Challenges in Rare Variant Association Studies for Complex Kidney Traits: *CFHR5* and IgA Nephropathy

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With the first successful genome–wide application of linkage disequilibrium mapping in 2005,[1] genome–wide association studies (GWASs) have now surpassed their 10th anniversary. GWASs have been applied extensively to dissect contributions of common variants to complex disease, and thousands of robust disease associations have been identified using this approach.[2] Important discoveries have also been made in nephrology, where GWASs provided new insights into the regulation of BP,[3] renal function,[4] and albuminuria.[5] In addition, several landmark studies have shown strong contribution of common variants to the risk of glomerular disease, providing novel clues about human biology of these disorders. For example, the discovery of African *APOL1* risk alleles explained a large fraction of racial disparities in kidney disease and pointed to a completely new disease mechanism for FSGS.[6] GWAS findings for IgA nephropathy (IgAN) established the pathogenic role of the intestinal network for IgA production and the alternative complement pathway.[7–9] These findings led to a significant refinement of the disease pathogenesis model and provided novel clues about the disease geoepidemiology.[10–12] Similarly, the genetic interaction between variants in *PLA2R1* and *HLA* arising from GWAS solidified the pathogenesis model for membranous nephropathy, highlighting the antigen-HLA interplay as central to the disease process.[13]

Despite this progress, however, a large portion of the genetic contribution to many complex traits remains unexplained, including traits for which very large GWAS meta–analyses have already been performed. This issue has been identified as the missing heritability problem. The missing heritability has many potential explanations, including the possibility that low-frequency variants substantially contribute to the inherited risk of disease. Such rare alleles are not well captured on popular microarray genotyping platforms and thus, have been largely ignored by traditional GWASs. However, rare alleles can be accurately detected by direct DNA sequencing. Rapid progress in sequencing technology now enables investigations of the role of rare genetic variants in complex traits through sequence–based association studies. Although the jury is still out on whether such studies can account for the missing heritability, this design has already been deployed for several complex disorders, and we can certainly expect its broader application to kidney traits in the near future.

Sequence–based association studies present substantial challenges that relate to the detection, analysis, and interpretation of rare variants. Unless sample sizes or variant effect sizes are very large, these studies are usually limited by low statistical power. Moreover, the requisite multiple test corrections are poorly understood, creating difficulties in the interpretation of findings. In GWASs, the standard approach involves a single-variant test with the significance threshold of 5×10^{-8} that accounts for the estimated number of common haplotype blocks in the genome.[14] Given sufficient sample size, a similar approach can also be applied to rare variants, but its power is inversely related to allelic frequency. For example, to achieve 80% power to detect a disease association for a rare variant with a frequency of 1:1000 (0.1%) and an odds ratio of 2.0, one would require >60,000 patients (and an equal number of controls) to detect a statistically significant association for a disease with a population prevalence of 5%. In addition, single-variant tests on the basis of standard regression methods might not be accurate if the number of subjects with the variant is very small. Numerous alternative strategies have, therefore, been proposed to circumvent these issues. Most strategies aggregate variants over genomic regions or genes and evaluate their cumulative effects, increasing power when multiple variants in the group are associated with a trait.

The burden tests represent the simplest class of aggregation tests; they collapse information for multiple variants into a single genetic score, which is then regressed against the disease status. The genetic score captures genotype information by counting the number of minor alleles across all variants in a gene or region. These scores can also be weighted by allelic frequency (to up weight rare variants) or on the basis of functional predictions (to up weight potentially deleterious variants). One intuitive way to consider the significance for gene–based burden tests is by the application of a simple Bonferroni correction for the number of independent tests. In this case, the number of independent tests corresponds to the number of genes in the human genome; assuming 20,000

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genes, $P<0.05/20{,}000$ or 2.5×10^{-6} can be considered as statistically significant. Therefore, collapsing rare variants over genes provides a clear power advantage, because one pays a smaller penalty for multiple tests compared with a single-variant analysis.

The burden tests perform reasonably well if a region of interest harbors a large fraction of causal variants with the same direction of effect. However, the tests lose power if both risk and protective variants coexist in a region or when the majority of variants have no effect. To handle these more complex situations, a number of alternatives have been developed, including adaptive burden tests and variance component tests (refer to the work by Lee *et al.*[15] for a recent review). One of the most popular tests in this category is the sequence kernel association test (SKAT), which uses a weighted sum of squares of single–variant score statistics, thus accommodating variants with opposed directions of association.[16]

Given the complexities of rare variant tests, independent replication of new associations remains one of the most critical aspects of a good study design. However, the best practices for proper validation of rare variant associations are not as well developed as for traditional GWAS. Effective strategies for replication depend on the characteristics of the discovered variants, including their frequencies and effect sizes, and may involve targeted genotyping of selected variants or preferably, resequencing studies of genes or regions of interest. One must also bear in mind that successful replication of rare variant associations is not equivalent to showing causality, and experimental validation is usually needed to confirm the biologic relevance of an implicated gene.

The study by Zhai *et al.*[17] in this issue of the *Journal of the American Society of Nephrology* illustrates some of the challenges in the execution and interpretation of sequence–based rare variant associations. The study is motivated by the initial GWAS findings for IgAN, which established a disease association within the *complement factor H* (*CFH*) locus on chromosome 1q32 encompassing the *CFH* gene and five *CFH*-related genes (*complement factor H–related protein 1* [*CFHR1*] through *CFHR5*).[7] Subsequently, the GWAS signal at this locus has been replicated across diverse cohorts of different ethnicities[8,9] and recently, fine mapped to a single most likely causal variant—a common combined deletion of *CFHR1* and *CFHR3* genes that is protective against IgAN.[18]

In parallel, rare internal duplication within *CFHR5* has been shown to cause a familial form of C3GN.[19] This disease, also known as CFHR5 nephropathy, is endemic to Cyprus, where the founder mutation likely arose in a common ancestor approximately 16 generations ago. The disease shares some clinical features of IgAN, including microscopic hematuria and synpharyngitic flares with characteristic episodes of macrohematuria. Similar to IgAN, the risk of progressive renal impairment is more common in men, and the disease has been reported to recur after kidney transplantation.[20] CFHR5 is a universal component of complement deposits *in vivo*, suggesting its function in complement regulation.[21] Indeed, recent studies show that CFHR5, CFHR2, and CFHR1 share a dimerization motif that enables the formation of homo- and heterodimers enhancing the avidity of these proteins for C3b, allowing them to function as competitive antagonists of factor H.[22]

Given these findings, Zhai *et al.*[17] hypothesized that rare genetic variation in *CFHR5* may also be contributing to the risk of IgAN. By applying SKAT to *CFHR5* sequence data from 500 patients with IgAN and 576 controls, Zhai *et al.*[17] observed a difference in rare variant distributions with $P=2\times10^{-3}$. Careful analysis of the 28 detected rare variants identified nine as potentially functional, including three variants altering protein length and three variants increasing C3b binding to recombinant CFHR5 by *in vitro* assays. On the basis of these promising data, Zhai *et al.*[17] conclude that these rare variants contribute to the genetic risk of IgAN and suggest *CFHR5* as a new "IgAN susceptibility gene."

Considering multiple challenges with the design and interpretation of rare variant association studies, one must critically assess the provided evidence in support of this claim. Certainly, *CFHR5* represents an excellent candidate gene for IgAN on the basis of its established involvement in C3GN. However, the SKAT *P* value for rare variants in *CFHR5* is only suggestive, falling short of the conservative genome–wide significance for gene-based tests. Moreover, population stratification may still be confounding this association, because rare variant tests are particularly sensitive to even subtle ancestry differences between patients and controls. Finally, it is not yet clear if the detected differences in the distribution of rare variants are truly independent or merely shadowing the effects of the nearby *CFHR3* and *CFHR1* deletion. Additional studies that combine sequencing and copy number variant typing to jointly analyze the full spectrum of genetic variation across this region will be needed to establish the precise haplotype relationships between protective and risk alleles. In this regard, important lessons can be learned from the recent studies of age–related macular degeneration, where carefully conducted conditional and haplotype analyses of the *CFH* locus defined several independent common and rare alleles with opposing effects on the disease risk.[23,24]

In summary, although *CFHR5* represents a biologically plausible candidate, more evidence is still needed before unequivocally declaring *CFHR5* as a new IgAN susceptibility gene. As noted by Zhai *et al.*,[17] independent replication is needed to confirm that these findings are not caused by chance or an artifact because of uncontrolled biases. Moreover, validation studies in non-Asian cohorts would also help to solidify the evidence and provide more information on the generalizability of these intriguing findings to other populations.

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DISCLOSURES

None.

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