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Relevance of the *ACTN4* gene in African Americans with non-diabetic ESRD

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Abstract

Background—African Americans (AAs) are predisposed to non-diabetic (non-DM) end-stage renal disease (ESRD) and studies have shown a genetic component to this risk. Rare mutations in *ACTN4* (α-actinin-4) an actin binding protein expressed in podocytes cause familial focal segmental glomerulosclerosis.

Methods—We assessed the contribution of coding variants in *ACTN4* to non-DM ESRD risk in AAs. Nineteen exons, 2800 bases of the promoter and 392 bases of the 3' untranslated region of *ACTN4* were sequenced in 96 AA non-DM ESRD cases and 96 non-nephropathy controls (384 chromosomes). Sixty-seven single nucleotide polymorphisms (SNPs) including 51 novel SNPs were identified. The SNPs comprised 33 intronic, 21 promoter, 12 exonic, and 1 3' variant. Sixty-two of the SNPs were genotyped in 296 AA non-DM ESRD cases and 358 non-nephropathy controls.

Results—One SNP, rs10404257, was associated with non-DM ESRD (p<1.0E-4, odds ratio (OR)=0.76, confidence interval (CI)=0.59–0.98; additive model). Forty-seven SNPs had minor allele frequencies less than 5%. These SNPs were segregated into risk and protective SNPs and each category was collapsed into a single marker, designated by the presence or absence of any rare allele. The presence of any rare allele at a risk SNP was significantly associated with non-DM ESRD (p = 0.001, dominant model). The SNPs with the strongest evidence for association (n = 20) were genotyped in an independent set of 467 non-DM ESRD cases and 279 controls. Although, rs10404257 was not associated in this replication sample, when the samples were combined rs10404257 was modestly associated (p=0.032, OR=0.78, CI=0.63–0.98; dominant model). SNPs were tested for interaction with markers in the *APOL1* gene, previously associated with non-DM ESRD in AAs and rs10404257 was modestly associated (p = 0.0261, additive model).

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The authors have no conflicts of interest to disclose.

Conclusions—This detailed evaluation of *ACTN4* variation revealed limited evidence of association with non-DM ESRD in AAs.

Keywords

ACTN4; non-diabetic ESRD; FSGS; kidney; hypertensive nephrosclerosis; African Americans

Introduction

Alpha-actinin-4 (*ACTN4*) is an actin-bundling protein which is widely expressed in a number of tissues, most notably glomerular podocytes [1]. *ACTN4* binds actin in the cell cytoskeleton, contributes to cell structure, and regulates cell motility [2, 3]. Deficiency in the protein in mice has been shown to be directly related to a loss of normal podocyte function[4]. In humans, mutations in *ACTN4* have been shown to cause familial focal segmental glomerulosclerosis (FSGS) [1, 5, 6]. Most mutations led to increased actin binding by α-actinin-4 with subsequent formation of actin aggregates [7–9]. This form of familial FSGS is an autosomal dominant disorder which is believed to be rare in the general population.

Non-diabetic (non-DM) end-stage renal disease (ESRD) is the second most common cause of advanced kidney disease in African Americans after diabetic nephropathy. African Americans are particularly susceptible to FSGS, even when hypertension and socioeconomic factors are considered [10]. Studies in African American families have shown that there is a genetic component to this predisposition to kidney disease [11, 12].

As rare variants in *ACTN4* have been shown to lead to familial forms of FSGS, we hypothesized that similar rare variants in *ACTN4* may also lead to increased susceptibility to non-DM ESRD in African Americans and thus be more readily detectable. To test this possibility, we sequenced 19 (of 21 total) exons in the *ACTN4* gene in African American cases with ESRD and controls.

Methods

Subjects

Non-DM ESRD cases self-reporting their race as African American were collected from dialysis clinics in Network 6, from North Carolina, South Carolina, Georgia, Virginia and Tennessee. Patients were classified as having non-DM ESRD if they had hypertension or chronic glomerular disease listed as their primary cause of renal disease. Cases confirmed the onset of high blood pressure prior to development of ESRD, in the absence of other risk factors for kidney disease such as diabetes mellitus. Non-DM ESRD was typically diagnosed in the presence of proteinuria ≤ 1.5 gm/day, urinalysis ≤ 100 mg/dl protein, or spot urine protein:creatinine ratio ≤ 1.5 gm/gm (when data available); with either EKG evidence of left ventricular hypertrophy or presence of hypertensive retinopathy. Chronic glomerulonephritis was diagnosed in those with kidney biopsy evidence or higher levels of proteinuria. Patients with polycystic kidney disease, Alport's syndrome, urologic disease or surgical nephrectomy were excluded. Non-nephropathy controls were self-identified healthy African Americans born in North Carolina, age ≥ 18 years, and denying a personal or family history of kidney disease in 1st degree relatives. Each participant provided 40mL blood for DNA isolation. DNA was isolated from whole blood using an AutoPure LS automated DNA extraction robot (Gentra Systems, Minneapolis, MN). Recruitment and sample collection procedures were approved by the Institutional Review Board at Wake Forest School of Medicine. All of the subjects in the Wake Forest case control cohort were tested for

relatedness prior to inclusion in the study via identity by descent statistics. Individual which appear to be first or second degree relatives are removed from the sample set.

Subjects were sampled in two groups. Set 1 comprised 296 non-DM ESRD cases and 358 non-nephropathy controls. For the exon sequencing SNP discovery stage, 96 cases and 96 controls were randomly selected from Set 1 samples. We followed up the initial analysis with replication in Set 2 samples, which comprised 467 non-DM ESRD cases and 279 non-nephropathy controls. Finally, we performed a combined association analysis of all the samples, 763 non-DM ESRD cases and 637 non-nephropathy controls.

Sequencing

We sequenced 19 exons, 2800 bases of the promoter region and 392 bases of the 3' untranslated region in the *ACTN4* gene in 96 African American non-DM ESRD cases and 96 African American non-nephropathy controls. PCR primers were designed to amplify regions approximately 500 bases at a time, and nested to provide complete coverage of the promoter region and exons. Primer sequences are available upon request. Each 500 base region was PCR amplified and the product was purified. DNA sequencing was performed using Big Dye Ready Reaction Mix (Applied Biosystems, Foster City, CA) on an ABI3730xl sequencer (Applied Biosystems, Foster City, CA). Sequence data was visualized using Sequencher Software Version 4.6 (GeneCodes Corporation, Ann Arbor, MI). Exons 20 and 21 and a portion of the 3' untranslated region of the *ACTN4* gene could not be sequenced due to a region on chromosome 4 with a highly similar sequence.

SNP genotyping

Forty-one of the 67 SNPs were genotyped using the Sequenom MassARRAY genotyping system (Sequenom, San Diego, CA). Genotyping primers were designed using the MassARRAY Assay Design 3.4 Software (Sequenom, San Diego, CA); sequences will be provided upon request. Twenty-one SNPs could not be designed using the MassARRAY system due to close proximity of other SNPs or other assay design issues. These SNPs were sequenced using the primer sequences for the initial screen as described above. The 21 SNPs for sequencing were chosen based on their location with the intent to maximize the number of SNPs that could be captured using the least number of primers. Five SNPs that were not genotyped by sequenom or sequencing (see SNPs shaded in gray in Table 2). Four of these SNPs (C-2752T, C58279T, C61663G, and G76310A) were extremely rare, with only 1 or 2 individuals identified, and we elected not to pursue them. The fifth SNP, G61675T was in high LD with another SNP which was genotyped, G61672A and was not genotyped separately.

To calculate percentage of African ancestry, 70 ancestry informative markers were genotyped on all cases and controls. Percentage of African ancestry was calculated using the computer program FRAPPE as described previously [13].

Statistical Analysis

Age, BMI and percentage of African ancestry for the non-DM ESRD cases and healthy controls was compared using an unpaired t-test or a Mann-Whitney Rank Sum Test where appropriate (SigmaStat 3.5, SYSTAT Software, San Jose, CA). Departures from Hardy-Weinberg Equilibrium (HWE) were tested for each SNP using chi-square goodness-of-fit statistics in our association analysis program SNPGWA [14]. Tests for association included the overall 2 degree of freedom genotype test of association and the three *a priori* genetic models (i.e. dominant, additive, and recessive).

To test for association at rare alleles, (MAF < 0.05), we collapsed these variants into a single marker using the following technique. For each individual, the presence of any rare allele (at any SNP), the individual was coded with the genotype “1 2”. Individuals with no rare alleles at any SNP were coded with the genotype “2 2”. Therefore, all samples were coded with either a 1 2 or 2 2 genotype. This single marker was then tested for association with case status using SNPGWA as described above. This technique was used to test for association at SNPs in the promoter region only and the promoter region combined with sequenced exons. We collapsed 47 of the 62 SNPs genotyped in Set 1 into a single marker. In addition, we also collapsed just the 17 rare SNPs that were located in the promoter region (which were also included in the 47 SNPs).

For the genotyping of the Set 2 DNA samples, 20 SNPs were selected from the list of 62 SNP genotyped in Set 1 (Table 3). The SNPs were ranked by the 2 degree of freedom p-value and were combined into the largest possible single assay in succession. If a SNP did not plex with the assay, the next ranked SNP would be chosen for assay design. As a result, there are SNPs included in the assay that were not necessarily the most associated SNP by rank (Table 4).

We tested the 20 SNPs that were genotyped on both sets of samples as well as the collapsed rare alleles for interaction with risk at the apolipoprotein L1 (*APOL1*) risk variants. The G1 and G2 risk variants (as described by [15]) were genotyped on all samples using the Sequenom MassArray system (Sequenom, San Diego, CA). Case only and case control tests of association were performed for gene-gene interaction between the 20 SNPs and the *APOL1* G1 and G2 risk haplotypes. This test models *APOL1* risk as the response for all individuals with recessive haplotypes at either G1 or G2 or heterozygosity at both G1 and G2. Interaction analyses were adjusted for age, gender and percentage of African ancestry.

Results

Table 1 summarizes characteristics of the study samples. Two sample sets were evaluated. Set 1 consisted of 296 African Americans with non-diabetic ESRD and 358 non-nephropathy controls in which the non-DM ESRD cases were older, had lower BMI and were slightly higher in percentage of African ancestry (Table 1) than controls. There was no significant difference in gender proportions between cases and controls in this group. The replication sample, designated as set 2 consisted of 467 African Americans with non-diabetic ESRD and 279 non-nephropathy controls. Similar to set 1, the set 2 cases were older, had fewer females, lower BMI and higher percentage of African ancestry than the set 2 controls. These trends remained consistent when the samples were combined in a final set of 763 cases and 637 controls.

The variants identified by sequencing the 384 chromosomes in 96 non-DM ESRD cases and 96 non-nephropathy controls are listed in Table 2. Known variants are identified by their dbSNP ID and novel variants are identified by position relative to the first nucleotide of exon 1. We identified 67 variants in the α-actinin-4 gene, 51 of which were novel. Twenty-one SNPs were in the promoter region, 12 were coding SNPs, 33 were intronic and 1 SNP was in the 3’ untranslated region. Of the 12 coding SNPs, only C58409T in exon 5, was non-synonymous, resulting in a proline to leucine amino acid change. The majority of the SNPs (n = 47) were rare, with minor allele frequencies (MAF) less than 0.05 in the control samples. One SNP, rs2112649, had three alleles in our samples. As the A allele has not been previously reported for this SNP, we sequenced this individual in the forward and reverse directions to confirm the presence of the third allele. A single case individual possessed an A allele at this locus, where as all other individuals tested had a G or C. For association tests, which require 2 alleles, we coded the A allele as unknown.

Sixty-two SNPs were genotyped in set 1 cases and controls. As set 1 samples comprised the 96 cases and controls that made up the SNP discovery set, we compared the genotypes derived from sequencing with those derived from Sequenom genotyping and all genotypes were 100% concordant. Association analysis revealed significance at rs10404257 ($p < 0.0001$, additive model), A-98C ($p = 0.001$, dominant model), and rs2112649 ($p = 0.010$, additive model) (Table 3). Another SNP, C62517T, was significantly associated; however, this SNP deviated significantly from Hardy-Weinberg proportions in all groups of control samples. This SNP is a coding, synonymous variant and was eliminated from further analysis. Both rs10404257 and rs2112649, remained significantly associated after adjustment for age, BMI, gender and African ancestry, ($p = 0.004$, odds ratio (OR) = 0.60, confidence interval (CI) = 0.43 – 0.85, dominant model and $p = 0.026$, OR = 1.31, CI = 1.03–1.67, additive model, respectively).

A number of SNPs were rare (MAF < 0.05) and our current association tests are not powerful at such low frequencies. To overcome this limitation, we collapsed rare alleles together and tested for association. Rare SNPs in the promoter region only (17 SNPs) and throughout the entire gene including the promoter (47 SNPs) were collapsed into a single marker where the presence of a rare allele was coded as “1 2” and the absence of a rare allele was coded as “2 2” (see Methods for complete details). A total of 251 individuals had at least 1 rare allele whereas 404 individuals had only the common alleles. Neither the rare SNPs in the promoter region ($p = 0.981$), nor those throughout the entire gene ($p = 0.166$) were associated with non-DM ESRD in the set 1 samples.

One major drawback to this method of collapsing the rare alleles is that directionality of the alleles (ie risk vs protective) is not taken into consideration. To overcome this limitation, we have segregated the SNPs into “protective” and “risk” categories based on their allele frequencies in cases and controls. Sixteen SNPs were categorized as protective whereas 24 SNPs were categorized as risk. The two categories were collapsed and tested for association as described above. The SNPs collapsed into the protective category were not significantly associated with ESRD, $p = 0.260$. However, the SNPs collapsed into the risk category were significantly associated with ESRD, $p = 0.006$, two degree of freedom test, and 0.001, dominant model.

Twenty of the SNPs from the set 1 analysis which had the greatest evidence of association were genotyped in the set 2 samples, 467 cases and 279 controls (Table 4). A single SNP, A-98C, was associated, ($p = 0.0002$, OR = 0.23, CI = 0.08 – 0.64, additive model). When the two sample sets were combined, one SNP rs10404257 was modestly associated ($p = 0.032$, OR = 0.78, CI = 0.63 – 0.98, dominant model). Adjustment for age, gender, BMI and African ancestry did not change the p-value, ($p = 0.033$). As rs10404257 was not associated in set 2 samples, the significant p-value in the combined sample set is driven by association in set 1. This result does not meet evidence of association accounting for multiple comparisons. The other modestly significant SNP, rs2112649, failed genotyping in set 2 samples.

Recently, two markers on *APOL1* were shown to significantly contribute to risk of ESRD in AAs [15], These risk variants have been designated as G1 (a haplotype consisting of rs73885319 and rs60910145) and G2 (rs71785313). Despite the highly significant contribution to risk, second hits (gene-gene or gene-environment interactions) are required for initiation of renal disease. We hypothesize that there are other genetic markers that may interact with *APOL1* G1 and G2 risk variants. Therefore, we tested rs10404257 and the collapsed rare alleles for interaction with *APOL1* risk alleles G1 and G2 [15]. There was no evidence of interaction with *APOL1* risk alleles for the marker derived from collapsed rare

alleles (p = 0.16, recessive model); however, rs10404257 was modestly associated (p = 0.026, additive model).

Discussion

We sequenced 19 of the 21 exons in the *ACTN4* gene and identified a total of 67 variants in 96 African American non-diabetic ESRD cases and 96 African American non-nephropathy controls. Although initial genotyping of 62 markers revealed some evidence of association in set 1 samples, when set 2 samples (with a combined total of 763 cases and 637 controls) were included these SNPs were no longer associated, leading us to conclude that they are the result of spurious association.

The majority of the identified variants are rare (MAF < 0.05), as would be expected from exon sequencing. Tests for association in these rare variants are low in power and largely limited to variants of large effect. Given the impact of other *ACTN4* mutations, this remained a reasonable possibility. In order to analyze these variants for association we collapsed the rare variants into a single variant. We were able to compare the presence or absence of rare variants in cases and controls using this method. We did not find any association when the rare variants were collapsed, although once samples were segregated based of directionality (risk ve protective) the variants which were categorized as risk were significantly associated with increased risk of ESRD. While it appears as though the presence of rare “risk” alleles contributes to ESRD, it should be noted that the two individuals who possessed the largest numbers of rare risk alleles (one individual had 7 risk alleles, and one had 6) were both non-nephropathy controls. Clearly the role of these rare variants is more complicated that the initial analysis indicates. We propose that additional investigation using a larger sample size into is warrented, with some additional consideration for the potential functional roles of these variants.

The absence of variants in the promoter and exons of the *ACTN4* gene associated with non-DM ESRD in AAs is surprising but not entirely unlikely. This gene is ubiquitously expressed and is important for maintaining cell structure and motility. It is possible that defects in this gene result in significant alterations of function that would be catastrophic and unable to be retained across generations. Indeed the familial FSGS cases that have been identified are isolated in individual families and afflicted members suffer a range of kidney diseases at young ages. We have scanned the available literature for previous examples of association at the variants we have identified, however none of the variants we have noted are described as being associated with FSGF previously. This outcome is not entirely unexpected considering that little to none work has been done in African American samples and highlights the need for continuing research on this sample group.

Through the genotyping of the promoter region and 19 exons of α-actinin-4 we have identified a total of 62 variants, most of which are in the non-coding regions of the gene. Despite the large number of variants identified, few were common and none were associated with non-DM ESRD in AA cases and controls. Collapsing of the risk variants into a single marker, did result in significant association, which should be followed up in future studies. Despite the role of *ACTN4* in familial FSGS, the function of the *ACTN4* gene is clearly important and variants in this gene do not appear to contribute significantly to non-DM ESRD in AAs.

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Table 1

Demographic data for African American non-diabetic ESRD cases and African American non-nephropathy controls for set 1, set 2, and all samples combined. African ancestry is presented as a percentage calculated from 70 ancestry informative markers.

	N	Age (yr)	Gender (F)	BMI (kg/m^2)	Age at ESRD (yr)	% African ancestry
Set 1						
non-nephropathy control	358	49.9 ± 10.1	45.3%	29.4 ± 6.7	–	78.0 ± 11.0
non-DM ESRD	296	53.4 ± 14.8*	42.6%	26.7 ± 7.3*	47.6 ± 15.7	79.9 ± 10.0*
Set 2						
non-nephropathy control	279	46.5 ± 12.0	62.7%	31.2 ± 7.2	–	77.6 ± 10.4
non-DM ESRD	467	54.7 ± 14.4*	44.6%*	27.0 ± 6.7*	49.3 ± 15.5	80.1 ± 10.6*
Combined						
non-nephropathy control	637	48.4 ± 11.1	52.7%	30.2 ± 7.0	–	77.8 ± 10.8
non-DM ESRD	763	54.2 ± 14.5*	44.0%*	26.6 ± 6.9*	48.7 ± 15.5	80.1 ± 10.4*

* significant difference between cases and controls ($p < 0.05$)

Table 2

Variants identified from sequencing the exons and the promoter region of ACTN4. Position is shown relative to the Hg19 assembly of the human genome (column 3) and the first nucleotide of exon 1 (column 4). Samples highlighted in grey were only genotyped on 96 cases and 96 controls from Set 1, whereas the remaining SNPs were identified by sequencing and subsequently genotyped in the entire Set 1 samples, 296 non-DM ESRD cases and 358 non-nephropathy controls. Total alleles shows the number of copies of the minor allele present in cases and controls. MAF = minor allele frequencies

					Total Alleles		MAF		
Variant	**Location**	**Hg19 assembly**	**Position (exon 1)**	**Alleles**	**Control**	**Case**	**Control**	**Case**	
C-2752T	promoter	39135575	−2752	C/T	2	0	0.010	0.000	
rs12052046	promoter	39135874	−2453	C/T	42	29	0.067	0.053	
A-2318T	promoter	39136009	−2318	A/T	28	27	0.044	0.052	
A-2243G	promoter	39126084	−2243	A/G	8	5	0.013	0.010	
C-2222T	promoter	39136105	−2222	C/T	47	30	0.075	0.058	
G-1969A	promoter	39136358	−1969	G/A	4	7	0.006	0.015	
C-1962T	promoter	39136365	−1962	C/T	29	27	0.046	0.058	
C-1921T	promoter	39136406	−1921	C/T	1	2	0.002	0.004	
G-1846A	promoter	39136481	−1846	G/A	4	0	0.006	0.000	
rs10404257	promoter	39136486	−1841	G/A	202	125	0.320	0.264	
C-1547T	promoter	39136486	−1547	C/T	6	4	0.009	0.008	
C-1291T	promoter	39136780	−1291	C/T	1	2	0.002	0.004	
G-1201A	promoter	39137036	−1201	G/A	17	5	0.027	0.014	
C-993T	promoter	39137126	−993	C/T	3	2	0.005	0.004	
C-850T	promoter	39137334	−850	C/T	24	28	0.039	0.053	
G-655A	promoter	39137477	−655	G/A	24	28	0.039	0.053	
G-197C	promoter	39137672	−197	G/C	3	0	0.005	0.000	
G-181A	promoter	39138130	−181	G/A	1	1	0.002	0.002	
A-163C	promoter	39138146	−163	A/C	11	11	0.019	0.026	
A-98C	promoter	39138164	−98	A/C	3	8	0.005	0.021	
T-85A	promoter	39138229	−85	T/A	3	0	0.005	0.000	
C52997T	exon 2	39191323	52997	C/T	12	14	0.019	0.026	Synonymous
rs2112649	intron 2	39191383	53059	G/C/A	265	252	0.418	0.481	
G53218A	intron 2	39191544	53218	G/A	47	25	0.073	0.048	

Variant	Location	Hg19 assembly	Position (exon 1)	Alleles	Total Alleles		MAF		
					Control	Case	Control	Case	
rs2287728	intron 3	39195558	57232	C/T	29	28	0.045	0.055	
G57359A	intron 4	39195685	57359	G/A	2	0	0.003	0.000	
rs2368475	intron 4	39195735	57409	C/T	12	14	0.019	0.026	
C58279T	intron 4	39196605	58279	C/T	0	1	0.000	0.005	
A58329T	intron 4	39196655	58329	A/T	0	1	0.000	0.002	
C58409T	exon 5	39196735	58409	C/T	6	3	0.009	0.006	Non-synonymous pro-leu
rs11553600	exon 5	39196736	58410	G/A	51	33	0.082	0.063	synonymous
rs3745859	exon 5	39196745	58419	C/T	177	118	0.276	0.227	synonymous
G61654T	intron 6	39199980	61654	G/T	64	58	0.099	0.112	
C61663G	intron 6	39199989	61663	C/G	0	1	0.000	0.005	
G61672A	intron 6	39199998	61672	G/A	20	21	0.111	0.112	
G61675T	intron 6	39200001	61675	G/T	20	21	0.111	0.112	
G62427A	intron 7	39200753	62427	G/A	3	3	0.005	0.006	
C62517T	intron 7	39200843	62517	C/T	89	41	0.138	0.075	
T62682G	intron 8	39201008	62682	T/G	0	1	0.000	0.002	
G62719A	intron 8	39201045	62719	G/A	1	3	0.002	0.006	
G62777A	intron 8	39201103	62777	G/A	3	3	0.005	0.006	
T66655A	intron 8	39204981	66655	T/A	2	0	0.003	0.000	
rs2306196	intron 8	39205015	66689	C/T	9	7	0.014	0.014	
G69271A	intron 9	39207597	69271	G/A	1	0	0.002	0.000	
C70315A	exon 11	39208641	70315	C/A	4	5	0.006	0.009	synonymous
C70387T	exon 11	39208713	70387	C/T	1	2	0.002	0.004	synonymous
rs7248577	intron 11	39208795	70469	C/T	39	27	0.064	0.052	
C73985T	exon 12	39212311	73985	C/T	1	0	0.002	0.000	synonymous
C74066T	intron 12	39212392	74066	C/T	53	29	0.083	0.057	
rs2306197	intron 12	39212400	74074	C/T	14	11	0.024	0.021	
C76085T	intron 13	39214411	76085	C/T	28	30	0.048	0.056	
G76310A	exon 14	39214636	76310	G/A	1	1	0.005	0.005	synonymous
C76548T	exon 15	39214874	76548	C/T	4	6	0.006	0.011	synonymous

Variant	Location	Hg19 assembly	Position (exon 1)	Alleles	Total Alleles		MAF		
					Control	Case	Control	Case	
rs12986337	exon 16	39215172	76846	T/C	27	29	0.043	0.055	synonymous
G76867A	exon 16	39215193	76867	G/A	1	2	0.002	0.004	synonymous
rs3786851	intron 16	39215333	77007	C/T	156	108	0.244	0.213	
G77865A	intron 16	39216191	77865	G/A	31	31	0.050	0.057	
rs12979947	intron 16	39216203	77877	T/C	30	28	0.047	0.055	
G77942A	intron 16	39216268	77942	G/A	3	2	0.005	0.004	
C78043T	exon 17	39216369	78043	C/T	3	3	0.005	0.006	synonymous
rs1105759	intron 17	39217507	79181	G/A	37	31	0.060	0.061	
G79206A	intron 17	39217532	79206	G/A	0	1	0.000	0.002	
C79225T	intron 17	39217551	79225	C/T	6	4	0.010	0.008	
C80196T	intron 18	39218522	80196	C/T	2	0	0.003	0.000	
rs12981131	intron 19	39218694	80368	A/G	48	35	0.076	0.067	
rs12974733	intron 19	39219560	81234	G/T	30	30	0.046	0.055	
T82862A	3' UTR	39221188	82862	T/A	5	9	0.008	0.017	

Table 3

Results of genotyping 62 SNPs identified by sequencing in Set 1 samples (296 cases and 358 controls). InterSNP linkage disequilibrium is shown by r^2 for each SNP and the SNP immediately following. Association is shown for the 2 degree of freedom test (2DF) followed by the three genetic models, dominant (Dom), additive (Add), and recessive (Rec). MAF = minor allele frequency, HWE = Hardy Weinberg Equilibrium

	MAF				Genotypic Association			
Marker	**Cases**	**Controls**	**Prob HWE**	**r^2**	**2 DF**	**Dom**	**Add**	**Rec**
rs12052046	0.055	0.071	0.735	0	0.260	0.187	0.282	0.496
A-2318T	0.053	0.043	0.642	0.001	0.742	0.440	0.447	0.845
A-2243G	0.009	0.012	1.000	0.001	0.915	0.714	0.758	0.914
C-2222T	0.062	0.079	0.539	0.001	0.394	0.210	0.273	0.808
G-1969A	0.014	0.006	1.000	0.002	0.348	0.152	0.183	0.873
C-1962T	0.055	0.044	1.000	0	0.441	0.278	0.346	0.633
C-1921T	0.004	0.001	1.000	0	0.705	0.421	0.489	0.876
G-1846A	0.000	0.006	1.000	0.001	0.344	0.207	0.330	0.870
rs10404257	0.262	0.320	0.323	0.004	**0.027**	**0.008**	**0.034**	0.763
C-1547T	0.009	0.010	1.000	0	0.980	0.893	0.924	0.914
C-1291T	0.003	0.002	1.000	0	0.825	0.563	0.633	0.954
G-1201A	0.013	0.026	0.211	0	0.526	0.257	0.272	0.748
C-993T	0.004	0.004	1.000	0.002	0.982	0.909	0.949	0.920
C-850T	0.052	0.037	0.623	1	0.457	0.211	0.219	0.930
G-655A	0.052	0.037	0.623	0.003	0.463	0.216	0.224	0.931
G-197C	0.002	0.005	1.000	0	0.860	0.685	0.785	0.862
G-181A	0.002	0.002	1.000	0	0.948	0.744	0.758	0.871
A-163C	0.025	0.017	1.000	0	0.685	0.385	0.394	0.862
A-98C	0.022	0.005	1.000	0	**0.049**	**0.001**	**0.025**	0.842
T-85A	0.000	0.005	1.000	0	0.511	0.350	0.503	0.845
C52997T	0.025	0.017	1.000	0.016	0.609	0.321	0.335	0.911
rs2112649	0.484	0.425	0.936	0.054	0.113	0.101	**0.010**	**0.077**
G53218A	0.050	0.077	0.741	0.001	0.161	0.099	0.069	0.199
rs2287728	0.055	0.043	1.000	0	0.384	0.245	0.312	0.591
G57359A	0.000	0.003	1.000	0.082	0.613	0.423	0.571	0.918
rs2368475	0.025	0.017	1.000	0	0.615	0.325	0.340	0.912

	MAF				Genotypic Association			
Marker	**Cases**	**Controls**	**Prob HWE**	**r^2**	**2 DF**	**Dom**	**Add**	**Rec**
A58329T	0.002	0.000	1.000	0	0.664	0.420	0.534	0.898
C58409T	0.007	0.010	1.000	0.001	0.902	0.700	0.753	0.899
rs11553600	0.066	0.085	0.158	0.027	0.511	0.254	0.248	0.617
rs3745859	0.222	0.266	1.000	0.038	0.163	0.057	0.074	0.547
G61654T	0.111	0.097	0.829	0.042	0.638	0.361	0.409	0.957
G62427A	0.005	0.004	1.000	0	0.968	0.799	0.807	0.929
C62517T	0.074	0.129	**0.000**	0	**3.12E-4**	0.166	**0.008**	**1.03E-4**
T62682G	0.002	0.000	1.000	0	0.685	0.443	0.558	0.915
G62719A	0.007	0.001	1.000	0	0.297	0.137	0.199	0.899
G62777A	0.005	0.004	1.000	0	0.954	0.758	0.768	0.914
T66655A	0.000	0.003	1.000	0	0.596	0.403	0.549	0.932
rs2306196	0.013	0.013	1.000	0	0.992	0.962	0.943	0.902
G69271A	0.000	0.001	1.000	0	0.842	0.674	0.796	0.926
C70315A	0.009	0.008	1.000	0	0.993	0.928	0.914	0.919
C70387T	0.004	0.003	1.000	0.001	0.965	0.789	0.799	0.913
rs7248577	0.051	0.064	1.000	2	0.519	0.449	0.361	0.322
C73985T	0.000	0.001	1.000	0	0.859	0.714	0.835	0.901
C74066T	0.060	0.085	0.069	0.002	0.194	0.078	0.112	0.947
rs2306197	0.019	0.023	0.239	0	0.820	0.794	0.696	0.534
C76085T	0.056	0.046	1.000	0.002	0.492	0.372	0.460	0.531
C76548T	0.011	0.006	1.000	0.002	0.618	0.334	0.373	0.914
rs12986337	0.055	0.042	0.389	0	0.512	0.248	0.253	0.915
G76867A	0.003	0.001	1.000	0	0.794	0.521	0.591	0.932
rs3786851	0.207	0.238	0.819	0.016	0.375	0.161	0.187	0.686
G77865A	0.057	0.048	0.684	0.967	0.284	0.322	0.475	0.311
rs12979947	0.055	0.045	1.000	0	0.510	0.356	0.432	0.593
G77942A	0.003	0.006	1.000	0	0.884	0.676	0.745	0.917
C78043T	0.005	0.004	1.000	0	0.970	0.805	0.813	0.931
rs1105759	0.065	0.058	0.717	0	0.645	0.509	0.588	0.579
G79206A	0.002	0.000	1.000	0	0.704	0.465	0.581	0.931

Marker	MAF		Prob HWE	r^2	Genotypic Association			
	Cases	Controls			2 DF	Dom	Add	Rec
C79225T	0.007	0.009	1.000	2	0.965	0.838	0.878	0.913
C80196T	0.000	0.003	1.000	0	0.618	0.429	0.577	0.914
rs12981131	0.068	0.071	0.530	0.625	0.793	0.994	0.880	0.508
rs12974733	0.055	0.045	1.000	0.001	0.453	0.310	0.384	0.572
T82862A	0.016	0.007	1.000	2	0.337	0.146	0.175	0.915

Table 4

Genotypic Association results from 20 SNPs in the Set 2 cases alone and then combined with the Set 1 cases. RAF = relative allele frequency, 2DF = 2 degree of freedom test, Dom = dominant model, Add = additive model, Rec = recessive model

	Set 2 Cases (n = 467) and Controls (n = 279)						Set 1 and 2 Cases (n = 763) and Controls (n = 637)					
	RAF		Genotypic Association				RAF		Genotypic Association			
Marker	**Cases**	**Ctrls**	**2 DF**	**Dom**	**Add**	**Rec**	**Cases**	**Ctrls**	**2 DF**	**Dom**	**Add**	**Rec**
G-1969A	0.006	0.008	0.836	0.555	0.551	0.787	0.009	0.007	0.825	0.554	0.587	0.954
C-1962T	0.054	0.048	0.398	0.585	0.743	0.255	0.054	0.046	0.193	0.270	0.386	0.208
C-1921T	0.001	0.006	0.284	0.124	0.177	0.780	0.002	0.003	0.865	0.599	0.636	0.949
G-1846A	0.001	0.002	0.852	0.572	0.593	0.778	0.001	0.004	0.243	0.111	0.168	0.951
rs10404257	0.264	0.268	0.988	0.885	0.875	0.914	0.263	0.296	0.097	0.032	0.068	0.659
G-197C	0.001	0	0.883	0.781	0.902	0.858	0.002	0.003	0.915	0.694	0.741	0.996
G-181A	0.001	0.002	0.929	0.701	0.717	0.848	0.002	0.002	0.999	0.965	0.967	0.986
A-163C	0.025	0.022	0.455	0.599	0.806	0.332	0.024	0.020	0.485	0.416	0.526	0.475
A-98C	0.004	0.028	0.001	**2.00E-04**	**2.00E-04**	0.309	0.011	0.015	0.687	0.526	0.452	0.465
T-85A	0.004	0.008	0.600	0.315	0.351	0.827	0.003	0.006	0.439	0.217	0.267	0.983
G53218A	0.063	0.078	0.512	0.259	0.248	0.665	0.058	0.078	0.088	0.055	0.037	0.163
G57359A	0*	0	NA	NA	NA	NA	0	0.002	0.443	0.255	0.376	0.947
A58329T	0.001	0	0.904	0.874	0.993	0.800	0.001	0	0.823	0.636	0.757	0.951
rs3745859	0.25	0.226	0.556	0.287	0.287	0.620	0.242	0.247	0.886	0.664	0.750	0.925
G62719A	0.001	0.005	0.318	0.142	0.200	0.800	0.004	0.003	0.991	0.925	0.947	0.950
T66655A	0	0.002	0.534	0.300	0.403	0.800	0	0.002	0.245	0.124	0.213	0.933
G69271A	0	0	NA	NA	NA	NA	0	0.001	0.692	0.451	0.566	0.920
C73985T	0.001	0.002	0.880	0.613	0.633	0.801	0.001	0.002	0.820	0.557	0.627	0.951
G79206A	0	0	NA	NA	NA	NA	0.001	0	0.833	0.656	0.777	0.939
C80196T	0.002	0.004	0.827	0.537	0.557	0.802	0.001	0.003	0.637	0.363	0.425	0.944