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ORIGINAL ARTICLE

Polymorphisms Within RYR3 Gene Are Associated With Risk and Age at Onset of Hypertension, Diabetes, and Alzheimer's Disease

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BACKGROUND
Hypertension affects 33% of Americans while type 2 diabetes and Alzheimer's disease (AD) affect 10% of Americans, respectively. Ryanodine receptor 3 gene (RYR3) codes for the RYR which functions to release stored endoplasmic reticulum calcium ions (Ca^{2+}) to increase intracellular Ca^{2+} concentration. Increasing studies demonstrate that altered levels of intracellular Ca^{2+} affect cardiac contraction, insulin secretion, and neurodegeneration. In this study, we investigated associations of the RYR3 genetic variants with hypertension, AD, and diabetes.

METHODS
Family data sets were used to explore association of RYR3 polymorphisms with risk and age at onset (AAO) of hypertension, diabetes, and AD.

RESULTS
Family-based association tests using generalized estimating equations (FBAT–GEE) showed several unique or shared disease-1 associated variants in the RYR3 gene. Three single nuclear polymorphisms (SNPs; rs2033610, rs2596164, and rs2278317) are significantly associated with risk for hypertension, diabetes, and AD. Two SNPs (rs4780174 and rs7498093) are significantly associated with AAO of the 3 diseases.

CONCLUSIONS
RYR3 variants are associated with hypertension, diabetes, and AD. Replication of these results of this gene in these 3 complex traits may help to better understand the genetic basis of calcium-signaling gene, RYR3 in association with risk and AAO of these diseases.

Keywords: blood pressure; diabetes and Alzheimer's disease, hypertension, RYR3 gene, shared genetic variants, SNP.

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Worldwide, hypertension, type 2 diabetes, and Alzheimer's disease (AD) are common public health problems estimated to cause millions of premature deaths. In recent decades, hypertension and diabetes, as vascular risk factors, increase the risk of cognitive impairment and dementia.[1] Hypertension alone affects 33% of Americans presently. In 2015, diabetes was estimated to affect 9.4% of the US adult population. However, exclusively above 65 years, the percentage increases to 25%. Regarding AD, 10% of individuals above 65 years suffer from the disease. There is a close link among these 3 diseases based on their risk factors, causes, pathological bases,[2–5] and genetic factors, including lipoprotein lipase gene in these 3 diseases.[6] More evidence supports the link type 2 diabetes and hypertension with AD, the most common form of dementia. These 3 traits are caused by multiple susceptibility genes, whose effects are modulated by gene-environment and gene–gene interactions. Twin and family studies show low-to-moderate heritability for AD,[7,8] hypertension,[9] and diabetes.[10]

Mendelian hypertension has elucidated certain biological pathways contributing to hypertension over dietary salt intake or directly through increased peripheral vascular resistance. The Mendelian mutation/genes exercise large effects on blood pressure–related phenotypes. However, genome wide association study (GWAS) and meta-analysis for blood pressure have yielded many signals with small effects.[11] Thus far, few loci have been validated. Both genetic strategies are necessary, and much remains to be explored.

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Genetic/genomic insights and analyses of Mendelian hypertension syndromes and GWAS on essential hypertension have contributed to the depth of understanding of the genetics origins of hypertension.[12] The results from several large-scale studies have clearly shown that blood pressure–related single nuclear polymorphisms (SNPs) are associated with a difference in blood pressure and cardiovascular disease outcome.[13]

Type 2 diabetes is characterized by insulin resistance, obesity, and high blood pressure, each influenced by both genetic and environmental factors.[14] Advanced diabetes is characterized with abnormal pancreatic islet β-cell function in presence of insulin.[15] Insulin secretion is triggered by glucose, which is transported into the β-cells and metabolized. This increases the concentration of adenosine triphosphate (ATP), which in turn leads to closure of ATP-sensitive K^+ channels and depolarization of cellular membrane. Depolarization activates voltage-gated Ca^{2+} channels, allowing entry of extracellular Ca^{2+} into β cells which triggers an even greater release of ER Ca^{2+} which is mediated by ryanodine receptors (RYRs). This suggests calcium-signaling pathway is involved in pathophysiology of diabetes.

The third complex trait is AD. It is characterized clinically by progressive deterioration of cognitive functions,[16] and physiologically by β-amyloid peptides (Aβ) aggregates and intracellular neurofibrillar tangles composed of hyperphosphorylated microtubule-associated tau protein. While aging is the major risk factor, a high number of cases are characterized by earlier onset and are inherited in an autosomal dominant manner.[17,18] In neurons, higher Ca^{2+} concentration, released by RYRs, leads to the release of neurotransmitter at synaptic junctions and affects dendritic action potential.[19] The results from research studies suggest adults with type 2 diabetes have a higher risk of late-onset AD. Numerous investigations have reported that risk for late-onset AD is increased in the presence of type 2 diabetes[2,3] and hypertension.[4] An estimated 54 million US adults have prediabetes and most of these people will develop type 2 diabetes within the next 10 years. Diabetes and hypertension raise the risk of heart disease and stroke. The pathophysiological factors include, but are not limited to, damaged blood vessels in the brain, increased insulin levels, unbalanced chemical changes, and high blood glucose. These changes may cause inflammation, damage brain cells, and increases risk for AD (https://www.alz.org/national/documents/latino_brochure_diabetes.pdf). A recent study focus on evaluation of amyloid-protective factors showed that hypertension, diabetes, and metabolic conditions were also associated with AD-like neurodegeneration.[5]

The critical roles of calcium-signaling pathway in all 3 phenotypes lead us to think shared genetic variants in the genes involved in calcium signaling may be associated with these complex traits. We are interested in gene *RYR3* located at 15q13.3. The protein encoded by *RYR3* (Gene ID: 6263) is a RYR, which functions to release calcium from the ER. As a large intracellular homotetrameric protein (>2 MDa) that comprises 4,780 amino acids,[20,21] RYRs reside on the sarcoplasmic reticulum membrane and release Ca^{+2} from intracellular stores to regulate concentration.[22]

Increased genes and mutations were reported to be associated with these traits based on the results of GWAS, candidate genes, meta-analysis, and next-generation sequencing studies. In the cardiovascular system, Ca^{+2} is essential for cardiac muscle contraction and relaxation, and acts as a second messenger in signal transduction pathways. Complex mechanisms regulate intracellular free calcium levels in the heart and vasculature, and a failure of these systems to maintain normal Ca^{+2} homeostasis has been linked to hypertension and other cardiovascular disease outcomes.[22]

Studies of the *RYR3* have reported association with AD. For example, a meta-analysis based on 4 GWAS identified *RYR3* association with AD risk using generalized multifactor dimensionality reduction.[23] Another study observed a significant interaction between *RYR3* and *CACNA1C* (genes coding for calcium channels that mediate the influx of calcium ions into the cell upon membrane polarization) in all 3 independent data sets of Alzheimer's Disease Neuroimaging Initiative cohorts.[24] Functional studies of *RYR3* suggest that upregulated RYR levels are found in human AD brains,[25] and RYR3 isoforms are upregulated at early and late stages of AD in animal models.[26]

Limited study of shared SNPs has been reported in hypertension, diabetes, and AD. Based on previous findings and reasoning above, we hypothesized SNPs in *RYR3* are involved in development of hypertension, diabetes, and AD.

MATERIALS AND METHODS

Subjects

A family-based sample was available from the National Institute on Aging–Late Onset Alzheimer's Disease (NIA-LOAD) family study: 2,545 individuals (1,266 cases including 1,070 with age at onset [AAO] values) were available for our current study. Family Study: GWAS for Susceptibility Loci—Study Accession: phs000168.v1.p1. This study is to identify and recruit families with 2 or more siblings with the late-onset form of Alzheimer's disease and a cohort of unrelated, nondemented controls similar in age and ethnic background, and to make the samples, the clinical and genotyping data and preliminary analyses available to qualified investigators world-wide. Genotyping by the Center for Inherited Disease Research (CIDR) was performed using the Illumina Infinium II assay protocol with hybridization to Illumina Human 610Quadv1_B Beadchips. The details about these subjects were described elsewhere.[27] Overall, 1,266 AD cases and 1,279 non-AD individuals (including 1,070 with AAO values) were from 1386 pedigrees (including 589 nuclear families) (Table 1).

Hypertension is defined as systolic blood pressure of 140 mm Hg or greater, diastolic blood pressure of 90 mm Hg or greater, or taking antihypertensive medication (phs000168.v1.p1). There were a total of 1,036 patients with hypertension and 1,240 control subjects.

Diabetes is defined by a history of diabetes or high blood sugar, or treatment of diabetes or high blood sugar reported by the subject. There are 247 patients with diabetes and 2,028 control subjects. There are 279 SNPs within the RYR3 gene available for patients with diseases and control subjects.

Table 1. Descriptive characteristics of cases and controls

Variable	AD patients	Controls	Hypertension	Controls	Diabetes	Controls
Sample size (*n*)	1,266	1,279	1,036	1,240	247	2,028
Sex						
Male	435	466	382	462	120	727
Female	831	813	654	778	127	1,301
Mean AAO (years ± SD)	76.4 ± 6.7	–	60.3 ± 13.1	–	61.9 ± 12.2	–
Median AAO (years)	77	–	60	–	60	–
Range of age at onset (years)	50–98	–	22–91	–	35–89	–

Abbreviations: AAO, age at onset; AD, Alzheimer's disease.

Statistical methods

Genotype quality control: Hardy–Weinberg equilibrium was tested for all SNPs using the controls by using HAPLOVIEW version 4.1 software.[28] Minor allele frequency was determined for each SNP and pairwise linkage disequilibrium statistics (D′ and r^2) were assessed using HAPMAP Caucasian data.

Family-based study: family-based association analyses for 3 traits were performed using PBAT version 36.1,[29] which can handle nuclear families with missing parental genotypes, extended pedigrees with missing genotypic information, analysis of SNPs, haplotype analysis, quantitative traits, and time-to-onset phenotypes. For the affection status of hypertension, diabetes, and AD, family-based association tests using generalized estimating equations (FBAT–GEE) was used to perform family-based association analysis For testing time-to-onset trait (AAO), FBAT–Wilcoxon statistics were employed.[30] The AAO values for healthy siblings were censored and age at entry into the study was used. Haplotype analysis was conducted in 2-SNP or 3-SNP sliding windows. For multiple comparison, Bonferroni correction ($\alpha = 0.05/279 = 1.79 \times 10^{-4}$) was used for statistical significance. Descriptive statistics were conducted with SAS 9.4 (SAS Institute, Cary, NC). Three genetic models were used (allele-dose, dominant, and recessive).

In silico analysis

We evaluated potential function of the disease-associated SNP. We examined if these variants were located within the regions of the gene that might have potential functional importance. The sequences containing the associated SNPs were examined for microRNA-binding sites, splicing sites, regulatory gene regions, and species-conserved regions using NIH-SNP Function Prediction (http://snpinfo.niehs.nih.gov/cgi-bin/snpinfo/snpfunc.cgi). To determine disease-associated SNPs with RYR3 expression levels in different human tissues, we used publicly available data from the Genotype-Tissue Expression (GTEx) project[31] in which, there is RNA sequencing on brain tissue from healthy donors available, resulting in genotype and expression phenotype data for ~100–120 normal individuals in multiple different brain regions. The information about subjects and RNA quality can be found in the GTEx website (www.gtexportal.org).

RESULTS

Genotype quality control and descriptive statistics

Out of 279 SNPs in *RYR3*, 3 SNPs with $P < 10^{-4}$ for Hardy–Weinberg equilibrium (HWE) were removed for further analysis. The demographic characteristics of the subjects in the study are shown in the Table 1. The mean AAO for AD, hypertension, and diabetes were 76.4, 60.3, and 61.9 years.

Single-marker and haplotype analyses based of risk of hypertension, diabetes, and AD using FBAT–GEE

We found shared disease-associated SNPs among these complex traits. A number of SNPs were associated with each individual disease (Table 2), 5 SNPs (rs16973062, rs2288609, rs965471, rs4780167, rs10519874, and rs7498093) were associated with diabetes and also survived after the correction for multiple testing using the Bonferroni correction. Single-marker analysis showed that 3 SNPs (rs2033610, rs2596164, and rs2278317, $P < 0.05$) were associated with hypertension, diabetes, and AD in various genetic models (additive, dominant, and/or, recessive) as well as 3 SNPs (rs1390158, rs11637619, and rs8037864) also showed associations with hypertension and AD and 2 SNPs (rs4780167 and rs8028974) showed associations with hypertension and diabetes ($P < 0.05$) without correction for multiple testing. Four disease-associated SNPs (rs965471, rs10519874, rs7498093, and rs17236525) were shared between diabetes and AD in various genetic models. These results suggest shared genetic susceptibility among these 3 diseases in the RYR3 gene (Table 2).

We also identified haplotypes in association with 2 of 3 traits, hypertension, and diabetes. The A-T haplotype from rs4780118 and rs11072471 (D′ = 0.87) and the A-C haplotype from rs2291736-rs937303 (D′ = 0.63) was significantly associated with hypertension in the family-data ($P = 0.00254$ and 0.00667, respectively) (Table 3). Moreover, we also observed the G-G haplotype from rs12906709–rs4780167 SNPs ($P = 2.71 \times 10^{-5}$), A-T haplotype of rs2288609–rs4780174 SNPs ($P = 5.44 \times 10^{-6}$), and the A-G haplotype from rs10519875–rs11855625 ($P = 6.71 \times 10^{-5}$) were significantly associated with diabetes in the family-data (Table 3).

Table 2. Single-marker analysis of risk of hypertension, diabetes, and AD based on FBAT–GEE ($P < 0.05$)

SNP	Position[a]	AL[b]	MAF[c]	HWE[d]	Fam#[e]	$P_{\text{FBAT–GEE}}$[f] for hypertension	$P_{\text{FBAT–GEE}}$[h] for diabetes	$P_{\text{FBAT–GEE}}$[i] for AD
rs16973323	31694920	T	0.17	0.722	145	0.00379(a)0.00215(d,r)[g]	0.697(a) 0.516(d,r)	0.556(a) 0.466(d,r)
rs2291736	31726787	A	0.21	0.438	186	0.0046(a) 0.0221(d,r)	0.534(a) 0.142(d,r)	0.875(a) 0.0534(d,r)
rs4780118	31774633	C	0.29	0.704	216	0.00746(a) 0.0382(d,r)	0.703(a) 0.276(d,r)	0.194(a) 0.128(d,r)
rs1390158	31744649	A	0.06	0.173	72	0.0151(a) 0.0437(d,r)	0.846(a) 0.223(d,r)	0.0459(a) 0.0207(d,r)
rs11072471	31507915	C	0.27	0.740	208	0.0169(a) 0.0191(d,r)	0.143(a) 0.154(d,r)	0.403(a) 0.292(d,r)
rs11637619	31743447	C	0.14	0.185	132	0.0171(a) 0.0255(d,r)	0.887(a) 0.155(d,r)	0.162(a) 0.041(d,r)
rs2033610	31542203	T	0.47	0.031	260	0.0226(a) 0.0384(d,r)	0.0306(a) 0.0344(d,r)	0.098(a) 0.0245(d,r)
rs2596164	31546180	G	0.47	0.041	258	0.0237(a) 0.0526(d,r)	0.0227(a) 0.0391(d,r)	0.00963(a) 0.0178(d,r)
rs12441112	31921018	G	0.21	0.703	207	0.0266(a) 0.933(d,r)	0.876(a) 0.711(d,r)	0.519(a) 0.26(d,r)
rs12906709	31797258	A	0.41	0.003	241	0.0293(a) 0.317(d,r)	0.725(a) 0.27(d,r)	0.231(a) 0.172(d,r)
rs2339273	31528153	T	0.26	0.217	207	0.0301(a) 0.812(d,r)	0.244(a) 0.364(d,r)	0.349(a) 0.438(d,r)
rs2088143	31770837	C	0.22	0.737	188	0.0344(a) 0.0636(d,r)	0.403(a) 0.369(d,r)	0.627(a) 0.173(d,r)
rs8037864	31801713	G	0.21	0.178	173	0.036(a) 0.0846(d,r)	0.797(a) 0.374(d,r)	0.129(a) 0.0323(d,r)
rs2278317	31848032	G	0.31	0.737	223	0.0385(a) 0.779(d,r)	0.106(a) 0.0208(d,r)	0.163(a) 0.0255(d,r)
rs17236476	31784069	C	0.07	0.945	85	0.0392(a) 0.0267(d,r)	0.701(a) 0.223(d,r)	0.146(a) 0.081(d,r)
rs8034012	31687997	C	0.37	0.239	239	0.0438(a) 0.101(d,r)	0.614(a) 0.541(d,r)	0.499(a) 0.576(d,r)
rs16973062	31679589	C	0.06	0.523	70	0.0451(a) 0.0438(d,r)	0.884(a) 0.365(d,r)	0.376(a) 0.226(d,r)
rs2288609	31821738	A	0.27	0.319	214	0.602(a) 0.0516(d,r)	6.82×10^{-5}(a) 0.365(d,r)	0.50(a) 0.38(d,r)
rs965471	31868429	G	0.34	0.361	213	0.982(a) 0.634(d,r)	1.21×10^{-4}(a) 0.00229(d,r)	0.118(a) 0.0326(d,r)
rs4780167	31799489	G	0.41	0.905	266	0.111(a) 0.00388(d,r)	1.43×10^{-4}(a) 1.22×10^{-4}(d,r)	0.116(a)0.150(d,r)
rs10519874	31863494	G	0.38	0.507	247	0.786(a) 0.77(d,r)	1.62×10^{-4}(a) 0.365(d,r)	0.101(a) 0.0.0266(d,r)
rs7498093	31808568	G	0.43	0.208	249	0.83151(a) 0.901(d,r)	1.63×10^{-4}(a) 0.00208(d,r)	0.0494(a) 0.0254(d,r)
rs10519873	31862443	A	0.36	0.749	243	0.869(a) 0.848(d,r)	2.38×10^{-4}(a) 0.00292(d,r)	0.112(a) 0.068(d,r)
rs8028974	31785766	G	0.39	0.865	245	0.453(a) 0.0175(d,r)	3.21×10^{-4}(a) 0.00172(d,r)	0.748(a) 0.223(d,r)
rs10519875	31864956	A	0.37	0.557	248	0.77(a) 0.749(d,r)	5.16×10^{-4}(a) 0.00387(d,r)	0.14(a) 0.065(d,r)
rs12440440	31829188	A	0.30	0.134	244	0.283(a) 0.368(d,r)	5.50×10^{-4}(a) 0.00224(d,r)	0.522(a) 0.739(d,r)
rs17236525	31813550	T	0.37	0.978	248	0.663(a) 0.679(d,r)	6.16×10^{-4}(a) 0.0017(d,r)	0.0298(a) 8.17×10^{-4}(d,r)
rs7165052	31851908	T	0.43	0.823	258	0.825(a) 0.849(d,r)	7.13×10^{-4}(a) 0.00659(d,r)	0.615(a) 0.574(d,r)[g]

P values in bold are the ones retained statistical significant after correction for multiple testing using Bonferroni correction ($a = 0.05/279 = 1.79 \times 10^{-4}$) since a total of 279 SNPs in the RYR3 gene were used.
Abbreviations: AD, Alzheimer's disease; FBAT–GEE, family-based association tests using generalized estimating equations; SNP, single nuclear polymorphism.

[a]Physical position is based on NCBI Genome Build 36.3.

[b]Minor allele.

[c]Minor allele frequency.

[d]*P* value of Hardy–Weinberg equilibrium test.

[e]The number of informative families for risk of hypertension using an additive model.

[f]*P* value based on FBAT–GEE analysis for risk of hypertension.

[g]Letters in parentheses indicate the genetic models used for analysis (a, additive; d, dominant; r, recessive model).

[h]*P* value based on FBAT–GEE analysis for risk of diabetes.

[i]*P* value based on FBAT–GEE analysis for risk of Alzheimer's disease.

Table 3. Haplotypes associated with risk of hypertension and diabetes based on FBAT–GEE

SNPs	r^{2a}	Haplotype[b]	Frequency[c]	Fam#[d]	$P_{\text{FBAT–GEE}}$[e]
Hypertension					
rs4780118–rs11072471	0.87	A–T	0.59	222	0.00254
rs2291736–rs937303	0.63	A–C	0.20	190	0.00667
Diabetes					
rs12906709–rs4780167	0.44	G–G	0.33	282	2.71×10^{-5}
rs2288609–rs4780174	0.41	A–T	0.27	216	5.44×10^{-6}
rs10519875–rs11855625	0.34	A–G	0.32	266	6.71×10^{-5}

Abbreviations: FBAT–GEE, Family-based association tests using generalized estimating equations; SNP, single nuclear polymorphism.
[a]Linkage disequilibrium measure (r^2).
[b]Haplotype inferred from 2 SNPs.
[c]Haplotype frequency.
[d]Fam# refers to the number of informative families.
[e]*P* value based on FBAT–GEE analysis.

Single-marker analysis of age at onset of hypertension, diabetes, and AD in NIA-LOAD Family Study

In addition to exam RYR3 SNPs in association with the risks of 3 traits, we also tested these SNPs in association with AAO. The most significant association was revealed for rs10519818 which was shared by patients with hypertension ($P = 0.000227$ at both dominant and recessive models) and patients with diabetes ($P = 0.0119$ at additive genetic model, Table 4). Another disease-associated SNPs shared by hypertension and diabetes was rs12909478. Moreover, we also observed disease-associated SNPs shared by 3 diseases, they were rs7498093 and rs4780174 at the various genetic models (Table 4). Three disease-associated SNPs (rs17236525, rs12440440, and rs4238567) were also shared by diabetes and AD at the different genetic models (Table 4). These results, once again support the disease-risk SNPs and haplotypes in associations with AAO of hypertension and diabetes. The *P* values of all disease-associated SNPs in Table 4 were before correction for multiple testing. A common haplotype of A–C from rs2291736-rs937303 was associated with hypertension observed in 190 families ($P = 0.00667$, Table 5) and the common haplotype of G–G of rs2288609–rs4780174 SNPs showed an association with diabetes ($P = 5.68 \times 10^{-3}$).

Shared disease-associated SNPs and haplotype between the risk and AAO

We observed different disease-associated SNPs with disease risk and AAO; however, in the current study, we found several SNPs were associated not only with the disease risk, but also with AAO. SNP rs12440440 was found to be associated with diabetes risk (Table 2) and AAO for both diabetes and AD (Table 4). While SNP, rs17236525 was associated with both risk and AAO for diabetes and AD (Table 2 and 4). SNP rs2288609 was found to increase susceptibility for AAO and risk of diabetes. Finally, rs7498093 was observed association with risk of diabetes and AD as well as AAO for 3 diseases at various genetic models (Table 2 and 4).

In silico analysis

To test if the associated SNPs were located at regulatory gene regions and species-conserved regions, we used NIH-SNP Function Prediction. We found hypertension-associated SNPs, rs11072471 and rs16973323, were located at the species-conserved region and the gene regulatory region, respectively, which suggests the regions containing biological function.[32] Having established strong associations of disease-associated SNPs with AAO and risk of AD, we tested if the genotype at these SNPs are associated with levels of gene expression in various tissues, including brain and adrenal gland based on the data from the Genotype-Tissue Expression. We hypothesized the effects of the SNP genotypes on these 3 disease-risks may reflect genotype-based differences in levels of the gene expression. To investigate this, we analyzed recently released GTEx Consortium data. In postmortem samples from 100 to 120 normal individuals from the GTEx data set, among the SNPs we tested, the homozygous genotypes (T/T) for minor alleles of all 3 diseases (AD, hypertension and diabetes) associated SNP, rs2033610 were shown significantly decreased gene expression as compared with C/C and C/T genotypes in the adrenal glands ($P = 2.6 \times 10^{-7}$, Figure 1a). For rs2596164, GG genotype carriers showed the lowest levels of expression ($P = 2.8 \times 10^{-7}$) as compared with AG and AA genotypes in the adrenal glands (G is minor allele, Figure 1b). Finally, hypertension- and AD-associated SNP, rs8037864, was also shown statistically significantly decreased gene expression of homozygous GG genotype as compared with T/G and T/T genotypes in the cells of transformed fibroblasts ($P = 4.7 \times 10^{-5}$).

DISCUSSION

Supporting our hypothesis, we identified unique and shared SNPs of *RYR3* with hypertension, diabetes, and AD. In addition, the results of haplotype analyses and

Table 4. Single-marker analysis of age at onset of hypertension, diabetes, and Alzheimer's disease based on FBAT–Wilcoxon test ($P < 0.05$)

SNP	Position[a]	AL[b]	MAF[c]	HWE[d]	Fam#[e]	P-$_{FBAT-GEE}$[f] for hypertension	P-$_{FBAT-GEE}$[h] for diabetes	P-$_{FBAT-GEE}$[i] for AD
rs10519818	31499690	C	0.12	0.228	62	0.00186(a)0.000227(d,r)[g]	0.0119(a) 0.0174(d,r)	0.573(a) 0.0943(d,r)
rs2596230	31508018	C	0.12	0.275	65	0.00309(a) 0.00498(d,r)	0.113(a) 0.139(d,r)	0.946(a) 0.643(d,r)
rs2018899	31546496	T	0.09	0.592	50	0.0419(a) 0.00484(d,r)	0.206(a) 0.298(d,r)	0.879(a) 0.836(d,r)
rs7498093	31808568	G	0.42	0.208	125	0.255(a) 0.0381(d,r)	0.00273(a) 0.0138(d,r)	3.6×10^{-4}(a) 0.00345(d,r)
rs4780174	31822821	T	0.48	0.176	121	0.103(a) 0.0316(d,r)	0.00388(a) 0.0336(d,r)	0.00355(a) 0.00343(d,r)
rs1036006	31900157	G	0.36	0.566	111	0.399(a) 0.406(d,r)	0.00457(a) 0.00599(d,r)	0.828(a) 0.589(d,r)
rs12914825	31902573	T	0.09	0.203	52	0.679(a) 0.811(d,r)	0.0116(a) 0.00218(d,r)	0.926(a) 0.581(d,r)
rs2288609	31821738	A	0.27	0.319	116	0.777(a) 0.0346(d,r)	0.0126(a) 0.0256(d,r)	0.0981(a) 0.24(d,r)
rs17236525	31813550	T	0.37	0.978	119	0.152(a) 0.865(d,r)	0.0144(a) 0.0183(d,r)	0.00447(a) 0.00351(d,r)
rs2059956	31910824	T	0.46	0.228	129	0.423(a) 0.509(d,r)	0.0197(a) 0.0159(d,r)	0.5(a) 0.397(d,r)
rs12440440	31829188	A	0.30	0.134	130	0.468(a) 0.494(d,r)	0.0305(a) 0.556(d,r)	0.00256(a) 0.0153(d,r)
rs11072687	31895202	T	0.27	0.983	100	0.193(a) 0.295(d,r)	0.0357(a) 0.0353(d,r)	0.799(a) 0.605(d,r)
rs7165389	31589238	C	0.12	0.327	55	0.75(a) 0.617(d,r)	0.0365(a) 0.0706(d,r)	0.627(a) 0.574(d,r)
rs4780144	31741944	C	0.05	0.015	23	0.136(a) 0.136(d,r)	0.0389(a) 0.0389(d,r)	0.603(a) 0.621(d,r)
rs12909478	31616542	T	0.19	0.831	76	0.389(a) 0.0269(d,r)	0.0407(a) 0.0288(d,r)	0.988(a) 0.84(d,r)
rs4238567	31714681	T	0.49	0.064	129	0.834(a) 0.865(d,r)	0.0411(a) 0.473(d,r)	0.00443(a) 0.0043(d,r)
rs12901404	31862592	C	0.14	0.719	64	0.511(a) 0.786(d,r)	0.0498(a) 0.0737(d,r)	0.628(a) 0.543(d,r)

Abbreviations: AD, Alzheimer's disease; FBAT–GEE, family-based association tests using generalized estimating equations; SNP, single nuclear polymorphism.
[a]Physical position is based on NCBI Genome Build 36.3.
[b]Minor allele.
[c]Minor allele frequency.
[d]*P* value of Hardy-Weinberg equilibrium test.
[e]The number of informative families for age at onset of hypertension using an additive model.
[f]*P* value based on FBAT–Wilcoxon test for age at onset of hypertension.
[g]Letters in parentheses indicate the genetic models used for analysis (a, additive; d, dominant; r, recessive model).
[h]*P* value based on FBAT–Wilcoxon test for age at onset of diabetes.
[i]*P* value based on FBAT–Wilcoxon test for age at onset of Alzheimer's disease.

Table 5. Haplotypes associated with age at onset of hypertension and diabetes based on FBAT–Wilcoxon test

SNPs	r^2[a]	Haplotype[b]	Frequency[c]	Fam#[d]	$P_{FBAT-GEE}$[e]
Hypertension					
rs2596240–rs8023659	0.30	A–G	0.03	22	0.00888
rs2291736–rs937303	0.63	A–C	0.20	190	0.00667
Diabetes					
rs16970801–rs16970823	0.70	C–A	0.06	20	0.00949
rs2288609–rs4780174	0.41	G–G	0.43	44	0.00568

Abbreviations: FBAT–GEE, Family-based association tests using generalized estimating equations; SNP, single nuclear polymorphism.
[a]Linkage disequilibrium measure (r^2).
[b]Haplotype inferred from 2 SNPs.
[c]Haplotype frequency.
[d]Fam# refers to the number of informative families.
[e]*P* value based on FBAT–Wilcoxon test.

disease-risk SNP function predictions of *RYR3* support the role of its variants in these complex and multifactorial diseases. Furthermore, hypertension risk SNPs rs11072471 and rs16973323 were found located at the species-well-conserved region indicating functional importance of *RYR3*, which supports how important it is to identify potentially important conserved noncoding sequences in association with these complex diseases. A recent study reports an identification of a major quantitative trait locus on chromosome 15q26 for systolic blood pressure (A logarithm of the odds [LOD] = 3.36), which is close to RYR gene location (15q13.3) in Mexican-Americans.[33] Furthermore, 2 disease-associated SNPs (rs2033610 and rs2596164) also showed alterations of gene expression in the adrenal gland tissue, which plays an important role in secreting hormones that regulate both blood sugar and pressure. Additionally, a recent study has confirmed their previous findings of gene–gene interactions of *RYR3* and *CACNA1C* in late-onset of AD using endo-phenotype analysis.[34]

Overall, results strongly imply the existence of shared SNPs associated of the RYR3 gene across these 3 diseases. A number of previous studies identified common genes and variants among diabetes, hypertension, and other phenotypes,[35] e.g., lipoprotein lipase gene is associated with hypertension, AD, type 2 diabetes, and coronary heart disease.[6] There might be a pathophysiology mechanism of sharing genetic variants for these 3 traits, as previous studies suggest that high glucose level and high blood pressure affect AD through multiple mechanisms, including reduction in cerebral blood flow[36] and breakdown of the blood–brain barrier.[37] A recent study (39) has also showed that excess sugar in the blood can lead to organ and even brain damage, which can lead to dementia as well as early onset of AD. It has been demonstrated that alterations of calcium (Ca^{2+})-signaling pathway are involved in hypertension, diabetes, and AD based on functional studies.[24,25] To the best of our knowledge, this is the first study of investigating the association of RYR3 gene with the risk and AAO of hypertension, diabetes, and AD.

The strengths of this study include the comparatively medium sample size used which is of relatively large size for this type of study. The sample was also ethnically homogenous (US European decent), which gives indication that within this ethnic community the same genetic evidence may be replicated. Multiple analyses were performed using single-marker analysis FBAT–GEE and FBAT–Wilcoxon. The RYRs associated variants were also supported by the results of haplotype and *in-silico* analyses. We used a family-based design, which can reduce the type 1 error rate arising from population stratification. Especially, the FBAT–GEE approach in the PBAT software can easily be adapted to scenarios with multiple offspring per family and missing parental information, and testing for linkage disequilibrium under the assumption of linkage.[38] We are also aware of some limitations: among disease-associated SNPs, only 5 diabetes-risk SNPs were retained after multiple testing using the Bonferroni correction, other unique and shared SNPs did not; however, it is well known that Bonferroni correction is a very conservative method. Moreover, we cannot exclude contribution from environmental or behavioral factors or other nongenetic correlations. Additional replication of these results is also necessary. Finally, our current findings might be spurious or subject to type I error. Future confirmatory studies of the *RYR3* in these 3 traits in independent samples or targeted genome sequencing of the gene for these diseases may provide an opportunity to dissect the genetic complexity of this gene more accurately.

In conclusion, we provide genetic evidence of unique and shared disease-associated *RYR3* polymorphisms among hypertension, diabetes, and AD. Suggesting an etiologic relationship between them, calcium disturbances and certain calcium-signaling pathways may be involved in pathophysiology of these traits. These results will provide a basis for replication in larger samples and/or other populations to elucidate the potential role of these genetic variants.

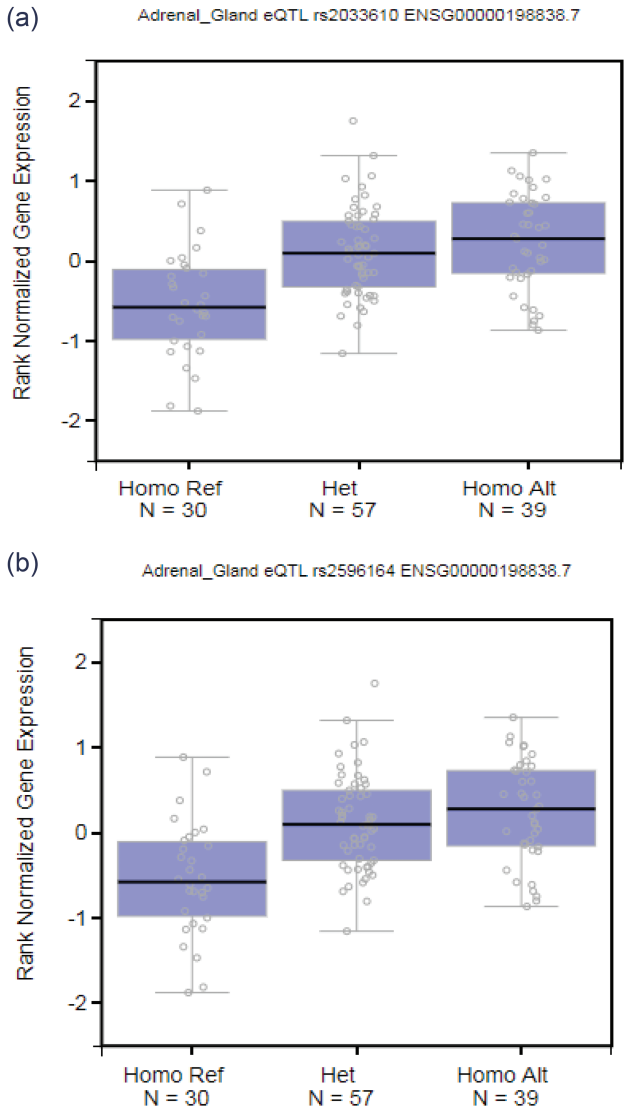

Figure 1. Based on the GTEx Consortium data of postmortem samples from 100 to 120 normal individuals,the homozygous genotypes (T/T) for minor alleles of all three diseases (AD, hypertension and diabetes) associated SNP, rs2033610 were shown significantly decreased gene expression as compared with C/C and C/T genotypes in the adrenal glands (P = 2.6 x 10-7, [**a**]). For rs2596164 GG genotype carriers showed the lowest levels of expression ($P = 2.8 \times 10^{-7}$) as compared with AG and AA genotypes in the adrenal glands (G is Pminor allele, [**b**]). (**a**) Genotypes at 3 disease risk variant, rs2033610, were also associates with gene expression in the adrenal glands in eQTLBoxplot ($P = 2.6 \times 10^{-7}$) from the GTEx Consortium, with TT (Home Ref, N = 30) genotype carriers showing the lowest levels of expression. Medians and interquartile ranges are indicated. CT genotype (Het, N = 57) and CC genotype (Homo Alt, N = 39) are shown. (**b**) Genotypes at 3 disease risk variant, rs2596164, of the RYR3 gene were also associates with gene expression in the adrenal glands in eQTLBoxplot ($P = 2.6 \times 10^{-7}$) from the GTEx Consortium, with GG (Home Ref, N = 30) genotype carriers showing the lowest levels of expression. Medians and interquartile ranges are indicated. AG genotype (Het, N = 57) and AA genotype (Homo Alt, N = 39) are shown.

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DISCLOSURE

The authors declared no conflict of interest.

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