Fine Mapping Implicates a Deletion of *CFHR1* and *CFHR3* in Protection from IgA Nephropathy in Han Chinese

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ABSTRACT

An intronic variant at the *complement factor H* (*CFH*) gene on chromosome 1q32 (rs6677604) associates with risk of IgA nephropathy (IgAN), but the association signal has not been uniformly replicated in Han Chinese populations. We investigated whether the causal sequence variant resides in the *CFH* gene or the neighboring *complement factor H–related 1* (*CFHR1*) gene and *CFHR3*, which harbor an 84-kb combined deletion (*CFHR3,1Δ*) in linkage disequilibrium with rs6677604. Imputation of 1000 Genomes Project data did not suggest new causal single–nucleotide variants within the *CFH* cluster. We next performed copy number analysis across the *CFH* locus in two independent Han Chinese case-control cohorts (combined *n*=3581). The *CFHR3,1Δ* and rs6677604-A alleles were rare (4.4% in patients and 7.1% in controls) and in strong linkage disequilibrium with each other (r^2=0.95); of these alleles, *CFHR3,1Δ* associated more significantly with decreased risk of IgAN (odds ratio [OR], 0.56; 95% confidence interval [95% CI], 0.46 to 0.70; $P=8.5 \times 10^{-8}$ versus OR, 0.61; 95% CI, 0.50 to 0.75; $P=1.6 \times 10^{-6}$ for rs6677604-A). Moreover, *CFHR3,1Δ* explained all of the association signal at rs6677604 and remained significant after conditioning on rs6677604 genotype (*P*=0.01). Exploratory analyses of clinical and histopathologic parameters using the Oxford classification criteria revealed a suggestive association of *CFHR3,1Δ* with reduced tubulointerstitial injury (OR, 0.46; 95% CI, 0.25 to 0.79). These data indicate that dysregulated activity of the alternative complement pathway contributes to IgAN pathogenesis in both Asians and Europeans and implicate *CFHR3,1Δ* as the functional allele at this locus.

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IgA nephropathy (IgAN), a common form of GN, is characterized by mesangial proliferation and mesangial IgA deposits. In two recent genome–wide association studies (GWASs),[1,2] we detected a major susceptibility locus for IgAN within the *complement factor H* (*CFH*) gene cluster on chromosome 1q32. The top single–nucleotide polymorphism (SNP) in this region, rs6677604, is located in intron 12 of *CFH* and protective in IgAN. However, the causal allele driving the association signal at the *CFH* locus has not been identified. A common 84-kb deletion of the *complement factor H–related 3* (*CFHR3*) and *CFHR1* genes (*CFHR3,1Δ*), which is in linkage disequilibrium (LD) with rs6677604, has been considered as the likely causal allele,[1–4] but this has not been formally tested by direct genotyping of *CFHR3,1Δ* in large case-control cohorts. Moreover, several rare copy number variants (CNVs), such as

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solitary deletions of *CFHR1* and *CFHR3* (*CFHR1Δ* and *CFHR3Δ*, respectively), have been detected in this region, but their functional role remains uncertain.[5–7]

The detection of *CFHR3,1Δ* and other CNVs is complicated by the extensive sequence homology across the *CFH* gene cluster on chr1q32 (called the Regulator of Complement Activation [RCA] locus), and these CNVs are, therefore, not discernible with standard DNA microarrays.[8,9] Consequently, we performed multiplex ligation–dependent probe amplification (MLPA) analysis and quantitative PCR genotyping specifically targeting the *CFH* gene cluster to comprehensively evaluate CNVs in two large independent Han Chinese case-control cohorts (combined n=3581). The goals of this study were threefold: (*1*) to directly genotype *CFHR3,1Δ* in Han Chinese patients with IgAN and healthy controls and formally test its association with IgAN, (*2*) to determine whether *CFHR3,1Δ* and/or other rare CNVs explain the association between rs6677604 and IgAN at the *CFH* locus, and (*3*) to determine if *CFHR3,1Δ* is associated with clinical disease parameters and Oxford biopsy scores.

RESULTS

To find causal variants driving the GWAS association signal at the *CFH* locus (rs6677604), we first searched for novel coding single–nucleotide variants (SNVs) in LD with rs6677604 in the 1000 Genomes Project data but did not identify any SNVs that could potentially account for the association. To detect potentially causal noncoding variants, we next imputed the *CFH* gene cluster of the Beijing cohort (which was genotyped genome wide[1]) using the 1000 Genomes Project reference data and performed systematic conditional analyses to detect novel, previously untyped SNVs that may explain the rs6677604 association. We detected multiple loci with significant nominal association, but conditional analysis confirmed that rs6677604 is the top SNV signal within this interval (P=1.4 × 10^{-5}). Consultation of expression quantitative trait loci in the Genotype-Tissue Expression (GTEx) Project[10] confirmed that rs6677604-A is associated with significantly lower expression of *CFHR1* and *CFHR3* but not *CFH* or any other neighboring genes (Supplemental Figure 1, Supplemental Table 1).

The above data motivated a systematic search for CNVs that are in LD with rs6677604 as potentially causal variants within the RCA locus. Therefore, we performed MLPA–based CNV analysis across the RCA interval in a total of 1929 patients with biopsy-proven IgAN and 1652 healthy controls across two independent Han Chinese cohorts (Supplemental Table 2). We detected multiple rare CNVs across this region. The most frequent CNV was the known 84-kb deletion of both *CFHR3* and *CFHR1* (*CFHR3,1Δ*), with a frequency of 7.0% in the Han Chinese controls, which is considerably lower compared with allelic frequencies observed in Europeans (22.5%–27.5%).[7,11] Consistent with prior reports,[5,6] we also detected additional rare CNVs involving single-gene deletion of *CFHR1* or *CFHR3* (frequencies of 1.9% and 0.7%, respectively) (Supplemental Figure 2, Table 1). In accordance with prior studies in Europeans,[5] the rs6677604-A allele nearly perfectly tagged *CFHR3,1Δ* in our Asian cohorts (r^2=0.95; D'=0.98) (Supplemental Table 3). Only a few individuals with the rs6677604-G allele harbored *CFHR3,1Δ*, and none carried single-gene deletions.

CFHR3,1Δ Is Strongly Protective against IgAN in Two Han Chinese Cohorts

In the Beijing cohort (1194 patients and 902 controls), *CFHR3,1Δ* was strongly protective against IgAN (odds ratio [OR], 0.51; 95% CI, 0.38 to 0.67; P=2.3 × 10^{-6}), and this effect was stronger compared with its tag SNP, rs6677604 (OR, 0.55; 95%CI, 0.42 to 0.72; P=1.4 × 10^{-5}) (Table 1). Because the Beijing cohort was previously genotyped at a genome-wide level, we phased multi-SNP haplotypes containing *CFHR3Δ* and *CFHR1Δ* and identified 10 distinct haplotypes across this interval. We next performed haplotype–based association tests (Supplemental Table 4A) using the H1 haplotype as reference, because it has equal frequency in the patients with IgAN and controls (7.4%). We identified two distinct haplotypes that carried *CFHR3,1Δ*: the common haplotype H2 (overall frequency =5.0%; OR, 0.54; 95% confidence interval [95% CI], 0.38 to 0.77) and the rare haplotype H3 (overall frequency =0.3%; OR, 0.14; 95% CI, 0.02 to 1.25). The combined protective OR for these two haplotypes was 0.52 (95% CI, 0.36 to 0.74). Choosing a different reference haplotype (H8) did not alter the results of this analysis (Supplemental Table 4B).

The analysis of the CNV types in the Shanghai cohort (735 patients and 750 controls) confirmed the protective effect of *CFHR3,1Δ* (OR, 0.65; 95%CI, 0.48 to 0.90; P=8.3 × 10^{-3}), which was also stronger compared with rs6677604 (OR, 0.71; 95% CI, 0.52 to 0.96; P=0.02) (Table 1). In the combined Beijing and Shanghai cohorts, *CFHR3,1Δ* achieved near genome–wide significance (OR, 0.56; 95% CI, 0.46 to 0.70; P=8.5 × 10^{-8}) (Table 1). This effect was significantly stronger compared with the pooled effect of the tag SNP, rs6677604 (OR, 0.61; 95% CI, 0.50 to 0.75; P=1.6 × 10^{-6}). None of the other rare CNVs were associated with IgAN susceptibility, although our power was limited by the rarity of these alleles. Meta-analysis of the two cohorts also showed that the *CFHR3,1Δ* association is nearly an order of magnitude more significant than the rs6677604 association (Supplemental Table 5A).

CFHR3, 1Δ Accounts for the GWAS Association Signal at the RCA Locus

To determine if the GWAS signal at rs6677604 can be fully explained by *CFHR3,1Δ*, we performed conditional association analyses (Figure 1B, Table 2). When we conditioned the association of rs6677604 on *CFHR3,1Δ*, the protective effect of rs6677604 was no longer discernible (conditioned OR, 1.25; 95% CI, 0.69 to 2.27; P=0.46). Conversely, the estimated effect of *CFHR3,1Δ* remained statistically significant after controlling for rs6677604 (conditioned OR, 0.45; 95% CI, 0.25 to 0.84; P=0.01). Meta-analysis of the two cohorts also showed

Table 1. Allelic associations for rs6677604 and different CNVs within *CFHR3* and *CFHR1*

Variants	Beijing Cohort, *n*=2067, 1185 Patients/882 Controls			Shanghai Cohort, *n*=1472, 722 Patients/750 Controls			Cohorts Combined, *n*=3539, 1907 Patients/1632 Controls		
	MAF Patients/Controls	OR (95% CI)	*P* Value	MAF Patients/Controls	OR (95% CI)	*P* Value	MAF Patients/Controls	OR (95% CI)	*P* Value
rs6677604 (A)	0.040/0.072	0.55 (0.42 to 0.72)	1.4×10^{-5}	0.051/0.070	0.71 (0.52 to 0.96)	0.02	0.044/0.071	0.61 (0.50 to 0.75)	1.6×10^{-6}
CFHR3-CFHR1									
Del-Del	0.037/0.071	0.51 (0.38 to 0.67)	2.3×10^{-6}	0.046/0.069	0.65 (0.48 to 0.90)	8.3×10^{-3}	0.040/0.070	0.56 (0.46 to 0.70)	8.5×10^{-8}
Wt-Del	0.0076/0.0085	0.89 (0.45 to 1.78)	0.74 (NS)	0.012/0.011	1.10 (0.56 to 2.16)	0.78 (NS)	0.0094/0.0098	0.96 (0.59 to 1.56)	0.87 (NS)
Del-Wt	0.0025/0.0034	0.74 (0.24 to 2.31)	0.61 (NS)	0.006/0.004	1.57 (0.55 to 4.42)	0.40 (NS)	0.0039/0.0037	1.07 (0.50 to 2.29)	0.86 (NS)
Wt-Dup	0.0042/0.0034	1.24 (0.45 to 3.43)	0.68 (NS)	0.003/0.005	0.59 (0.17 to 2.03)	0.40 (NS)	0.0037/0.0040	0.92 (0.43 to 1.97)	0.83 (NS)

MAF, minor allele frequency; Del, deletion; Wt, wild type; Dup, duplication.

similar results (Supplemental Table 5B). These findings indicate that the protective effect of *CFHR3,1Δ* fully explains the observed effect of rs6677604 on IgAN.

Suggestive Association of *CFHR3,1Δ* with Reduced Glomerulosclerosis and Tubulointerstitial Injury by Oxford MEST Scoring

We next performed exploratory analyses of the effect of *CFHR3,1Δ* on clinical parameters and kidney histopathology as defined by the most recent Oxford classification system (Table 3). In total, 604 patients had biopsy slides available for Oxford scoring. In this dataset, carriers of *CFHR3,1Δ* had nominal associations for lower occurrence of glomerular segmental adhesions and sclerosis (*S*-score OR, 0.59; 95% CI, 0.36 to 0.98; P=0.04) as well as interstitial fibrosis and tubular atrophy (*T*-score OR, 0.46; 95% CI, 0.25 to 0.79; $P=6.9 \times 10^{-3}$). These associations were again more significant compared with rs6677604 (Supplemental Table 6). *CFHR3,1Δ* was not associated with any other clinical parameters, including eGFR, systolic BP, diastolic BP, hyperuricemia, gross hematuria, or degree of proteinuria (Supplemental Table 7). The association with the *T* score remains suggestive after correction for 12 independent tests (Bonferroni-corrected P=0.08).

DISCUSSION

The complement system plays a critical role at the interface of hemostasis and immunity. In this study, we performed fine mapping of the *CFH* gene cluster, which had been detected at genome-wide significance in three multiethnic genetic studies of IgAN.[1–3] In those studies, the top signal rs6677604 was highly significant in European populations but was not uniformly significant in Asian cohorts.[1–3] In addition, the evidence of association for rs6677604 was very modest in the most recent GWAS involving >8000 Chinese patients with IgAN (meta–analytic P value =0.001).[12] Here, we show that the combined deletion of *CFHR1* and *CFHR3* explains the rs6677604 signal at the *CFH* and locus, indicating that this deletion is the likely functional allele in this interval. Furthermore, by directly genotyping this allele, we achieved near genome–wide significance in two modestly sized Han Chinese cohorts. These findings are further supported by the GTEx Project data showing that rs6677604-A is associated with significantly lower expression of *CFHR1* and *CFHR3* across multiple tissues, including the liver, which is a major source of complement production (Supplemental Figure 1, Supplemental Table 1). Altogether, these data indicate that the *CFHR3,1Δ* association was poorly detectable in East Asians–only GWASs, because this allele is rare in this population, and the functional allele was not directly genotyped.[12] Our findings identify the functional variant at this interval, and by confirming the association in Han Chinese cohorts, we also show that dysregulated activity of the alternative complement pathway contributes to disease pathogenesis in both Asians and Europeans.

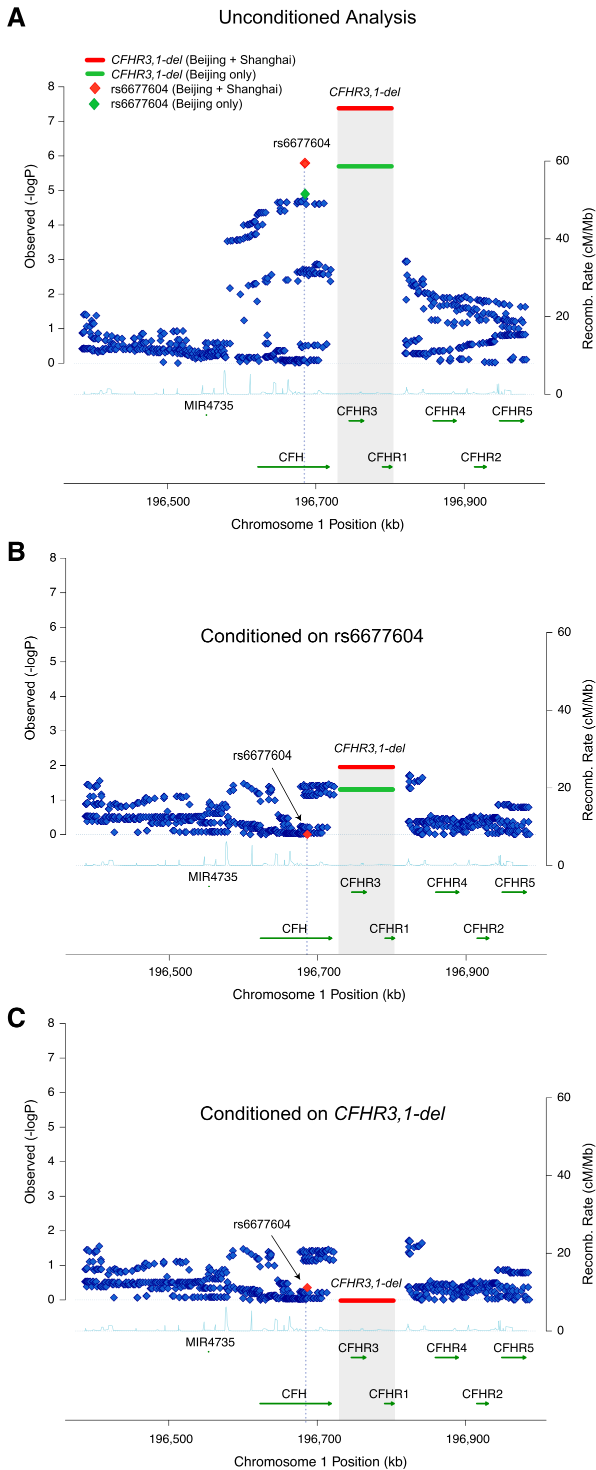

Figure 1. High–resolution regional plots for the *CFH* locus, including a complete set of the 1000 Genomes Project phase 3 imputed markers. (A) First–pass association analysis: *CFHR3,1Δ* polymorphism represents the top association in the region. (B) Conditional association results after adjusting for rs6677604 genotypes: the association of *CFHR3,1Δ* persists after accounting for the effect of rs6677604. (C) The reciprocal conditioning on *CFHR3,1Δ* completely abrogates any association of rs6677604. The *y* axis corresponds to the level of statistical significance (−log–transformed *P* values for the test of association); the shaded area represents the deletion of *CFHR3* and *CFHR1* genes, with the horizontal bar indicating the level of statistical significance for the MLPA-typed deletion. Green symbols represent the association results for the Beijing cohort (1185 patients and 882 controls); red symbols correspond to combined results for the Beijing and Shanghai cohorts (1907 patients and 1632 controls).

In IgAN, the mesangial deposits in glomeruli contain components of the alternative (C3 and Properdin) and terminal pathways (C5, C9, and membrane attack complex) in the absence of initiating elements of the classic pathway (C1q or C4), suggesting activation of the alternative pathway.[13,14] Although IgA is not thought to activate complement and circulating C3 and C4 levels are within normal limits in patients with IgAN, several studies have described increased plasma levels of C3 proteolytic fragments (*e.g.*, iC3b and C3d), which are produced through the alternative pathway.[15–17] The polymeric form of IgA and the Fab fragment of immobilized human IgA may be able to activate C3 in alternative pathway–specific conditions,[18–20] but the mechanisms of the alternative pathway activation remain unclear.

CFH is a major regulator of the alternative complement pathway, protecting against its excessive activation.[8,21,22] The function of *CFHR* genes, which share significant sequence homology with *CFH*, is less well understood. Recent data indicate that *CFHR1*, *CFHR2*, and *CFHR5* can form heterodimers *via* their N-terminal domains and essentially act as CFH antagonists by competing with CFH for C3b binding without exerting any intrinsic complement–regulatory activity.[21,22] Hence, genetic rearrangements that generate hybrid *CFHR* genes result in competitive deregulation of the alternative complement pathway and inhibit CFH action, leading to GN.[21–24] Conversely, in the setting of genetic inactivation of *CFHR* genes (*e.g.*, *CFHR3,1Δ*), the unopposed action of CFH leads to more pronounced downregulation of the alternative complement pathway.[21,22] Other studies have shown that variants within the *CFH* locus affect circulating CFH, C3, and C3a levels, again pointing to their functional relevance.[4,25] Additional studies have shown that C3a and C5a, cleaved products of complement activation, induce tubulointerstitial injury *via* the C3a and C5a receptors.[16,26] Consistent with these data, we observed a suggestive association of *CFHR3,1Δ* with reduced tubulointerstitial injury in IgAN—the Oxford parameter most consistently associated with progression of kidney disease in IgAN. In this study, this association does not seem to be driven by glomerular injury or proteinuria, because we did not detect any association with these parameters. Although these data are intriguing, these findings will require confirmation in larger multiethnic cohorts, with detailed analysis of parameters, such as proteinuria, glomerulosclerosis scores, and progression rates.

Genetic variation in the *CFH* gene clusters has been associated with susceptibility to multiple immune and infectious disorders, including age–related macular degeneration,[5,27,28] atypical hemolytic uremic syndrome,[29–31] C3 glomerulopathy,[21–24] SLE,[32]

Table 2. Conditional analysis rs6677604 and *CFHR3,1Δ*

Tested Variant	Conditioning Variant	Beijing Cohort, *n*=2067, 1185 Patients/882 Controls		Shanghai Cohort, *n*=1472, 722 Patients/750 Controls		Cohorts Combined, *n*=3539, 1907 Patients/1632 Controls	
		OR (95% CI)	*P* Value	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value
rs6677604	None	0.55 (0.42 to 0.72)	1.4×10^{-5}	0.71 (0.52 to 0.96)	0.02	0.61 (0.50 to 0.75)	1.6×10^{-6}
rs6677604	*ΔCFHR3/CFHR1*	1.20 (0.52 to 2.80)	0.67	1.38 (0.59 to 3.21)	0.46	1.25 (0.69 to 2.27)	0.46
ΔCFHR3/CFHR1	None	0.51 (0.38 to 0.67)	2.3×10^{-6}	0.65 (0.48 to 0.90)	8.3×10^{-3}	0.56 (0.46 to 0.70)	8.5×10^{-8}
ΔCFHR3/CFHR1	rs6677604	0.42 (0.18 to 1.02)	0.05	0.48 (0.20 to 1.15)	0.09	0.45 (0.25 to 0.84)	0.01

and susceptibility to meningococcal disease.[33] The *CFH* locus is also associated with variation in C3 and C4 levels[25] as well as myeloperoxidase levels.[34] The interplay between *CFHR3,1Δ* and risk of immune-mediated diseases is complex: *CFHR3,1Δ* is protective against IgAN and age–related macular degeneration[5] but increases susceptibility to atypical hemolytic uremic syndrome[29] as well as SLE.[32] Interestingly, patients with atypical hemolytic uremic syndrome homozygous for *CFHR3,1Δ* alleles may be susceptible to developing autoantibodies to CFH, suggesting that complete absence of CFHR1 and CFHR3 proteins may promote autoimmunity.[35,36] *CFHR3,1Δ* frequency also varies significantly among world populations, reaching ≤40% in some African populations.[2] Recently, we have shown that variation in risk allele frequencies for IgAN may represent adaptation to local pathogens.[2] The variable frequency of *CFHR3,1Δ* together with its opposing effects on different complex diseases suggest a role for balancing selection in determining genetic predisposition to IgAN.

Future studies may clarify the exact mechanisms accounting for activation of the alternative pathway in IgAN and contrasting effects of *CFHR3,1Δ* on different complex traits. Careful genotype-phenotype correlation studies in larger cohorts might better define phenotypic associations with *CFHR3,1Δ*, and animal studies may provide novel insight into the functional effect of individual complement factors on kidney injury, ultimately leading to better therapeutic options.

CONCISE METHODS

Study Cohorts

We studied a total of 1929 patients with biopsy–diagnosed unrelated primary IgAN and 1652 healthy unrelated controls of Han Chinese ancestry (Supplemental Table 2). Characteristics of these cohorts were previously described.[1,2] Patients and controls were recruited at the Renal Division, Peking University First Hospital (Beijing cohort: 1194 patients and 902 controls) and the Department of Nephrology, Shanghai Jiaotong University Ruijin Hospital (Shanghai cohort: 735 patients and 750 controls). All patients had IgAN defined by classic light microscopy findings together with dominant and mesangial staining for IgA by immunofluorescence (at least 2+ on a semiquantitative scale from 0 to 3+). Patients with systemic diseases, such as SLE, Henoch–Schonlein purpura, and chronic liver disease, were excluded from the study. Baseline demographic and clinical data were collected from all patients at the time of renal biopsy. All biopsies were reviewed and scored by experienced pathologists and underwent standardized scoring using the recently developed Oxford MEST system.[37,38] The controls were recruited among healthy blood donors from the same geographic region. In terms of demographics, 2128 (59.4%) were men, and 1453 (40.6%) were women; average age was 26 (range =11–80) years old. All study subjects provided informed consent to participate in our genetic studies, and the study was approved by the Columbia Institutional Review Board as well as local ethics committees. All subjects provided informed consent to participate in the study.

SNP Genotyping

The Beijing cohort was genotyped with the Illumina Human 610-Quad Bead Chip.[1,2] The Shanghai cohort was genotyped for top loci that emerged in a more recent multiethnic GWAS of IgAN[1,2] with the Sequenom MassARRAY (MALDI-TOF) and KASP Genotyping System. We implemented standard quality control measures as previously described.[1,2]

The 1000 Genomes Project Imputation

We performed imputation analysis on the Beijing cohort using Markov Chain Haplotyping (MACH) software, with phase 1 phased data from the 1000 Genomes Project panel for the reference panel (all

Table 3. Association analysis of Oxford pathology parameters with *CFHR3,1Δ*

Oxford Classification, %	*CFHR3,1Δ* Status		OR (95% CI)	*P* Value
	Wt/Wt, *n*=523	Wt/Del or Del/Del, *n*=81		
M1	228 (43.6)	23 (28.4)	0.77 (0.44 to 1.31)	0.34
E1	265 (50.7)	38 (46.9)	1.23 (0.74 to 2.02)	0.42
S1	381 (72.8)	50 (61.7)	0.59 (0.36 to 0.98)	0.04
T1 and T2	169 (32.3)	19 (23.5)	0.46 (0.25 to 0.79)	6.9×10^{-3}

Effect size and *P* value was adjusted by the cohort membership. Wt, wild type; Del, deletion; M, mesangial hypercellularity; E, endocapillary hypercellularity; S, segmental glomerulosclerosis; T, tubular atrophy/interstitial fibrosis.

Asian subpopulations) using MACH and Minimac2 softwares.[39–41] We applied strict quality control filters pre- and postimputation as described previously,[1,2] including $r^2>0.8$ for all imputed markers included in the analysis. Primary association analyses of the imputed data were performed using dosage association in PLINK.

MLPA

We performed the MLPA assay by using the SALSA MLPA Kit P236-A2 ARMD (MRC-Holland, Amsterdam, The Netherlands) according to the manufacturer's protocol. This kit contains 13 probes for the *CFH* gene, eight probes for *CFHR3*, five probes for *CFHR1*, and four probes for *CFHR2* as well as five probes in the flanking genes *KCNT2* and *CFHR5*. Briefly, we used 100 ng genomic DNA (20 ng/μl) per reaction. We used DNA pooled from 40 rs6677604-GG homozygotes (normal copy number of *CFHR3* and *CFHR1*) as a negative control and DNA pooled from 40 rs6677604-AG heterozygotes (heterozygous deletion of *CFHR3* and *CFHR1*) as a positive control. Blank, negative, and positive controls were included in each experiment. Fragment analyses were performed on an ABI Prism Model 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). CNVs were determined according to the manufacturer's instructions. The samples for MLPA were selected on the basis of the genotype at rs6677604. In total, using this approach, we confirmed the *CFHR3,1Δ* genotypes in all rs6677604-AA (n=16), all rs6677604-AG (n=376), and 207 (6.5%) of the randomly selected rs6677604-GG individuals. The *CFHR3,1Δ* statuses of the remaining rs6677604-GG homozygotes were determined by Taqman Quantitative Real–Time PCR (Applied Biosystems) as described below. All CNVs detected by quantitative PCR in rs6677604-GG homozygotes were confirmed by MLPA. The MLPA results underwent standard quality controls procedures according to the manufacturer's instructions. Briefly, visual examination of the peak patterns using a raw data checklist and a peak pattern flowchart was performed to evaluate each sample. Only data that passed the peak pattern filter were used in downstream analyses. After intra- and intersample normalization, we regarded probe ratios <0.7 as a copy loss and probe ratios >1.3 as a copy gain. The CopyCaller software generated two quality metrics for each analyzed sample: a confidence estimate (confidence score) and a deviation estimate (Z score). We only accepted copy number calls with confidence scores $\geq$95% and $|Z \text{ score}|<1.75$. The samples that failed these quality control requirements were repeated, at most, three times and discarded if conclusive genotypes could not be called (Supplemental Table 8).

Quantitative Real–Time PCR

Quantitative real–time PCR was carried out on genomic DNA using the Taqman Copy Number Real–Time Detection System (Applied Biosystems). Briefly, each sample of 20 ng DNA with a concentration of 5 ng/μl was plated along with a blank control (a mix of all PCR reagents without DNA), a negative control (40 pooled rs6677604-GG homozygotes), and a positive control (40 pooled rs6677604-AG samples with heterozygous deletion of both *CFHR3* and *CFHR1*). Two sets of predesigned primers, including *CFHR1* (Assay ID Hs04197581_cn; Applied Biosystems) and *RNaseP* (standard reference; Applied Biosystems), were used for quantitative real–time PCR. A duplex system was used, and quantitative real–time PCR was performed on the 7300 Real–Time PCR System (Applied Biosystems). CNV calls were determined using the Copy Caller Software (Applied Biosystems) with a known calibrator sample method. All CNVs detected using this method were also confirmed by MLPA as described above.

Primary Statistical Analyses

All allelic association tests for SNPs and CNVs were performed with PLINK v1.07.[42] The case-control analyses as well as association tests of *CFHR3,1-del* with clinical and histopathologic variables were first performed individually for the Beijing and Shanghai cohorts; then, combined statistics were derived using a cohort-stratified approach. The conditional analyses of rs6677604 and *CFHR3,1-del* were implemented using logistic regression in PLINK v1.07. For multimarker analysis in the Beijing cohort, we first phased all haplotypes and estimated their frequencies. Because extremely rare haplotypes increase the degrees of freedom while providing little power for detecting associations, all haplotypes with frequencies $<0.1\%$ were collapsed into a single group. For the purpose of global haplotype tests, these rare haplotypes were constrained to have the same pooled OR, and this estimate was treated as a nuisance parameter in the likelihood ratio tests, so that rare haplotypes are not individually tested for association.[43] For all other haplotypes, we estimated the ORs and the corresponding 95% CIs in reference to the most common haplotype that carried no putative functional alleles.

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DISCLOSURES

None.

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