conducted a genome-wide association study (GWAS) in Kenyan children, a population with a major disease burden, as part of the Wellcome Trust Case Control Consortium 2 (WTCCC2). Bacteremia is a heterogeneous phenotype and immune responses and genetic variants affecting susceptibility are likely to be at least partially pathogen specific. We therefore focused on bacteremia caused by *S. pneumoniae*, the most common bacteria found in our study. In addition, all-cause bacteremia was analyzed in order to assess the possible role of genetic risk factors for bacteremia regardless of its etiology.

**Subjects and Methods**

**Study Design**

To identify host genetic determinants of susceptibility to invasive pneumococcal disease in African children, we performed a two-stage GWAS of pneumococcal bacteremia in 542 Kenyan children with culture-confirmed disease and 4,013 healthy control subjects. 429 case subjects and 2,677 control subjects were included in the discovery phase analysis, with 113 case subjects and 1,336 control subjects included in the replication analysis. To identify determinants of invasive bacterial disease irrespective of the pathogen, we further performed a GWAS of culture-confirmed all-cause bacteremia in the same population of Kenyan children (discovery phase, 1,536 case subjects; replication phase, 434 case subjects). Adopting a Bayesian framework, we considered evidence for shared effects at loci associated with pneumococcal disease and all-cause bacteremia, across pathogens commonly causing bacteremia in this population. Finally, we characterized disease-associated genetic variation identified in the study, analyzing tissue-specific expression of implicated transcripts in immune cell subsets. A detailed study workflow is described in Figure S1.

**Study Participants**

All study participants were residents of Kilifi District on the coast of Kenya. Case subjects were recruited among children younger than 13 years of age who were admitted for elective procedures or because of minor acute diseases. However, the relevant genes for bacteremia susceptibility remain largely unknown.

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analyzed at 78,786 genotyped autosomal SNPs. Three main ethnicities—namely Chonyi, Giriama, and Kauma—were discernible with principal-components analysis (PCA) of the genome-wide data (Figure S5).

**Immunochip Genotyping**  
Approximately 2,000 SNPs out of the total 200,000 SNPs were selected to be included in the ImmunoChip array based on the initial association results of the bacteremia analyses. The replication set was genotyped with this array at the Wellcome Trust Sanger Institute. All the samples went through a similar QC process as described above for the discovery samples (Table S1) and 434 case subjects and 1,336 control subjects passed the QC. After excluding SNPs based on minor allele frequency <1%, SNP call rate <95% (<99% if MAF <5%), and Hardy-Weinberg equilibrium $p < 1 \times 10^{-10}$, 143,100 SNPs remained for the further analyses. The same ethnicities were detectable by PCA in the replication sample set as in the discovery analysis (Figure S6). As the ImmunoChip genotyping was performed before the imputation, these genotypes were mainly utilized to account for population stratification and relatedness in the later replication analyses.

**Imputation and Association Analyses**  
We performed whole-genome imputation using the 1000 Genomes Phase I data as a reference panel. Genotypes were pre-phased using SHAPEIT before imputation with IMPUTE2. Only samples and SNPs passing the QC were included for pre-phasing and imputation. SNPs with potentially unreliable imputation were filtered out based on MAF (<2%), imputation info value (<0.8), and Hardy-Weinberg equilibrium $p < 1 \times 10^{-10}$. 10,996,499 imputed autosomal SNPs that passed the QC were analyzed for additive and genotypic models using SNPTEST2, taking the imputed genotype uncertainty (frequentist score test) and the first two principal components (PCs) of genetic structure into account. The genometric control parameter $\lambda$ for bacteremia overall and pneumococcal bacteremia after imputation and QC were 1.043 and 1.013, respectively (see the QQ plots in Figure S7). At associated SNPs, statistical tests were also performed using a linear mixed model that uses genome-wide data to model the pair-wise relatedness among the individuals.

**Sequenom Replication and Confirmation of Imputation Accuracy**  
SNPs with $p < 1 \times 10^{-3}$ in the additive model or $p < 5 \times 10^{-7}$ in the genotypic model were directly genotyped in the discovery set to confirm imputation accuracy and in the replication sample set to confirm the associations using two Sequenom iPLEX assays. Five SNPs looked unreliable after inspection of the cluster plots, leaving 37 SNPs in the analysis (the cluster plot for the most significant SNP is shown in Figure S8). All of these SNPs had a call rate greater than 95% and the genotype distribution among controls obeyed Hardy-Weinberg equilibrium ($p > 0.05$). After removing the samples that were originally excluded from the discovery and Immunochip analyses, 102 and 80 samples were removed because of the low call rate (<80%) and 7 and 9 samples because of the mismatching gender from the first and second multiplexes, respectively. This left 1,514 case subjects (418 pneumococcal cases) and 2,642 control subjects in the discovery sample set and 407 case subjects (103 pneumococcal cases) and 1,333 control subjects in the replication analyses. Genotyping of these two iPLEXes was performed at the Wellcome Trust Sanger Institute. The functional SNP rs334 in HBB was not found in the discovery sample set, but was therefore genotyped separately using a Sequenom iPLEX at the Wellcome Trust Centre for Human Genetics, University of Oxford. The QC measures described above were applied to these samples, leaving 1,360 case subjects and 2,644 control subjects in the discovery set and 389 case subjects and 1,312 control subjects in the replication set.

Only the samples that were included in either the discovery set or Immunochip replication set were included in the final analysis to allow inclusion of the first two PCs in logistic regression analysis using PLINK and to model the pairwise relatedness in a linear mixed model. The combined statistics for the discovery and replication samples were obtained using fixed effects meta-analysis in GWAMA. The replication dataset had 80% power to detect an association ($p < 0.05$) with a common SNP (MAF 0.20) that has an effect size $\geq 1.3$, but for more rare SNPs (MAF = 0.05), an effect size $\geq 1.54$ was required (see Figure S9). Therefore, we did not have sufficient statistical power to reliably replicate associations with modest effect sizes.

**Approaches to Handle Relatedness**  
The SNPs chosen for replication were also analyzed via a linear mixed model that uses genome-wide data to model the pairwise relatedness among the individuals, and which also included the first two PCs as covariates, to better account for relatedness and possible population structure within the sampled individuals. This was done by including all relatives and also by including only distantly related individuals (r < 0.2). We further assessed whether the sample set with pneumococcal infection includes more pairs of close relatives than other bacteremia case subjects or than control subjects. This was assessed by comparing the observed number of relative pairs with estimated $r > 0.025$ among the pneumococcus case subjects to an equal-sized set of the rest of the case or control subjects that are matched with respect to manual clustering (Figure S11) by resampling 100,000 datasets.

**Bayesian Model Comparisons**  
To compare models of the similarity of effect across bacterial species at identified disease-associated loci, we took a Bayesian approach (for a similar approach, see Band et al. and Bellenguez et al.). The likelihood function is based on multinomial regression with strata corresponding to the control subjects and each of the seven most common bacterial subgroups (Figure 2; Table S3). Case subjects infected with more than one of these seven different bacterial species (2.1% of case subjects) are included in the analysis for each group.

The parameters of interest are the genetic effect sizes ($b_k$) on a log-odds scale for each of the case cohorts. We first find maximum likelihood estimates (with the corresponding observed information matrix) by including two PCs as covariates in the model, and then compute approximate Bayes factors using a multivariate normal approximation to the likelihood and the prior. The models are defined by prior distributions on the parameters $b_k$:

- **NULL**: $b_k = 0$ for all $k = 1, \ldots , 7$, i.e., no effects, all case groups are like the control group.
- **SAME**: $b_k \sim N(0,1)$ and $	ext{cor}(b_i,b_j) = 1$ for all pairs $i \neq j$, i.e., each $b_k$ is the same.
- **REL**: $b_k \sim N(0,1)$ and $	ext{cor}(b_i,b_j) = 0.96$ for all pairs $i \neq j$, i.e., $b_i$ and $b_j$ are correlated but not necessarily the same.
Additional models are defined after inspection of the observed association at each locus for each pathogen. Bacterial species hypothesized to be associated with a given locus are assumed to have the same non-zero effect with a prior of N(0,1), whereas for other pathogens the effect is 0.

Quantification of lincRNA Expression in Primary Immune Cell Subsets

Previous reports suggest that AC011288.2 encodes a lincRNA and is expressed in white blood cells and placental tissue. To identify which leukocyte population this lincRNA is expressed in, we isolated monocytes, B cells, and natural killer (NK) cells from consenting healthy adult European-ancestry donors using magnetic activated cell sorting (MACS, Miltenyi), as previously described.\textsuperscript{30} In addition, we isolated granulocytes (predominantly neutrophils) using Polymorphoprep (Allere) according to the manufacturer's instructions from eight individuals. The purity of cell subsets after cell separation was assessed by flow cytometry and was >90\% in a representative sample. Viability after sorting was assessed by the Trypan Blue dye exclusion method and observed to be >95\% in all cases. Total RNA was extracted with the RNeasy mini kit (QIAGEN) or TRIzol (Life Technologies) according to the manufacturer's instruction (QIAGEN). Total RNA was quantified by Nanodrop and Bioanalyzer for a subset according to the manufacturers' instruction (Bioanalyzer RNA 6000 Nano kit, Agilent).

To quantify levels of lincRNA expression, we performed quantitative real-time PCR (qPCR) using a relative quantification method. Beta-Actin (ACTB) was selected as a reference gene based on previous reports of its stable expression in neutrophils. Single-strand complementary DNA was synthesized by reverse transcription with the SuperScriptIII First-Strand Synthesis System (Invitrogen). Primers specific to each of the two reported transcripts for the lincRNA AC011288.2 were designed: AC011288.2-001 (for, 5'-GTCAGAAGCGGGTTCAAAG-3'; rev, 5'-TTTAACTCTTGAGTTCGAGGC-3') and AC011288.2-002 (for, 5'-GATGCTTAAGCCCTGGAACCC-3'; rev, 5'-TTCAGCTTCTATTCGCAGAAGG-3'). In addition we designed primers to AC006000.5 (for, 5'-ATCACGAGAGTATCGGACTCTG-3'; rev, 5'-TGACAGAGTAGGAGAC CCTGTTG-3') but consistent with previous reports that observed no expression in leukocytes, we did not identify any individuals that expressed this transcript and do not describe it further. To avoid potential amplification of genomic DNA, primers were designed to span exons. qPCR was performed using SYBR Green Supermix (BioRad) on a CFX96 Real-Time PCR Detection System (Bio-Rad). Reactions were run in duplicate with 1 cycle at 95°C (10 min), followed by 42 cycles consisting of denaturation at 95°C (10 s), annealing at 58°C (20 s), and extension at 72°C (20 s). Detection of the fluorescent products was carried out at the end of the 72°C extension period. To confirm amplification specificity, the PCR products were subjected to a melting curve analysis and agarose gel electrophoresis. Detection of a fluorescent product after cycle 38 (Ct \textsuperscript{38}) was considered evidence of expression beneath the detectable level. On the Ct value was reassigned to 38, so as to conservatively estimate the highest level of expression beneath the detectable level. On average, the Ct value for AC0011288.2-001 was 33 cycles in neutrophils. Relative gene transcript levels were determined by the [\textit{DELTAsub}][\textit{C.sub.T}] method expressed relative to ACTB. Comparisons of log10 transformed relative expression levels were made using a non-parametric Mann-Whitney test in GraphPad Prism.

Results

Genome-wide Association Results of Directly Genotyped Discovery and Immunochip Replication Sets

We identified several suggestive associations in the directly genotyped discovery data both in pneumococcal bacteremia and bacteremia overall analyses in Kenyan children (Table S4), but none of the SNPs reached established criteria for identifying novel associations (p < 5 \times 10^{-8}) in a combined analysis after replication.

Genome-wide Association Results of Bacteremia Caused by \textit{Streptococcus pneumoniae} after Imputation and Replication

After genome-wide imputation and quality control, nearly 10 million autosomal SNPs were included in the association analyses of pneumococcal bacteremia (Figure S10). In this analysis of 429 case and 2,677 control subjects, 17 SNPs in a single region on chromosome 7 were associated with disease at a level exceeding genome-wide significance (p < 5 \times 10^{-8}), with the peak of that association observed at rs140817150 (p imputed = 7.25 \times 10^{-8}; OR = 2.74) (Figures S10 and 1). This novel associated region includes two overlapping long intergenic non-coding RNA (lincRNA) genes: AC006000.5 and AC011288.2. The association at rs140817150 was confirmed by direct genotyping (p discovery = 3.58 \times 10^{-7}; OR = 2.39) and replication (p = 1.16 \times 10^{-3}; OR = 2.72), resulting in a combined OR estimate of 2.47 (95\% CI 1.84–3.31) and p value of 1.69 \times 10^{-9} (see Table S5 for a list of all suggestive associations in the analysis of pneumococcal bacteremia and Table S6 for the comprehensive list of associated SNPs in the chromosome 7 top associated region). Direct genotyping of the top imputed SNPs confirmed that imputation was generally accurate (average concordance between imputed and directly genotyped genotypes was 98.3\%). In order to protect against spurious associations due to possible cryptic relatedness, the SNPs chosen for replication were also analyzed using the mixed model approach (rs140817150; p combined = 1.5 \times 10^{-10}; OR = 2.66; Table S7) and by stratifying individuals on the basis of genetic background across the main ethnic groups (rs140817150; p discovery = 1.5 \times 10^{-7}; OR = 2.54; Figure S11). By either approach, the evidence for association remained strong. The pneumococcal case subjects do not have more close relatives than the control subjects in any of the four ancestry groups (p \geq 0.22). When compared to other case subjects, they show elevated levels of relatedness (p < 0.05) in group 1 only, which does not contribute strongly to the observed association signal (Figure S11). After conditioning on the top SNP, no associations were detected with p < 10^{-4} in the region.
Genome-wide Association Results of Bacteremia Overall after Imputation and Replication

In addition to the analysis of a more homogeneous phenotype, pneumococcal bacteremia, all-cause bacteremia overall (Figure S12) was also analyzed. Table S8 summarizes loci with the strongest evidence of association in bacteremia overall after imputation, including the replication results. The only genome-wide significant $\left(p < 5 \times 10^{-8}\right)$ association signal that replicated was under the genotypic model, which allows independent effects on risk for homozygotes and heterozygotes. It revealed a strong association at the previously identified $HBB$ locus (rs113892119, $p = 5.08 \times 10^{-13}$). The region of association included the functional rs334 polymorphism $\left(p = 1.33 \times 10^{-10}\right)$ that leads to the production of hemoglobin S (HbS) $^\text{21}$ and is located 25.6 kb upstream from the most associating SNP rs113892119.

As previously described, rs334 was associated with susceptibility among homozygotes $^\text{22}$ (HbSS versus HbAA; directly genotyped combined $p = 2.66 \times 10^{-12}$, OR = 4.9) and with protection from bacteremia among heterozygotes $^\text{7}$ (HbAS versus HbAA; directly genotyped combined $p = 4.67 \times 10^{-3}$, OR = 0.77). These same effects are seen in the most common bacterial subgroups (Figure S13).

Bayesian Model Comparison Results of rs140817150 and rs334 Associations with Common Causes of Bacteremia in Kenyan Children

Although the association with rs140817150 was discovered in the pneumococcal bacteremia analysis, we were able to utilize the all-cause bacteremia data at this locus to assess its effect on susceptibility to bacteremia caused by other species (Figure 2). To assess whether the data were consistent with the same effect among case subjects with different species of bacteremic pathogen, we compared models via a Bayesian approach (Figure 2). Assuming all models to be equally likely a priori, the most probable model is the one in which the susceptibility is confined to pneumococcus, *Acinetobacter* species, and *Haemophilus influenzae*. Removing the pneumococcal group, from which the association was ascertained, weakened the evidence for effect heterogeneity. The same effect in all subtypes was found to be the most probable model for rs334 association (heterozygote risk and homozygote protection) in $HBB$ (Figure S13).

**lincRNA Expression in Primary Immune Cell Subsets**

We assessed AC011288.2 RNA expression in the major leukocyte cell subsets and observed expression only in neutrophils. Expression levels were below the detection limit in monocytes, B cells, and natural killer (NK) cells (Figure 3). To verify that expression of this transcript is constitutive in neutrophils, we measured expression in an additional 75 donors, recruited in a separate study $^\text{23}$ and observed detectable expression in all 75 donors. We did not observe AC00600.5 expression in any leukocyte subsets.

**Discussion**

We report here a GWAS of bacteremia susceptibility, which is one of the few large-scale GWASs conducted in an...
related effects across subtypes (REL), or the same non-zero effect only in PNEUM, ACINET, and HAEMOP (P+), or in PNEUM (P). (See Subjects and Methods.) Models are a priori assumed to be equally likely. Bayes factors, which compare the evidence (marginal likelihood) between any pair of models, can be calculated as the ratio of the posterior probability assigned to each model as reported under each bar of the plot.

African population to date. We identified an association between polymorphisms in two overlapping long intergenic non-coding RNA (lincRNA) genes (AC006000.5 and AC011288.2) and pneumococcal bacteremia, the most common cause of bacteremia in our study set. Although immune responses and genetic variants affecting bacteremia susceptibility are likely to be at least partially pathogen specific, we also analyzed the bacteremia overall dataset to identify more universal risk factors. The only genome-wide significant hit for bacteremia overall was in a previously reported gene, HBB, including the well-known rs334 polymorphism associated with the production of sickle hemoglobin.21

The lincRNA risk allele at rs140817150 is derived (as reported on dbSNP) rather than ancestral, its frequency is low (2.7% in control subjects, 6.4% in pneumococcal bacteremia case subjects), and according to the 1000 Genomes project data (phase 3), it is polymorphic only in African populations. Consistent with the local recombination landscape and with the expectation that low-frequency derived alleles are relatively young, SNPs in linkage disequilibrium with rs140817150 extend over 500 kb (Figure 1). However, Bayesian analysis of the region of association24 in the imputed data suggests there is greater than 95% probability that one of the most associated SNPs (circled in Figure 1) is the causal variant, assuming there is a single causal variant, and it is imputed accurately in our dataset.

The association peak is located in the introns of two separate long intergenic non-coding RNAs (lincRNAs), annotated as AC011288.2 and AC006000.5. The importance of lincRNAs as key regulators of gene expression has only recently been recognized.25–27 It has been estimated that the human genome includes at least 10,000 lincRNAs but only a fraction of these has a known function.25–27 A recent study aiming to catalog the function of more than 8,000 human lincRNAs reported that lincRNA expression is significantly more tissue specific than expression of protein-coding genes.28 AC006000.5 is listed in the catalog (Figure 2). The closest protein-coding gene, ARL4A and ETV1 but there is no evidence that the associating lincRNAs regulate these two genes. However, data from a previous expression quantitative trait locus (eQTL) study29 suggest that there are some SNPs in the associating region that function as eQTLs in monocytes (rs1432496) and B cells (rs2568633) for PHF14 (PHD finger protein 14), a transcription factor that downregulates PDGFRα expression.34 However, neither is correlated with our most-associated SNP (r² < 0.01 in 1000 Genomes data). Although the role of lincRNAs in human infections is unknown, recent mouse studies have indicated that some lincRNAs can act in immune cells to regulate host susceptibility to bacterial and viral infections.35,36

Using the GWAS approach, we have identified an association between a genetic variation in a lincRNA gene and pneumococcal bacteremia. Furthermore, we have confirmed a previously reported association between HBB and bacteremia overall,22 with homozygotes associated with strong susceptibility but heterozygotes associated

Figure 2. rs140817150 lincRNA Association with the Main Bacterial Infections
(A) Log transformed combined odds ratios and 95% confidence intervals of directly genotyped discovery and replication samples. The dotted line represents the log OR of 0 (OR of 1; no difference between case and control subjects). The values of point estimates and standard errors (in parentheses) are also given. Bacterial infection abbreviations are as follows: PNEUM, Streptococcus pneumoniae (pneumococcus); ACINET, Acinetobacter species; HAEMOPH, Haemophilus influenzae; ECOLI, Escherichia coli; SALMON, Salmonella (non-typhoidal); STREPBH, Streptococcus beta hemolytic; SAUR, Staphylococcus aureus.
(B) The posterior probabilities on the models of association: no effect in any subtype (NULL), same effect in all subtypes (SAME), of association between any pair of models, can be calculated as the ratio of the posterior probability assigned to each model as reported under each bar of the plot.
were obtained (data not shown). Inspection of the melting curves. Similar results for AC011288.2-001 (Ct squares denote detection of a fluorescent product after cycle 38 (B) Unadjusted cycle number of amplification is shown. Filled (Mann-Whitney test).

AC011288.2-001 in neutrophils compared to other cell types denote the significance of the relative expression levels of association in the current study (p > 0.05). Given the likely importance of host-pathogen molecular interactions in bacteremia susceptibility, it is plausible that the effect of a risk allele will be dependent on bacterial species. Our data on the lincRNA locus provide initial evidence for this at the bacterial species level, and motivate approaches that stratify host genetic associations by pathogen species, serotype, or genotype. Understanding the molecular mechanisms leading to the doubled risk of pneumococcal bacteremia associated with this allele could provide new clues in the pressing search for new therapeutic targets.

Accession Numbers

Genotype data will be made available by the WTCCC access process, via the European Genotype Archive under the accession number EGAS00001001756.

Supplemental Data

Supplemental Data include 13 figures and 8 tables and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2016.03.025.

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Web Resources

1000 Genomes, http://www.1000genomes.org
European Genome-phenome Archive (EGA), https://www.ebi.ac.uk/ega
OMIM, http://www.omim.org/

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