Host Genetic and Gut Microbial Signatures in Familial Inflammatory Bowel Disease

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INTRODUCTION: The family history of inflammatory bowel disease (IBD) has been strongly associated with risk of developing IBD. This study aimed to identify the host genetic and gut microbial signatures in familial IBD.

METHODS: Genetic analyses using genome-wide single nucleotide polymorphism genotyping and whole exome sequencing were performed to calculate weighted genetic risk scores from known IBD-associated common variants and to identify rare deleterious protein-altering variants specific to patients with familial IBD in 8 Korean families that each included more than 2 affected first-degree relatives (FDRs) and their unaffected FDR(s). In parallel, gut microbial community was analyzed by 16S rRNA sequencing of stools from the sample individuals.

RESULTS: The risk of familial IBD was not well explained by the genetic burden from common IBD-risk variants, suggesting the presence of family-shared genetic and environmental disease-risk factors. We identified 17 genes (AC113554.1, ACE, AKAP17A, AKAP9, ANK2, ASB16, ASIC3, DNPH1, DUS3L, FAM200A, FZD10, LAMA5, NUTM2F, PKN1, PRR26, WDR66, and ZC3H4) that each contained rare, potentially deleterious variants transmitted to the affected FDRs in multiple families. In addition, metagenomic analyses revealed significantly different diversity of gut microbiota and identified a number of differentially abundant taxa in affected FDRs, highlighting 22 novel familial disease-associated taxa with large abundance changes and the previously reported gut dysbiosis including low alpha diversity in IBD and 16 known IBD-specific taxa.

DISCUSSION: This study identified familial IBD-associated rare deleterious variants and gut microbial dysbiosis in familial IBD.
small fraction of IBD risk (11). The large proportion of the missing heritability of complex diseases such as IBD have been suspected to be hidden behind large-effect rare variants whose disease association significance is generally weak because of a low statistical power in GWAS using unrelated subjects (12).

Gut dysbiosis has also been well documented to be critically associated with the pathogenesis of IBD (13). As the gut microbial community is affected by many factors including family-shared factors such as diet and a complex gene-microbiota interaction (14–17), FDRs tend to show a higher degree of similarity in gut microbial composition (18). Thus, the comparison of gut microbiota between familial patients with IBD and their unaffected FDRs with similar gut microbial communities provides a unique opportunity to pinpoint the IBD-risk gut microbial signature in less statistical noise.

Taken together, previous observations have illustrated the potential advantage of using familial IBD in identifying disease-risk signatures regarding host genetic variants and gut microbiome, although clinical and biological data of patients with familial IBD and their unaffected FDRs are scarce. In our study, we recruited 8 families with ≥2 IBD-affected FDRs and ≥1 unaffected FDR, obtaining their genomic DNAs in whole blood, gut bacterial DNA in stool samples, and clinical metadata. Genomic analysis of both common and rare variants and metagenomic analysis of gut microbiota revealed the landscape of disease-specific signatures in familial IBD.

METHODS

Study cohort
Patients with both a definite diagnosis of IBD (CD or UC) and a family history of IBD were eligible for this study. Unaffected FDRs (parents, full siblings, or children) of the patients with familial IBD were recruited to participate in this study. All patients were affiliated with a prospective single-center IBD cohort at Kyung Hee University Medical Center, and they met the diagnostic criteria of CD or UC based on clinical and endoscopic findings using histopathology and radiologic results (19,20). We excluded patients with IBD-unclassified or IBD-indeterminate type.

We identified 23 families with ≥2 FDRs of patients among the IBD cohort (n = 960). After excluding 15 families because of refusal for consent by the family members, we finally recruited 8 families including 16 patients with IBD (including 10 CD and 6 UC patients) and 9 unaffected FDRs (Figure 1). The pedigree of the study cohort is illustrated in Figure 2. Twelve patients with IBD had concordant type (4 CD families and 2 UC families), and the remaining 4 patients had discordant type (2 CD/UC families). All study participants provided written informed consent for this work. The study was approved by the Institutional Review Board of the Kyung Hee University Medical Center.

All the study participants provided whole blood for genetic analysis, serum for serologic tests, and/or fresh stool for microbiome analysis. Within 12 weeks before stool sampling, all participants were strictly withheld from treatment with antibiotics or probiotics. We collected basic demographic features from all participants and the clinical information from the patients (Table 1). All the study patients were in remission (defined as the CD activity index <150 or the Mayo score <3) and did not use proton-pump inhibitors at the time of stool collection. Serum C-reactive proteins, perinuclear antineutrophil cytoplasmic antibodies, and anti-Saccharomyces cerevisiae antibodies were measured in patients with familial IBD. About 1 g of stool was collected using the Stool Nucleic Acid Collection and Preservation Tube (NORGEN Bio-Tek) and immediately stored at −80 °C until DNA extraction.

Genome-wide variant analysis and calculation of weighted genetic risk scores

Genome-wide data of single nucleotide polymorphisms (SNPs) were generated from patients’ genomic DNA using the Korea biobank array (Affymetrix Axiom KORV1.1) (21) to calculate individual-level genetic risk scores for IBD, CD, and/or UC. Raw genotype data (with 827,783 variants) was filtered by a general quality control (QC) procedure (see Table S1, Supplementary Digital Content 1, http://links.lww.com/CTG/A324), retaining 406,422 variants with a call rate ≥95%, minor allele frequency ≥1%, and a P value for Hardy-Weinberg equilibrium ≥1 × 10−7 in the 25 individuals. Whole genome imputation was then performed by the prephaser SHAPEIT2 (22,23) and the imputator Minimac3 (24) with the imputation reference panel of the 1,000 Genomes Project phase 3. The imputation of variants in non-pseudosautosomal region in chromosome X was conducted in the same pipeline for male and female samples separately. SNP-level imputation QC retained ~8 million variants with INFO values ≥0.4.

The weighted genetic risk scores (wGRSs) value of each individual was calculated as a linear summation of the number of disease-associated alleles weighted by the effect estimates reported in previous large GWAS of IBD, CD, or UC (see Table S2, Supplementary Digital Content 1, http://links.lww.com/CTG/A324) (8,9,25–35). The wGRS distribution in a general population was estimated using 100,000 random wGRS simulated based on the Korean allele frequencies.

Whole exome sequencing and variant calling

The exome library was constructed from blood genomic DNA in the study subjects using the SureSelect V6 target enrichment system and was sequenced on an Illumina platform with 101-bp paired-end reads. Read alignment was performed using the Bowtie2 program with the reference human genome (hg38) (36). All procedures for QC and variant calling followed the GATK4 best practice pipeline (37) with a joint calling option (38). Common variants in dbSNP146 (minor allele frequency ≥1%) were excluded from subsequent analysis to identify rare exonic variants that were shared between the affected individuals (39). Deleterious effect on protein function was predicted based on the SIFT and PolyPhen-2 algorithms using the variant effect predictor program (40,41).

Sequencing of gut microbiome

The V3 and V4 amplicon of the 16S rRNA gene in stool DNA and sequencing library for the Illumina paired-end sequencing platform were prepared using Herculase II Fusion DNA Polymerase and Nextera XT library prep kit v2, respectively. Reads with poor base call quality or short insert sizes (excluding Nextera adapter sequence) were removed by using a Trimmomatic (42). Sequencing fragments merged from QC-passed paired-end reads of the V3 and V4 regions were further filtered according to the expected length (438–469bp) (43) of the target region. Operational taxonomic unit (OTU) clustering was performed using the QIME2 program (44) with the open reference OTU picking method at 97% sequence similarity using the vsearch plug-in (45), excluding chimeric or rare OTUs. Taxonomic classification and phylogenic annotation of OTUs were carried out using reference sequences (gg_13.8_97.fasta) from Greengenes (46) with classify-sklearn and q2-phylogeny plugins in QIME2 (47). The quality-filtered reads (n = 2,208,488) were clustered into 1,895 OTUs at 97% sequence similarity.
Alpha diversity (richness) of the gut microbial community in each individual was quantified by the Shannon index, and the association with disease status was tested using a Kruskal-Wallis test. Beta diversity (interindividual dissimilarity) of gut microbial composition in the study population was estimated using a principal coordinate analysis with the weighted UniFrac algorithm. Statistical significance for the difference in measured beta diversity among phenotypic groups was assessed by a permutational multivariate analysis of variance with adjustment for family and sex (48). Differentially abundant gut microbiota between case and control groups were identified using DESeq2 (49) in various taxonomic levels, adjusting for family and sex.

RESULTS

 Characteristics of the study cohort

The basic characteristics of the study cohort are listed in Table 1. There were no significant differences in demographic factors that could affect gut microbial composition, including the Bristol stool form scale (50), alcohol/cigarette consumption history, and breastfeeding in infancy. The enrolled nuclear families with familial IBD tended to have more unaffected FDRs in the parental generation. At the time of stool and blood sampling, all patients with familial IBD were in clinical remission (Mayo score = 1.0 ± 1.0 in UC; CD activity index = 87.5 ± 77.9 in CD). The relatedness of family members was genetically confirmed using genome-wide SNP array data and whole exome sequencing data.

Known common disease variants did not well explain risk of familial IBD

We investigated whether the known common variants associated with risk of IBD, CD, or UC were enriched in patients with familial IBD by calculating individual-level wGRS based on reported effect estimates (see Table S2, Supplementary Digital Content 1, http://links.lww.com/CTG/A324). Statistical significance of the increase in average wGRS in 2 affected members of each family was assessed by positioning the observed average into the distribution of the 100,000 wGRS of unaffected unrelated individuals simulated based on the known risk allele frequencies in the Korean population.
A single family with familial CD showed a significantly higher burden of common CD-risk variants than the expected distribution of unaffected individuals at a false-discovery rate of 5% (P-value 5.7 × 10^{-2}, Figure 3). There was, however, no significant increase in wGRS in the other families, raising the possibility that family-specific rare variants with large effects may drive risk of disease.

**Whole exome sequencing identified rare exonic variants that were transmitted to patients with familial IBD**

We sequenced exonic regions to profile rare missense variants in patients and their unaffected FDRs, achieving an average coverages of ≥92.3% for the protein-coding regions at 10x per-base depth on all samples (see Table S3, Supplementary Digital Content 1, http://links.lww.com/CTG/A324). A series of filtration procedures narrowed down all detected variants (n = 154,319) to 28,184 by retaining rare, potentially deleterious protein-altering variants that were transmitted preferentially to patients in a recessive or dominant genetic model.

We identified 17 genes (AC113554.1, ACE, AKAP17A, AKAP9, ANK2, ASB16, ASIC3, DNP11, DUS3L, FAM200A, FZD10, LAMA5, NUTM2F, PKNL, PRR26, WDR66, and ZC3H4) that each contained rare damaging missense variant(s) in at least 2 families. All the identified variants followed dominant inheritance. Damaging effects of the observed variants on the gene products were predicted in silico and described in Table 2. For example, the missense variant in LAMA5 resulting in a change from valine to methionine at amino acid position 3,111 (transcript ENST00000252999) in 2 independent families was...
one of the most deleterious variants according to the prediction methods (predicted as deleterious in SIFT and as probably damaging in PolyPhen-2). Several genes with multiple rare damaging variants were discovered in a disease subtype analysis (AC1135541, ACE, AKAP9, ANK2, ASIC3, DUS3L, NUTM2F, and ZCHH4) in CD; none in UC; Table S4, Supplementary Digital Content 1, http://links.lww.com/CTG/A324). Most of the listed genes excluding LAMA5 and ACE have been examined in few or no studies regarding their relationship with IBD or even their own function. However, interestingly, the gene expression levels of most genes were detectable (or relatively high) in disease-relevant tissues such as colon or small intestine and immune tissue (see Table S5, Supplementary Digital Content 1, http://links.lww.com/CTG/A324) (51).

Signatures of gut microbial profiles

Gut microbial community in all the subjects was profiled by 16S rRNA sequencing. We observed that the study patients in remission mostly had formed stool at the time of fecal sample collection. The microbial richness estimated by the Shannon diversity index showed a significantly lower alpha diversity in the patients than the unaffected FDRs ($P$ value $= 0.022$), highlighting the poor microbial richness in familial CD ($P$ value $= 0.0062$), but not UC ($P$ value $= 0.23$) compared with the unaffected FDRs (Figure 4a). Similarly, the beta diversity analysis using the weighted UniFrac algorithm did not reveal a significant difference in gut microbial composition between familial CD and controls ($P$ value $> 0.05$; Figure 4b).

Bacterial abundance was normalized for the sequencing library size and composition across the individuals to investigate the differential abundance of bacterial taxa in gut in patients with familial IBD. A total of 22 taxa at various taxonomic levels showed significant differences in relative abundance between the patients with familial IBD and the unaffected FDRs at a false discovery rate threshold of 5% (Figure 5 and see Table S6, Supplementary Digital Content 1, http://links.lww.com/CTG/A324). For example, familial IBD patients had significantly higher levels of the class Deltaproteobacteria, the order Desulfovibrionales, and the family Desulfovibrionaceae. Of the 22 detected differentially abundant taxa, 11 have been reported for their IBD-specific abundance in previous studies (17,52–56). The previously reported directions of bacterial abundance in patients with IBD were consistent with those in our analysis, except for 2 taxa with relatively weak fold changes in abundance. In addition, there were the other 11 familial IBD-specific taxa such as an unclassified species of Bacteroides and B. uniformis with >10-fold decreased abundance in patients with familial IBD.

We also compared the gut microbial profiles between IBD subtypes and unaffected FDRs. We identified 16 familial CD- and UC-specific taxa, 5 of which were reported to be deficient and abundant in patients in other studies (17,52–54,56), consistent with our analysis results. The other 11 unreported taxa were considered novel disease markers under a false discovery rate of 5% (Figure 5, see Tables S7 and S8, Supplementary Digital Content 1, http://links.lww.com/CTG/A324 and http://links.lww.com/CTG/A324).

DISCUSSION

This study first investigated both the host genetic and gut microbial signatures in familial IBD through an intensive analysis of common and rare variants in host genomic DNA and gut microbial bacteria composition in patients and unaffected FDRs. Our study yielded 3 important observations. First, known common disease-risk variants were not enriched in patients with familial IBD. We found a little evidence of the inflated genetic burden of IBD in patients with familial IBD because we detected a single trio (out of 8 families) in which 2 patients with CD had significantly higher wGRS based on GWAS-identified variants than expected. Similarly, a previous large-scale study that compared wGRS in patients with familial and sporadic IBD showed a statistically significant but very slight increase in wGRS in familial IBD (57). These 2 consistent results on the weak association of wGRS and familial IBD strongly indicate that the surprisingly high risk of IBD in individuals with a family history of IBD could not be simply explained by a genetic burden of common IBD-risk variants with small effect sizes, which may suggest the presence of
Table 2. Genes with rare deleterious alleles that were detected in multiple families with familial IBD and shared among affected FDRs only

<table>
<thead>
<tr>
<th>Gene</th>
<th>No. of families with any variant</th>
<th>Variant name</th>
<th>Variation location (hg38)</th>
<th>Allele frequency</th>
<th>Most affected transcript</th>
<th>Amino acid position</th>
<th>Amino acids</th>
<th>SIFT</th>
<th>PolyPhen-2</th>
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rare family-private variants with large effect sizes and/or family-shared environmental factors.

Second, we detected gene-wise recurrent family-specific missense variants that appeared among patients with familial IBD in 7 of 8 families. Those variants may explain a large portion of IBD risk, although these findings need to be replicated in independent families or validated in experimental approaches to link the disease-associated genes/variants to IBD. Interestingly, the expression

The effect of the amino acid substitution on protein function was predicted using SIFT and PolyPhen-2. In Polyphen-2, the predicted damaging impact increases in the order of “benign,” “possibly damaging,” and “probably damaging.” The population-level frequencies of potentially deleterious alleles were retrieved from the data in East Asians or all individuals of the 1,000 Genomes projects phase 3. (Higher frequencies were written.)

FDR, first-degree relative; IBD, inflammatory bowel disease.

Table 2. (continued)

<table>
<thead>
<tr>
<th>Gene</th>
<th>No. of families with any variant</th>
<th>Variant name</th>
<th>Variation location (hg38)</th>
<th>Allele (frequency)</th>
<th>Most affected transcript</th>
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<td>ENST00000601973</td>
<td>734</td>
<td>A/V</td>
<td>Deleterious</td>
<td>Probably damaging</td>
</tr>
</tbody>
</table>

The population-level frequencies of potentially deleterious alleles were retrieved from the data in East Asians or all individuals of the 1,000 Genomes projects phase 3. (Higher frequencies were written.)

FDR, first-degree relative; IBD, inflammatory bowel disease.

Figure 4. Taxonomic alpha and beta diversities in the gut microbial community in families with familial inflammatory bowel disease. (a) the alpha-diversity of gut microbial composition in each study subject was estimated based on Shannon index and box-plotted according the individual phenotypes-unaffected FDRs (denoted as unaffected) and patients with ulcerative colitis and Crohn disease. There was a significant difference in alpha diversity between inflammatory bowel disease patients and unaffected FDRs (P value = 0.022; Kruskal-Wallis test). (b) the beta-diversity of bacterial composition between individuals was measured using a principal coordinate analysis (PCoA) with the weighted UniFrac algorithm and plotted based on PCoA axis 1 and 2 that, respectively, explained 39.2% and 27.2% of the variance in bacterial community of the study subjects. The significant differences in bacterial community was not observed between the patients and unaffected FDRs (P value > 0.05; PERMANOVA). CD, Crohn disease; FDR, first-degree relative; PERMANOVA, permutational multivariate analysis of variance; UC, ulcerative colitis.
levels of the identified genes with deleterious missense variants were detectable or even relatively high in gastrointestinal tissues or immune cells, suggesting that they play pathological roles in disease-relevant tissues. For example, LAMA5, one of the identified genes, encodes a subunit of the extracellular matrix protein called laminin that is a key component in maintaining integrity of the intestinal basement membrane layer. A recent GWAS suggested the genetic association of common variants within the LAMA5 gene with pediatric IBD (58). Another identified gene ACE is highly expressed in small intestine (see Tables S5, Supplementary Digital Content 1, http://links.lww.com/CTG/A324), especially in patients with IBD (59). Recent studies described the potentially therapeutic effect of ACE inhibitors on IBD (60,61).

Third, we found distinct dysbiosis of gut microbiota at various taxonomic levels and reduced alpha diversity in the patients with familial IBD, compared with their unaffected FDRs. Our analysis confirmed several previously known IBD-specific taxa and the lowered alpha diversity in patients, indicating that there is some confidence in our samples and analysis. In addition, we discovered a large number of familial IBD-specific taxa that have not yet been suggested in sporadic patients with IBD. The newly identified taxa had large differences in abundance between patients and controls, which may shed light on the missing liability of familial IBD and may facilitate study on the interplay between their metabolites and host factors that requires much larger sample sizes and experimental validation.

All the affected participants with familial IBD in the study were registered in a single prospective IBD cohort characterized in Kyung Hee Medical Center, which helped to prepare the well-characterized clinical information and samples, eliminating potential recall and selection bias. However, although the collection of both genetic and gut microbial data from familial IBD families were highly challenging, we note that the major limitations in our study are the lack of a replication study using an independent cohort of familial IBD and the potentially low statistical power because of the relatively small sample size that might cause a large proportion of true disease signatures to be missing.

In summary, this study identified familial IBD-associated rare deleterious variants and gut microbial dysbiosis in Korean families with familial IBD, highlighting the presence of known and novel, large-effect factors and suggesting a complex interplay between host and gut microbiota involved in the immune response in familial IBD. The present results may have important clinical implications on prediction of IBD risk in asymptomatic relatives of patients with familial IBD. The unaffected relatives with family-specific deleterious genetic variants and/or gut
dysbiosis may require closer follow-up with longitudinal profiling of gut microbiota for early diagnosis of IBD.

CONFLICTS OF INTEREST
Guarantor of the article: Hyo Jong Kim, MD, PhD, and Kwangwoo Kim, PhD.


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Potential competing interests: None to report.

Ethics approval and consent to participate: All study participants provided written informed consent for this work. The study was approved by the Institutional Review Board of Kyung Hee University Hospital (KHUH-2018-03-009).

Study Highlights

WHAT IS KNOWN
✓ The family history of IBD is one of the most powerful predictors of developing IBD.
✓ Both genetic variants and gut dysbiosis confer the susceptibility to IBD.

WHAT IS NEW HERE
✓ Genomic analyses identifies several genes that each has rare, deleterious patient-specific variants in multiple families with familial IBD, but the high genetic load of common IBD-risk variants is not found in patients with familial IBD.
✓ The significant differences in gut microbial composition between patients with familial IBD and their unaffected first-degree relatives suggest the potential roles of large-effect novel and known IBD-risk taxa in the development of familial IBD.

TRANSLATIONAL IMPACT
✓ This study provides a catalogue of familial IBD-specific biomarkers including human genes with rare, deleterious variants, and gut microbial taxa with differential abundance in patients with familial IBD, which can be used to predict risk of familial IBD.
✓ This study will contribute to revealing the pathological mechanism by which the newly identified biomarkers increase a risk for developing IBD and presenting clinical symptoms.

REFERENCES

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