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Authors
Nelson, Peter T
Estus, Steven
Abner, Erin L
et al.

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ABCC9 gene polymorphism is associated with hippocampal sclerosis of aging pathology

Peter T. Nelson,
Department of Pathology, Division of Neuropathology, Rm 311, Sanders-Brown Center on Aging, University of Kentucky, 800 S. Limestone Avenue, Lexington, KY 40536-0230, USA

Steven Estus,
University of Kentucky, Lexington, KY, USA

Erin L. Abner,
University of Kentucky, Lexington, KY, USA

Ishita Parikh,
University of Kentucky, Lexington, KY, USA

Manasi Malik,
University of Kentucky, Lexington, KY, USA

Janna H. Neltner,
University of Kentucky, Lexington, KY, USA

Eseosa Ighodaro,
University of Kentucky, Lexington, KY, USA

Wang-Xia Wang,
University of Kentucky, Lexington, KY, USA

Bernard R. Wilfred,
University of Kentucky, Lexington, KY, USA

Li-San Wang,
University of Pennsylvania, Philadelphia, PA, USA

Walter A. Kukull,
University of Washington, Seattle, WA, USA

Kannabiran Nandakumar,
Mayo Clinic, Rochester, MN, USA

Mark L. Farman,
University of Kentucky, Lexington, KY, USA

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Correspondence to: Peter T. Nelson, pnels2@email.uky.edupeter.nelson@uky.edu; David W. Fardo, david.fardo@uky.edu.

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Wayne W. Poon,
University of California, Irvine, Irvine, CA, USA

Maria M. Corrada,
University of California, Irvine, Irvine, CA, USA

Claudia H. Kawas,
University of California, Irvine, Irvine, CA, USA

David H. Cribbs,
University of California, Irvine, Irvine, CA, USA

David A. Bennett,
Rush University, Chicago, IL, USA

Julie A. Schneider,
Rush University, Chicago, IL, USA

Eric B. Larson,
Group Health Research Institute, Seattle, WA, USA

Paul K. Crane,
University of Washington, Seattle, WA, USA

Otto Valladares,
University of Pennsylvania, Philadelphia, PA, USA

Frederick A. Schmitt,
University of Kentucky, Lexington, KY, USA

Richard J. Kryscio,
University of Kentucky, Lexington, KY, USA

Gregory A. Jicha,
University of Kentucky, Lexington, KY, USA

Charles D. Smith,
University of Kentucky, Lexington, KY, USA

Stephen W. Scheff,
University of Kentucky, Lexington, KY, USA

Joshua A. Sonnen,
University of Utah, Salt Lake City, UT, USA

Jonathan L. Haines,
Case Western Reserve University, Cleveland, OH, USA

Margaret A. Pericak-Vance,
University of Miami, Miami, FL, USA

Richard Mayeux,
Columbia University, New York, NY, USA

Lindsay A. Farrer,

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Boston University, Boston, MA, USA

Linda J. Van Eldik,
University of Kentucky, Lexington, KY, USA

Craig Horbinski,
University of Kentucky, Lexington, KY, USA

Robert C. Green,
Harvard University, Boston, MA, USA

Marla Gearing,
Emory University, Atlanta, GA, USA

Leonard W. Poon,
Institute of Gerontology, University of Georgia, Athens, GA, USA

Patricia L. Kramer,
Oregon Health and Sciences University, Portland, OR, USA

Randall L. Woltjer,
Oregon Health and Sciences University, Portland, OR, USA

Thomas J. Montine,
University of Washington, Seattle, WA, USA

Amanda B. Partch,
University of Pennsylvania, Philadelphia, PA, USA

Alexander J. Rajic,
University of California, Irvine, Irvine, CA, USA

KatieRose Richmire,
Group Health Research Institute, Seattle, WA, USA

Sarah E. Monsell,
University of Washington, Seattle, WA, USA

Alzheimer' Disease Genetic Consortium, **Gerard D. Schellenberg**, and
University of Pennsylvania, Philadelphia, PA, USA

David W. Fardo
Department of Biostatistics, University of Kentucky, 205E Multidisciplinary Science Building, 725 Rose Street, Lexington, KY 40536-0082, USA

Peter T. Nelson: pnels2@email.uky.edu; David W. Fardo: david.fardo@uky.edu

Abstract

Hippocampal sclerosis of aging (HS-Aging) is a high-morbidity brain disease in the elderly but risk factors are largely unknown. We report the first genome-wide association study (GWAS) with HS-Aging pathology as an endophenotype. In collaboration with the Alzheimer's Disease Genetics Consortium, data were analyzed from large autopsy cohorts: (#1) National Alzheimer's Coordinating Center (NACC); (#2) Rush University Religious Orders Study and Memory and

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Aging Project; (#3) Group Health Research Institute Adult Changes in Thought study; (#4) University of California at Irvine 90+ Study; and (#5) University of Kentucky Alzheimer's Disease Center. Altogether, 363 HS-Aging cases and 2,303 controls, all pathologically confirmed, provided statistical power to test for risk alleles with large effect size. A two-tier study design included GWAS from cohorts #1–3 (Stage I) to identify promising SNP candidates, followed by focused evaluation of particular SNPs in cohorts #4–5 (Stage II). Polymorphism in the ATP-binding cassette, sub-family C member 9 (*ABCC9*) gene, also known as sulfonylurea receptor 2, was associated with HS-Aging pathology. In the meta-analyzed Stage I GWAS, *ABCC9* polymorphisms yielded the lowest *p* values, and factoring in the Stage II results, the meta-analyzed risk SNP (rs704178:G) attained genome-wide statistical significance ($p = 1.4 \times 10^{-9}$), with odds ratio (OR) of 2.13 (recessive mode of inheritance). For SNPs previously linked to hippocampal sclerosis, meta-analyses of Stage I results show OR = 1.16 for rs5848 (*GRN*) and OR = 1.22 rs1990622 (*TMEM106B*), with the risk alleles as previously described. Sulfonylureas, a widely prescribed drug class used to treat diabetes, also modify human ABCC9 protein function. A subsample of patients from the NACC database ($n = 624$) were identified who were older than age 85 at death with known drug history. Controlling for important confounders such as diabetes itself, exposure to a sulfonylurea drug was associated with risk for HS-Aging pathology ($p = 0.03$). Thus, we describe a novel and targetable dementia risk factor.

Keywords

Oldest old; Neuropathology; KATP; CTAGE5; ADGC; Potassium channel

Introduction

In elderly individuals, hippocampal sclerosis of aging (HS-Aging) is a relatively common brain disease [41, 50]; for recent reviews, see Refs. [51, 80]. HS-Aging is defined by pathological manifestations: neuron loss, gliosis, and atrophy in the hippocampal formation, not deemed attributable to Alzheimer disease (AD)-type plaques and tangles [1, 44, 51]. The presence of HS-Aging pathology is associated with substantial cognitive impairment, independent of comorbid pathologies [48], yet this disease is generally misdiagnosed as AD in the clinical setting [9, 57].

Genetic risk factors of HS-Aging are not fully characterized. Hippocampal sclerosis pathology in AD cases has been linked to single nucleotide polymorphisms (SNPs) at genomic loci previously associated with frontotemporal lobar degeneration (FTLD), namely rs5848 (*GRN*) and rs1990622 (near *TMEM106B*) [17, 63, 76]. In contrast to AD, the risk of developing HS-Aging pathology is not associated with apolipoprotein E (*APOE*) alleles [9, 41, 50, 72].

There has been no prior published genome-wide association study (GWAS) focused on HS-Aging pathology. The endophenotype is deceptively complex [51, 80] and confers both potential obstacles and opportunities for a GWAS. Challenges include the requirement for reliable autopsy data on every research subject and the advanced old age of many patients with HS-Aging [50, 79]. Even archival neuropathology data must be interpreted with

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caution because HS-Aging has only recently been the subject of widespread attention by researchers [9, 51, 57, 62]. After accounting for these difficulties, there are characteristics of HS-Aging pathology that are conducive for GWAS. Most important is the high disease prevalence which enables analyses of diverse study cohorts including population-based samples [41, 51, 80]. HS-Aging pathology tends to co-occur with hippocampal TAR DNA-binding protein 43 (TDP-43) pathology [2, 36, 54], an important component of the pathological endophenotype [51]. Thus, a successful HS-Aging GWAS study would detect allele(s) associated with hippocampal sclerosis and TDP-43 pathologies across multiple cohorts of autopsied elderly individuals.

For the current study, genomic and pathologic data from dozens of different research centers were analyzed with the goal of identifying alleles associated with risk for HS-Aging pathology. Altogether these analyses incorporated 363 HS-Aging cases and 2,303 controls with neuropathologic evaluation. A two-tiered study design employed a GWAS to identify promising risk allele candidates, with subsequent evaluation of specific candidate alleles using separate cohorts. Our main findings are (1) a polymorphism in the ATP-binding cassette, sub-family C member 9 (*ABCC9*) gene, also known as sulfonylurea receptor 2 (SUR2), is associated with HS-Aging pathology, and (2) exposure to sulfonylurea drugs is associated with increased risk for HS-Aging pathology among individuals who died age 85 and older.

Materials and methods

Datasets

The Alzheimer's Disease Genetics Consortium (ADGC) accrued genomics data from 34 different research centers (US National Institute on Aging Alzheimer's Disease Centers, or ADCs) with multiple iterations of GWAS data (References [29, 39, 47] and see below), which were analyzed with neuropathological and clinical data obtained through the National Alzheimer's Coordinating Center (NACC; see Reference [4]). Research using NACC data was approved by the University of Washington Human Subjects Division; other protocols were supervised by local Institutional Review Boards. Neuropathologic evaluations were performed according to center-specific protocols–including whether neuropathologists studied left, right, or bilateral hippocampi [9]—and entered into a database in standardized format. Details of HS-Aging case/control operationalization have been discussed before [9] and are described in detail in Supplemental Methods. University of Kentucky submits data to NACC, but data from that center were excluded from the Stage I cohorts. In addition to the ADGC/NACC group, autopsy series included in the Stage I phase were the Rush University Religious Orders Study and Memory and Aging Project (ROS-MAP) with recruitment in Chicago, IL, USA [7], and the Adult Changes in Thought (ACT) study from the Group Health Cooperative and University of Washington with recruitment in Seattle, WA, USA [45].

Following the GWAS on Stage I datasets, additional studies were performed analyzing data from two separate cohorts (Stage II): the University of Kentucky Alzheimer's Disease Center (UK-ADC; included in this cohort were research subjects ($n = 49$) from the Georgia Centenarian Study [59] that were evaluated neuropathologically at the University of

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Kentucky [50]), and the University of California at Irvine 90+ Study (UCI90+). UCI90+ is a population-based longitudinal study of individuals aged 90 and older, with recruitment in Orange County, CA, USA; there is no overlap with the ADGC/NACC data. Descriptions of these autopsy cohorts were published previously [3, 15, 50, 65]. Immunohistochemical stains on hippocampal formation were performed using phosphorylated TDP-43 (P-TDP43) antibody 1D3 [53] as described previously [51]. The reason for using P-TDP43 immunostaining-based endophenotype for UK-ADC and not UCI90+ relates to a lack of analogous P-TDP43 immunohistochemical data.

For assessment of the prevalence of HS-Aging pathology in a population-based cohort, relative to other neuropathologically defined diseases, the Nun Study data [46, 74] were analyzed (n = 436 research subjects). These data were separate from the genomics analyses. Nun study neuropathologic methods were performed as previously described [50]. For the present study, neuropathologic observations were graded independently of each other: HS-Aging pathology [44, 51], AD pathology [stratified by Braak stages [8] using both a stringent criterion (Stages V/VI) and another that also incorporates milder cases (Stages IV–VI)], neocortical Lewy body pathology of a severity to indicate “high likelihood” that “the pathologic findings are associated with a DLB clinical syndrome” [43], and FTLD [42].

Statistical methods

In silico studies were performed for feasibility and power analyses. With the sample size of the Stage I cohorts (241 cases, 1,998 controls), the probability of determining a “true-positive” SNP associated with HS-Aging pathology, i.e. statistical power, was calculated. QUANTO software v.1.2.4 [25] was used to model the impact on statistical power of varying minor allele frequency (MAF) and the effect size of the risk allele. That effect size is operationalized by the odds ratio (OR) of HS-Aging pathology in risk SNP carriers versus non-carriers. Analyses were performed for an experiment that would meet criteria for genome-wide significance, pre-specified at $p < 5 \times 10^{-8}$ [11, 39]. This threshold corrects for multiple comparisons while also assuming that many SNPs are in linkage disequilibrium with each other and thus are not truly independent tests [20, 28]. A second model was performed relaxing the criteria for genome-wide statistical significance to $p < 10^{-5}$ to reflect the requirement of a risk allele candidate that could be tested separately in Stage II cohorts.

Stage I (GWAS) and Stage II (focused on individual SNP) studies

GWAS data access was coordinated through ADGC, providing quality-controlled genotypes using imputation to the Phase I v.3 1000 Genomes Project March 2012 release with imputation as previously described [39]. Additional quality control (QC) was applied within each Stage I dataset. To reduce the potential biases associated with population stratification, principal components (PCs) were iteratively calculated with normalized SNP data, and subjects with outlying values were removed after each of six iterations using SVS™ software. An additive mode of inheritance (MOI) was assumed in each cohort from the Stage I stage to balance statistical power with concerns of multiple testing, and logistic regression was performed for each SNP with correction for PCs. The number of PCs chosen for adjustment was based on examination of scree plots (Supplemental Fig. 1) for each Stage I cohort: four PCs for NACC and ROS-MAP; three PCs for ACT. For assessment of rs5848

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and rs1990622 SNPs, for which prior published studies derived from Mayo Clinic data [57, 61, 63], a dataset lacking that institution's participants was used for analyses of those alleles. Results across the three Stage I datasets were meta-analyzed using an inverse-variance-based statistic [70]. LocusZoom plots [60] reflect regional meta-analyzed *p* values from all the Stage I datasets.

In the Stage II cohorts, SNPs were characterized using Life Technologies' TaqMan-based SNP assays providing direct assessment of genotype. All tests for genotype/HS-Aging association were two-tailed. Meta-analyses were performed for the two Stage II cohorts, and then data were combined across all Stage I and Stage II cohorts, using an inverse-variance-based statistic as above. Forest plots displaying cohort-specific and aggregated ORs and confidence intervals were created reflecting these meta-analyzed results using R v.3.0.2 programming language [71].

Genomic *ABCC9* sequencing from HS-Aging cases and controls

Gene-specific genomic sequencing was performed on DNA from six individuals with pathologic and genomic information (three rs704178 G/G HS-Aging cases, three elderly rs704178 C/C controls). Data pertaining to all six individuals are provided in Supplemental Methods, as are details of the sequencing protocol. Briefly, *ABCC9* sequences were captured using the HaloPlex Target Enrichment System (Agilent Technologies) to amplify fragments spanning the human genomic region chr12:21950273-22094386 (GRCh37/hg19 genomic assembly). The six resulting fragment libraries were run in a single flow cell on a HiSeq 2500 (Illumina). The variant files were inspected both manually and with custom Perl scripts.

Western blots on hippocampal proteins from HS-Aging cases and controls

Sequential biochemical fractionation and immunoblot studies employed techniques modified from previous protocols [51, 54, 64] as described in detail in Supplemental Methods. Briefly, hippocampal tissue (CA1 and subiculum) was dissected from a convenience sample of postmortem brain specimens ($n = 9$) including subjects with or without the *ABCC9* polymorphism, and with or without HS-Aging pathology, which were extracted into separate low salt-, 1 % Triton-X100-, 1 % Sarkosyl+heat-, and 7M urea-extractable fractions. Equal amounts of protein were loaded to a denaturing SDS polyacrylamide gel prior to immunoblot analyses. Antibodies used for immunoblots were as follows: anti-SUR2B (C-15): sc-5793; anti-SUR2A (T-19): sc-32461; and anti-SUR2 (H-80): sc-25684, all three from Santa Cruz Biotechnology, and anti-SUR2B (N323A/31) and anti-SUR2A (N319A/14) from EMD Millipore. Control immunoblots using the same samples were performed using anti-PGRN/GRN (R&D Cat # AF 2420) and anti-β-Actin (Rockland Code 600-401-886) antibodies; see Supplemental Methods.

NACC neuropathology and drug data

The initial data source for these analyses was research volunteers evaluated after 2009 at an ADC with both reported clinical (including drug information) and autopsy data. Participants were excluded if they were younger than 85 at death to best match all participants to HS-Aging age range. Methods and rationale for the UDS clinical examination have been

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previously published [4, 5]. All participants received an initial in-person clinical evaluation and up to seven follow-up evaluations; data were collected on an approximately annual basis. In this analysis, HS-Aging pathology was defined as present if the neuropathologist recorded a "primary or contributing pathologic diagnosis of hippocampal sclerosis". Assessment of TDP-43 pathology was not collected on the NACC Neuropathology form, but allowed as a write-in; for individuals without FTLD, where some sort of medial temporal lobe TDP-43 pathology was noted, these cases were considered to be HS-Aging ($n = 4$).

For statistical analyses of the NACC data, longitudinal self-reported medication histories were reviewed for the 624 included subjects. Medication use was determined by asking subjects to provide a list of all medications taken within the last 2 weeks prior to their UDS assessment. Individuals who reported taking any sulfonylurea at any time during follow-up (September 2005 through April 2013) were coded as positive for use. Crude and adjusted ORs for the presence of HS-Aging at autopsy were calculated separately. Adjusted ORs were obtained via logistic regression, controlling for age at death (centered at 90) and year of death (centered at 2011). Possible group differences in mean age at death and interval between last evaluation and death were assessed using linear regression. Sensitivity analyses were conducted restricting the medication history to the last UDS assessment. Analyses were conducted using SAS/STAT 9.3®. Two-tailed tests were used to evaluate the hypothesis that use of sulfonylureas promotes HS-Aging pathology ($\alpha = 0.05$).

Results

Endophenotype for GWAS and subsequent studies

The endophenotype used for correlation with genomic information was HS-Aging pathology [9, 51]. Photomicrographs (Fig. 1) demonstrate features of HS-Aging pathology discernible after staining human hippocampal sections with hematoxylin and eosin (H&E) and using P-TDP-43 immunohistochemistry. A three-tier (0–2) semiquantitative scale of P-TDP-43 immunoreactive pathology was used in the UK-ADC dataset. Data about the prevalence of HS-Aging pathology, from the Nun Study, are shown in Fig. 2. Nun Study research volunteers were followed as a population-based cohort, mostly from normal status, and with high autopsy rate [46, 74]; this group lacked some of the recruitment biases of dementia clinic cohorts [6, 35, 46, 50]. Even when younger Nun Study research volunteers were included from the sample studied neuropathologically at University of Kentucky (total $n = 526$), no FTLD-TDP cases were observed (data not shown). By contrast, HS-Aging pathology is relatively prevalent in population-based cohorts, which are key sources of data in the current study (ROS-MAP, ACT, and UCI90+ studies).

Statistical and feasibility considerations

GWAS in Stage I cohorts was used to identify risk SNP candidates, followed by separate analyses of individual SNPs in Stage II cohorts (Fig. 3). This study design involved three key assumptions:

Assumption #1 (*central hypothesis being tested in the study*)> As there has been no prior published HS-Aging GWAS, polymorphisms remain to be described with

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strongly increased odds of HS-Aging pathology in risk allele carriers versus non-carriers, i.e. OR >1.5 for the risk allele;

Assumption #2> The Stage I cohort GWAS comprises adequate sample sizes to yield a limited number of credible candidate risk SNPs for evaluation in the Stage II cohorts; and

Assumption #3> The Stage II cohorts have sufficient numbers of HS-Aging cases and controls to adequately test for an HS-Aging risk SNP candidate.

Power analyses were performed to test whether Assumptions #2 and #3 were valid if the large effect size SNP (Assumption #1) exists. The effect size strongly influences statistical considerations (Fig. 4). Given sample sizes, the likelihood of uncovering an HS-Aging risk allele with genome-wide statistical significance ($p < 5 \times 10^{-8}$) in the Stage I GWAS was only modest, unless the effect size was quite large (Fig. 4a). However, the Stage I cohort was adequately powered for the purposes of identifying risk SNP candidates for downstream evaluation. In the presence of a strong HS-Aging risk allele (OR >1.5), there was a high chance that the risk allele would be identified in the Stage I cohort GWAS with $p < 10^{-5}$ (Fig. 4b). Definitive testing of the candidate risk SNPs would require downstream validation in independent cohorts. Stage II would be most effective in the case of a SNP with high MAF and with studies of individual SNPs performed serially so that the final cohort's analyses require no adjustment for multiple comparisons.

GWAS

As a positive control, a GWAS from AD pathology was performed initially on a sample from the ADGC cohort (1,443 AD cases and 99 controls) and as expected, only SNPs near the *APOE* gene reached genome-wide significance ($p = 4.2 \times 10^{-13}$; Supplemental Tables 1 and 2; Supplemental Fig. 2). We then performed a HS-Aging GWAS using data from the three Stage I datasets: ADGC/NACC, ROS-MAP, and ACT (Table 1 and Supplemental Table 3). The number of nominal SNPs that passed QC and were included in the GWAS was ~4.9 million (Table 2). HS-Aging cases and controls were classified according to pathology irrespective of AD or other prevalent pathologies; see Supplemental Methods for details. Manhattan plot and Q–Q plot for the HS-Aging GWAS are shown in Fig. 5; no Stage II cohort data were factored into these analyses.

The GWAS included individual SNPs previously linked to HS-type pathology [57], specifically, rs5848 [61] and rs1990622 [63]. For analyses of these alleles, a separate GWAS was performed using Stage I cohort cases and controls that did not overlap with prior studies of rs5848 and rs1990622. The SNPs previously linked to HS-Aging pathology can be tested with less stringent p value cutoffs due to targeted testing ($\alpha = 0.05$; two-tailed tests are reported without adjustment for the performance of two tests). Results are shown in Table 3 and Fig. 6. Meta-analyses of Stage I cohorts results show OR = 1.16 (95 % CI 0.92–1.46) for rs5848, OR = 1.22 (95 % CI 1.00–1.49) for rs1990622, with all cohorts tested demonstrating the same risk alleles (T allele for both SNPs) as previously described [61, 63].

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GWAS from meta-analyzed Stage I datasets detected no SNP associated with HS-Aging pathology so strongly as to achieve genome-wide statistical significance. The genomic locus bearing SNPs with the lowest *p* values in the GWAS data were in the *ABCC9* gene on chromosome 12p. The individual SNP that yielded the lowest *p* value in the GWAS itself was rs7966849 ($p = 1.0 \times 10^{-7}$, uncorrected, see Supplemental Fig. 3) in an intron of *ABCC9*. SNPs from only nine different genomic regions showed $p < 10^{-5}$ in the GWAS (Table 4, Supplemental Table 4), including *ABCC9* (15 nominal SNPs) and SNPs in and near *CTAGE5/FBOXO33* on chromosome 14 (50 nominal SNPs; Supplemental Fig. 4).

Stage II analyses

The initial criteria for deciding which candidate HS-Aging risk alleles to test in the Stage II cohorts were that the same alleles would yield $p < 0.05$ in all three Stage I cohorts. This led us to consider *ABCC9* putative risk SNPs including rs704178, a SNP with MAF ~0.45, in strong linkage disequilibrium with rs7966849 ($R^2 = 0.97$ and $D' = 1.0$) [23]. Genotype frequencies for rs704178 in HS-Aging cases and controls are displayed in Table 5. After accumulation of additional data, the rs704178 allele became the study's focal-point (Figs. 7, 8, 9). Primary data from *ABCC9* risk SNP rs704178 for HS-Aging cases and controls from the Stage II cohorts are presented in Table 6.

The UK-ADC was the first-stage Stage II cohort for evaluating HS-Aging candidate risk SNPs. For this cohort, the primary criterion for a positive "case" status was aberrant P-TDP-43 immunoreactivity in the hippocampus and subiculum, which we hypothesize provides (in the context of elderly individuals without FTLD) a sensitive and specific readout of HS-Aging pathology [2, 51]. From this convenience sample of 227 patients, 82 (36.1 %) were positive for P-TDP43 pathology. Ten initial SNP risk allele candidates from the Stage I datasets were evaluated in the UK-ADC cohort: rs7206273, rs1538842, rs4869959, rs5848, rs201825, rs2274567, rs6656401, rs2287910, rs4144545, rs704179, and rs704178. Of these, rs704178 was the only SNP showing positive association with the pathologic endophenotype.

Data from the UCI90+ study [15, 16], a population-based sample of 144 controls and 28 HS-Aging cases, was the second Stage II cohort. Here, H&E-based observation of hippocampal sclerosis pathology was used as the readout, since not all the cases were stained with the same P-TDP43 methods. The risk alleles (rs704178:G) trended toward positive association with HS pathology; no other SNP was tested in this dataset except rs704180, which was in complete linkage disequilibrium with rs704178 as expected (not shown). This trend was only statistically significant if the recessive MOI model was applied ($p < 0.03$ using two-tailed test); however, the effect size was still large at OR = 1.43 (95 % CI 0.80–2.6) even with additive MOI model and two-tailed test.

Combined meta-analyses of all Stage I and Stage II cohorts yielded a *p* value of 2.4×10^{-8} with an additive MOI model and a *p* value of 1.4×10^{-9} with recessive MOI (Fig. 8). Dominant MOI model had far weaker association as is shown in Supplemental Fig. 5. Having found the same *ABCC9* gene polymorphism is associated with hippocampal P-TDP43 pathology or HS-Aging pathology in two Stage II cohorts in addition to HS-Aging pathology in the Stage I cohorts, we focused on *ABCC9* in subsequent studies.

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Post-hoc studies related to ABCC9

To assess preliminarily whether the *ABCC9* risk alleles are proxies for as-yet uncharacterized genomic feature(s), *ABCC9* gene-specific sequencing was performed using genomic DNA (Supplemental Methods). For these studies, DNA was isolated from hippocampi of three autopsy-confirmed HS-Aging cases homozygous for *ABCC9* risk SNPs (rs704178: G/G), and the results were compared to three individuals over 95 at death (rs704178: C/C) and lacking HS-Aging pathology (Supplemental Methods). No previously uncharacterized genomic feature (including exonic SNP) was discovered for which rs704178 would be a proxy. As expected, SNPs in known linkage disequilibrium with rs704178 span a region over 10 exons (Fig. 9).

Western blots were performed on protein samples from snap-frozen human hippocampi to address whether differences in ABCC9 polypeptides can be observed in correlation with particular HS-Aging risk alleles or pathologic status. These experiments were designed to test for a qualitative change in ABCC9 protein such as insoluble aggregates, proteolytic fragments, or complete loss of expression. The convenience subsample from the UK-ADC comprised nine human hippocampi including HS-Aging and controls, and different homozygous *ABCC9* genotypes. Different protein extraction methods were utilized and five different commercially available anti-ABCC9 antibodies were applied. These experiments discovered no evidence of a specific qualitative change in hippocampal ABCC9 protein products, with caveats related to the small number of cases evaluated and to the inconsistency in the protein blotting characteristics observed for these antibodies (Supplemental Fig. 6). The imperfect antigenic specificity for the five putative ABCC9 antibodies also rendered immunohistochemistry technically unsatisfactory, although this was attempted (not shown).

The *ABCC9* gene is also known as sulfonylurea receptor 2, SUR2. Sulfonylureas are a widely prescribed class of drugs used to treat type II diabetes mellitus; these drugs antagonize the function of the ABCC9 protein product [10, 13, 33, 68]. We tested the hypothesis that exposure to sulfonylurea drugs is associated with altered risk for HS-Aging pathology (Table 7). Data were obtained from NACC database with both longitudinal drug history and postmortem data from individuals dying age 85 and above with autopsies between 2010 and 2013. HS-Aging pathology was observed in 17.3 % (108/624) of all autopsied cases in the sample, 30.6 % of sulfonylurea users and 16.5 % of non-users. Based on a two-tailed test and $\alpha = 0.05$, the crude OR for autopsy-confirmed HS-Aging pathology after use of any sulfonylurea versus no sulfonylurea use was 2.23 [95 % CI: 1.06–4.68, $p = 0.03$; age-adjusted OR = 2.19 (95 % CI: 1.04–4.63, $p = 0.04$)]. Use of any sulfonylurea was reported by 11 HS-Aging and 25 non HS-Aging subjects. When the analysis was restricted to the medication history given at the last assessment before death, the crude OR for use of any sulfonylurea was 2.52 [95 % CI: 1.10–5.76, $p = 0.02$; age-adjusted OR = 2.49 (95 % CI: 1.08–5.75, $p = 0.03$)]. There was neither positive nor negative association between sulfonylurea use and AD pathology, operationalized as AD-positive if Braak stage V or VI and AD-negative otherwise (not shown). These data are compatible with the hypothesis that sulfonylurea exposure is associated with HS-Aging pathology in this sample.

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Potential confounders include the clinical indication for sulfonylurea use, namely type II diabetes. Of subjects who reported taking any anti-diabetic agent (62/624), most reported use of a sulfonylurea (36/62). The association with HS-Aging pathology was not dependent upon diabetes itself because, in the NACC database overall, history of diabetes had a null association with HS-Aging pathology [52]. In this subsample, the odds of observing HS-Aging pathology were not increased for anti-diabetic agent users as a whole [age-adjusted OR = 1.43 (95 % CI: 0.75–2.71, $p = 0.28$)], for anti-diabetic agent users who never reported using a sulfonylurea [age-adjusted OR = 0.60 (95 % CI: 0.17–2.04, $p = 0.41$)], or for self-reported diabetes [age-adjusted OR = 1.08 (95 % CI: 0.58–2.03, $p = 0.81$)].

For a slightly younger group of research subjects, those dying age 80–84 ($n = 261$), among whom HS-Aging histopathology was relatively uncommon [9], post hoc sensitivity analyses indicated no association between sulfonylurea use and HS-Aging pathology [age-adjusted OR = 0.59 (95 % CI: 0.13–2.68)]. Effect estimates in this group are similar for use of any anti-diabetic medication [age-adjusted OR = 0.33 (95 % CI: 0.07–1.42)] and self-reported diabetes [age-adjusted OR = 0.43 (95 % CI: 0.15–1.29)]. Results in this slightly younger group of subjects, which included only 17 sulfonylurea users, did not support the hypothesis that sulfonylurea use is associated with HS-Aging pathology in this age group.

Discussion

In the large human autopsy cohorts studied, the risk of developing HS-Aging pathology was associated with polymorphism in the *ABCC9* gene. The effect size was relatively large (OR = 1.4–2.0 with additive MOI, 2.0–2.7 with recessive MOI) in all three Stage I cohorts and also in both Stage II cohorts. In the final Stage II cohort, only a single polymorphism was queried so statistical correction for multiple testing was obviated. The HS-Aging risk allele may be directly relevant to public health because aged human subjects in the NACC database with history of sulfonylurea exposure have increased risk for HS-Aging pathology.

There are potential pitfalls inherent to the study design. The Stage I GWAS did not produce a SNP that reached genome-wide statistical significance. Bias was thus inserted into the study by selecting which SNPs would be analyzed in the Stage II cohorts. Among the candidate SNPs from GWAS, *ABCC9* polymorphisms ultimately yielded the lowest p values by an order of magnitude, so that locus was an obvious choice to evaluate. Testing the association between sulfonylurea drug exposure and HS-Aging pathology in the NACC dataset is complicated by confounders and limitations. For example, the accuracy of medication self-report is imperfect since current medication use refers to the 2-week period prior to the clinical assessment, drug dosages were unknown, and some of the patients presumably used and discontinued sulfonylurea drugs prior to study enrollment as well as between annual study visits. Patient age was also a relevant factor; we found that HS-Aging pathology is relatively unusual among individuals younger than age 85 [9, 49-51] and found no evidence for correlation between the sulfonylurea drugs and HS-Aging pathologically in the post hoc analysis of younger patients. These analyses were also performed blind to patients' genotypes so the distribution of *ABCC9* SNPs versus sulfonylurea use is unknown. For these reasons, the data on HS-Aging linked to drug exposure should be interpreted with caution.

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The present study was not designed to evaluate polymorphisms that show weak associations with HS-Aging pathology. There were, however, some alleles from the GWAS that appear interesting despite failing to reach genome-wide statistical significance. For example, SNPs within and near the cutaneous T-cell lymphoma-associated antigen 5 (*CTAGE5*) gene yielded $p \sim 10^{-6}$ in the GWAS, and *CTAGE5* polymorphisms have been linked to familial idiopathic basal ganglia calcification (Fahr's disease) [40, 56]. Meta-analyses of Stage I cohort data also indicated that alleles linked previously to hippocampal sclerosis pathology —rs5848 [17] and rs1990662 SNPs [63]—trend toward association with HS-Aging pathology as operationalized in the current study, with the same risk alleles as previously described [57, 61, 63], and with OR ~1.2 (meta-analyzed data, additive MOI).

The main discovery of the present study is the association between HS-Aging pathology and polymorphism in the *ABCC9* gene. Meta-analyzed data indicate an overall OR = 1.62 with the additive MOI model—a relatively large effect size for a common allele [11, 24, 81]. Notably, the chromosomal region in which *ABCC9* resides (12p) has been linked to clinical late-onset AD risk [31, 66], which may be at least partially explained by *ABCC9*, because HS-Aging tends to be misdiagnosed clinically as AD [9, 57].

We can begin to develop hypotheses about mechanism(s) underlying the genomic associations. The presence of hippocampal TDP-43 pathology is associated with additive cognitive impairment [37, 48], and "unilateral" HS-Aging pathology usually shows TDP-43 pathology on the contralateral side [50]; the present study affirms that hippocampal TDP-43 pathology is linked to HS-Aging pathology, since risk for both is associated with the same allele, rs704178. The strength of the association between rs704178 and HS-Aging pathology with recessive model of MOI (OR = 2.13) indicates that the "non-risk alleles" may exert an adaptive function, even in the heterozygous state ("dominant" gain-of-function pattern).

The association between *ABCC9* and HS-Aging provides a novel insight into neurodegenerative diseases. Prior GWAS found that different polymorphisms of the *ABCC9* gene were associated with cardiac problems and altered sleep patterns [32, 34, 38, 55, 58], which could theoretically be pathogenetic factors "upstream" from HS-Aging. Alternatively, or in addition, *ABCC9* polymorphisms may exert influence through blood vessel pathology. The *ABCC9* polypeptide product *(SUR2)* is physiologically active in arteriolar smooth muscle [22, 55], and pathologically confirmed arteriolosclerosis is relatively severe in brains that also harbor HS-Aging pathology [52] (and see Ref. [18]). Stop-codon mutations in *ABCC9* lead to Cantú syndrome [26, 75], a rare condition with mostly uncharacterized neuropathology. Intriguingly, Cantú syndrome patients have demonstrated vascular anomalies, including in the brain [21, 27, 67].

What about the function of the HS-Aging risk SNPs themselves? These intronic SNPs span at least ten *ABCC9* exons. Some mRNA variants have been described with splice junctions near the HS-Aging risk SNPs [12, 78]. In the genotype-tissue expression project (GTEx) database [14] of expression quantitative trait loci, SNPs linked to HS-Aging pathology (particularly rs704192 which has $D' > 0.9$ with rs704178) also correlate with altered *ABCC9* expression specifically in both human hippocampus and amygdala (both $p < 0.05$) but not cerebellum. Using SNPExp web tool [30] where SNPs are correlated with gene expression

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using transformed lymphoblastoid cell lines, SNPs linked to HS-Aging pathology (particularly rs829080, with $D' = 1.0$ with rs704178) correlate strongly with *ABCC9* expression ($p < 0.0001$). Despite these interesting observations, it is not yet proven that the HS-Aging risk SNP variability leads directly to changes in gene splicing, mRNA expression, or polypeptide function in human brain.

Whatever the mechanism(s), *ABCC9* polymorphism in a relatively large number of human cases is associated with HS-Aging pathology. The differing neuropathologic findings and differing genetic vulnerabilities of HS-Aging and AD make it likely that prevention and treatment of HS-Aging, when achieved, will be different from prevention and treatment for AD. ABCC9 function is sensitive to pharmacologic manipulation, and we here report that in subjects dying after age 85, sulfonylurea drug exposure was associated with HS-Aging pathology. This study may herald progress of clinical relevance, because *ABCC9* agonists have been described, and may be neuroprotective [77, 82]. We conclude that the present study establishes a pathogenetic association between *ABCC9* and HS-Aging pathology—and perhaps the basis for a novel future therapeutic strategy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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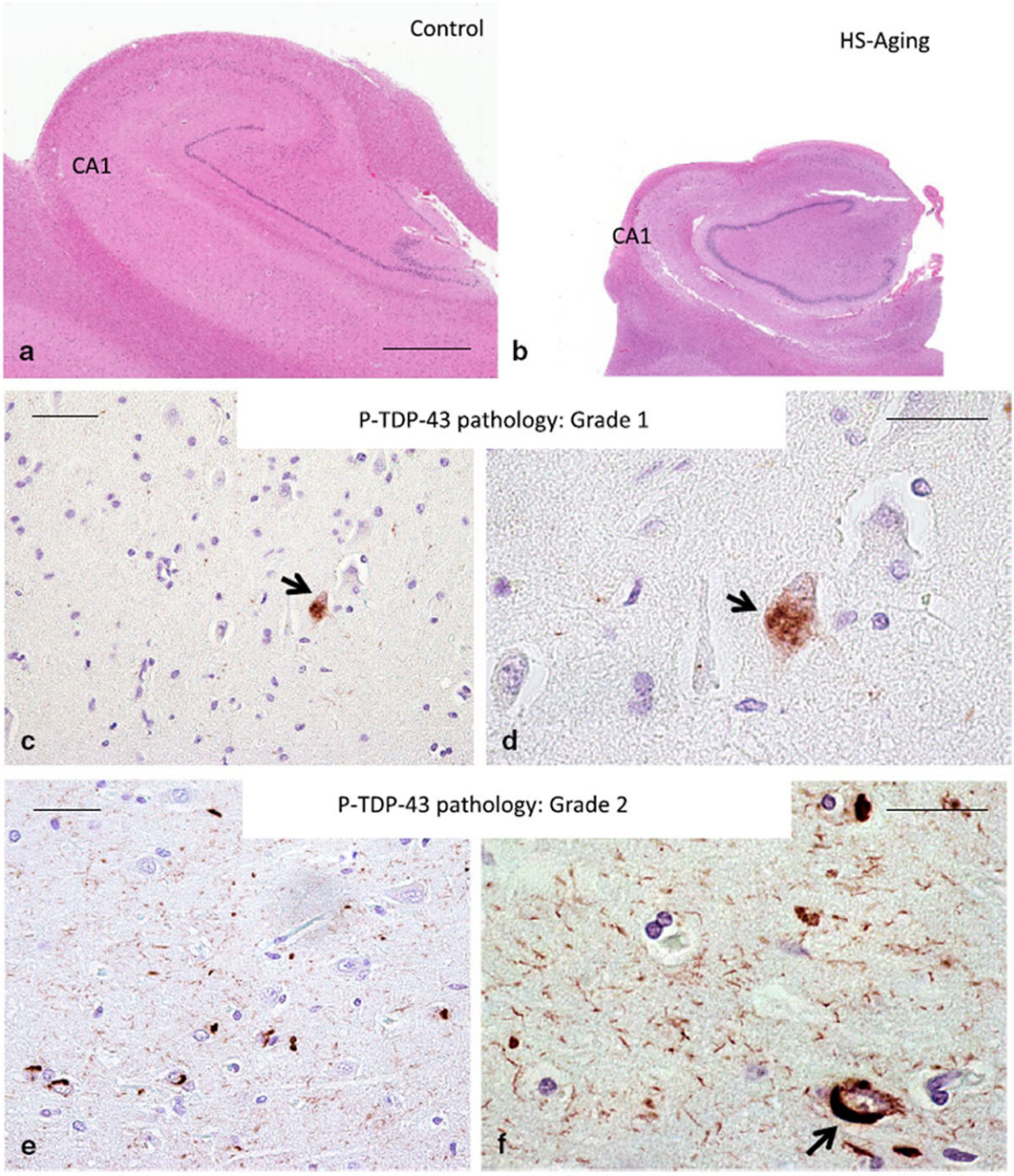

Fig. 1.

Hippocampal sclerosis of aging (HS-Aging) pathology is a complex endophenotype for genomic studies. Pathology defines the disease, illustrated in photomicrographs of hematoxylin and eosinstained sections to compare an aged control hippocampus (**a**) with a shrunken HS-Aging hippocampus (**b**); same *scale bar* 2 mm, CA1 subfield indicated. For grading P-TDP43 pathology in CA1 and subiculum of the hippocampal formation, a three-tier semiquantitative system was employed with “Grade 0” indicating no P-TDP43 pathology, “Grade 1” (**c, d**) indicating very sparse isolated pathology (P-TDP43 positive

neuronal inclusion at *arrow*) and "Grade 2" (**e, f**) indicating any more severe pathology, usually with intranuclear, intracytoplasmic (*arrow*), and neuritic P-TDP-43 pathology. *Scale bar* 60 microns (**c, e**) and 25 microns (**d, f**)

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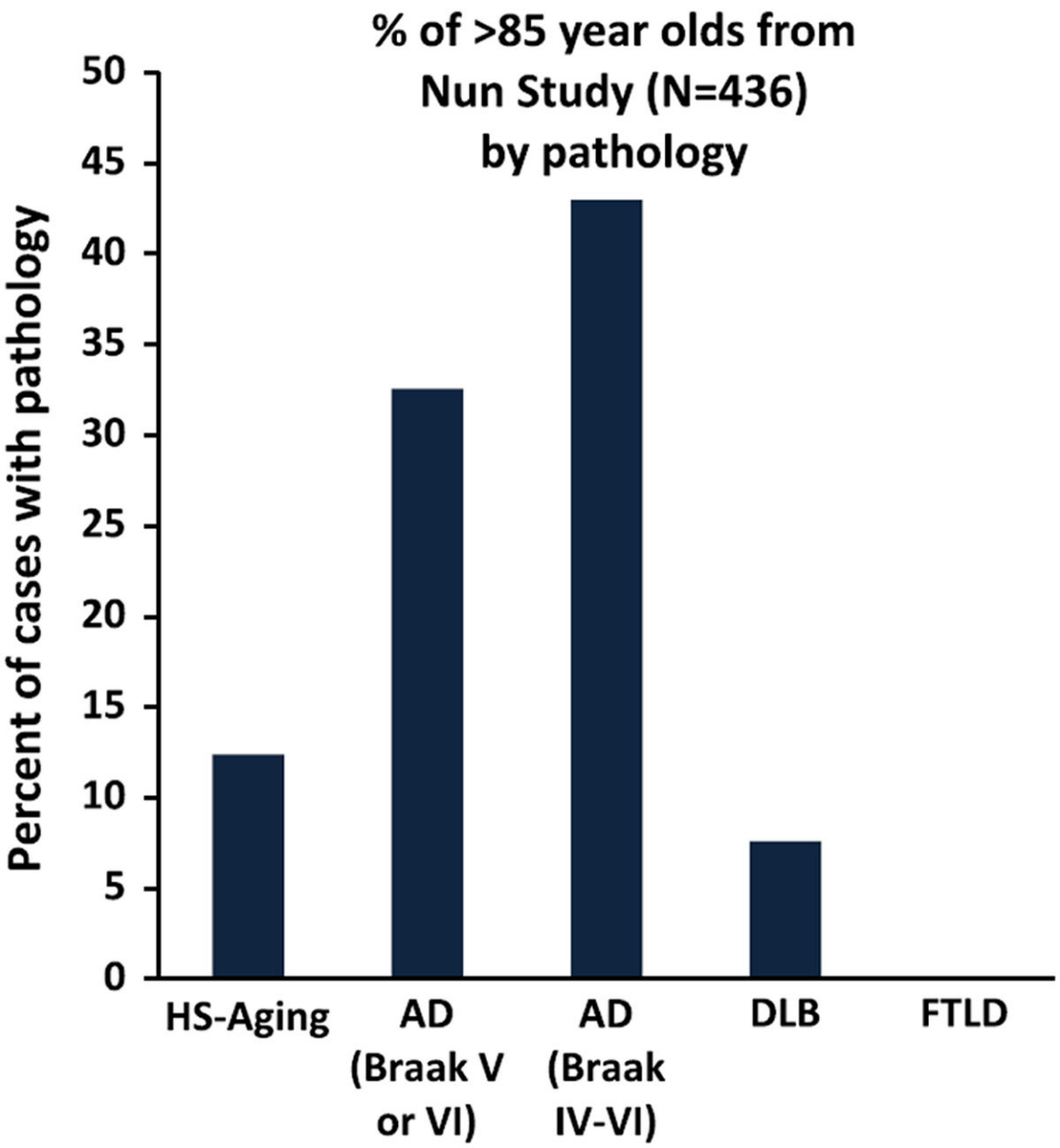

Fig. 2.

Prevalence of brain pathology subtypes in the Nun Study. The prevalence of HS-Aging pathology is germane to a study that incorporates different autopsy cohort types. Here, we show data about the prevalence of brain pathology subtypes in the Nun Study, a population-based cohort as previously described in detail [50]. As in most community-based or population-based cohorts (as opposed to cohorts connected with memory disorder clinics), frontotemporal lobar degeneration (FTLD) pathology is rare [19, 69, 73]

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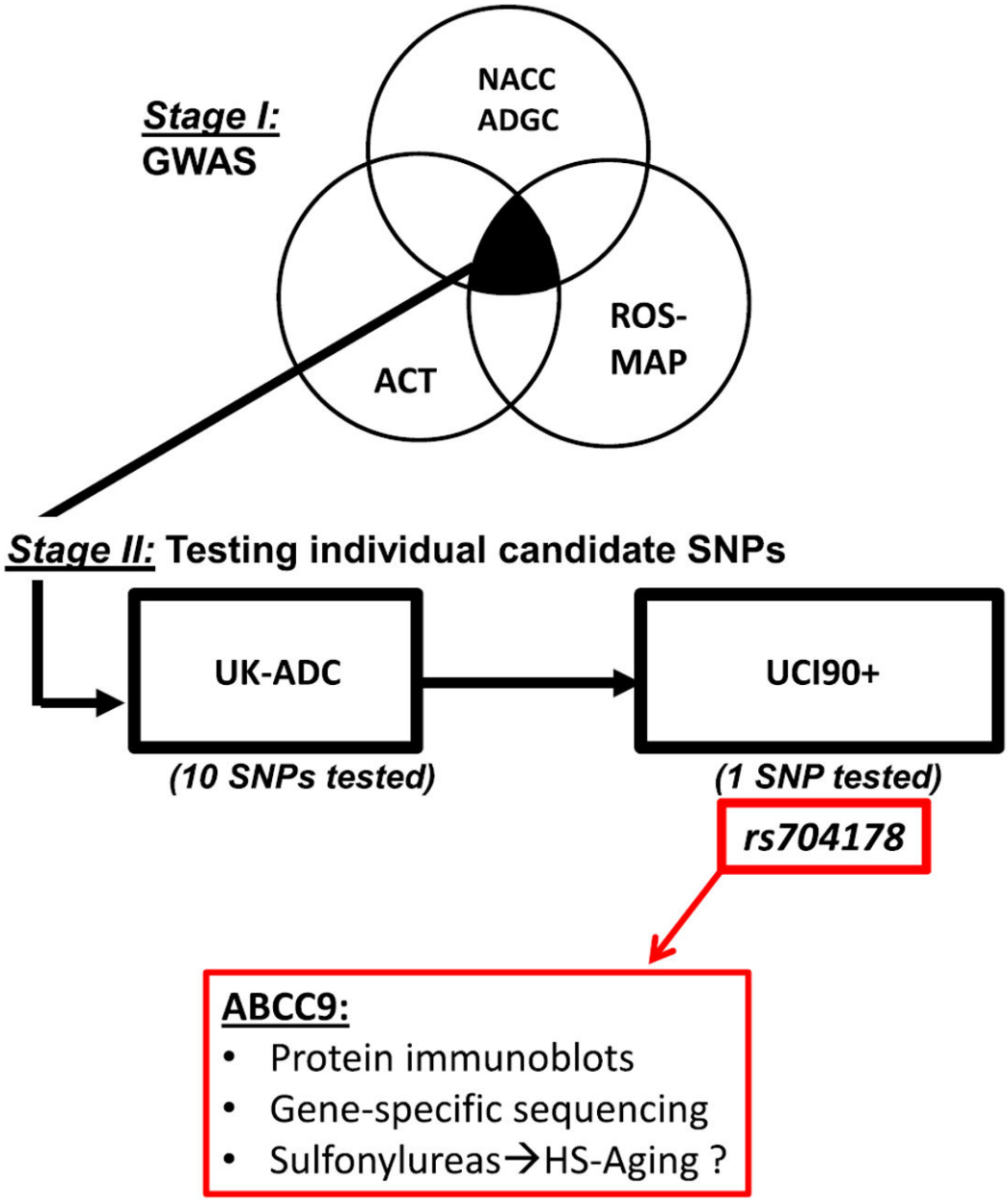

Fig. 3.

Study design for elucidating a large effect size human genomic polymorphism that is associated with HS-Aging pathology. The GWAS (Stage I) incorporated genomic data from Alzheimer's Disease Genetics Consortium (ADGC) and pathologic data from National Alzheimer's Coordinating Center (NACC) derived from 34 different US Alzheimer's Disease Centers; Rush University Religious Orders Study and Memory and Aging Project (ROS-MAP); and the Group Health Research Institute Adult Changes in Thought study (ACT). Subsequent evaluation of particular risk alleles was then performed (Stage II) using data from two additional cohorts: University of Kentucky ADC (UK-ADC), and University of California at Irvine 90+ Study (UCI90+). Post-hoc studies were performed on the *ABCC9* allele (rs704178) showing the most robust association with HS-Aging pathology

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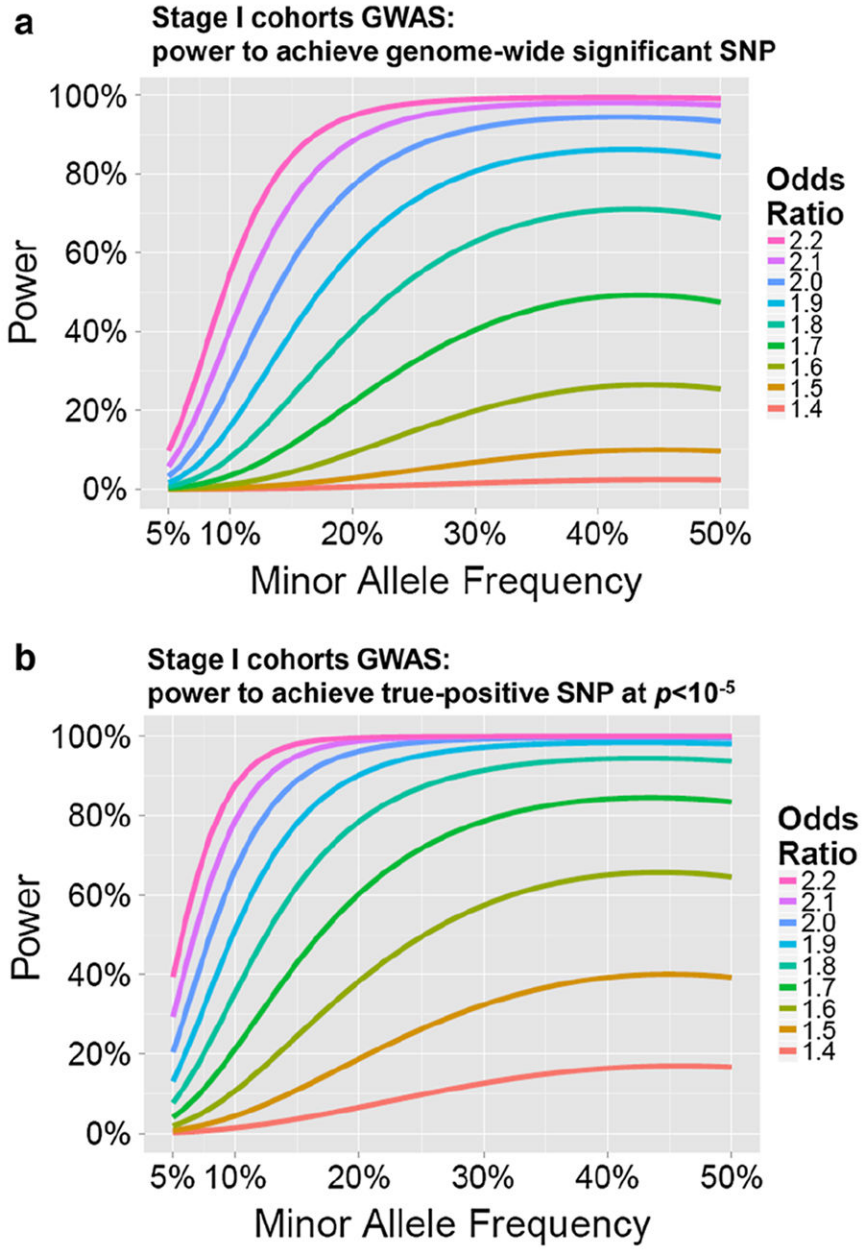

Fig. 4.

Statistical power analyses. With the sample size of the Stage I cohorts (241 cases, 1,998 controls), the probability of elucidating a "true-positive" SNP associated with HS-Aging pathology was modeled. Two different genome-wide significance thresholds were modeled, each with varying minor allele frequency and risk allele effect sizes operationalized by the odds ratio of HS-Aging pathology in risk SNP carriers versus non-carriers. To reflect the study design, the *p* values were set at $p < 5 \times 10^{-8}$ (**a**) and $p < 10^{-5}$ (**b**)

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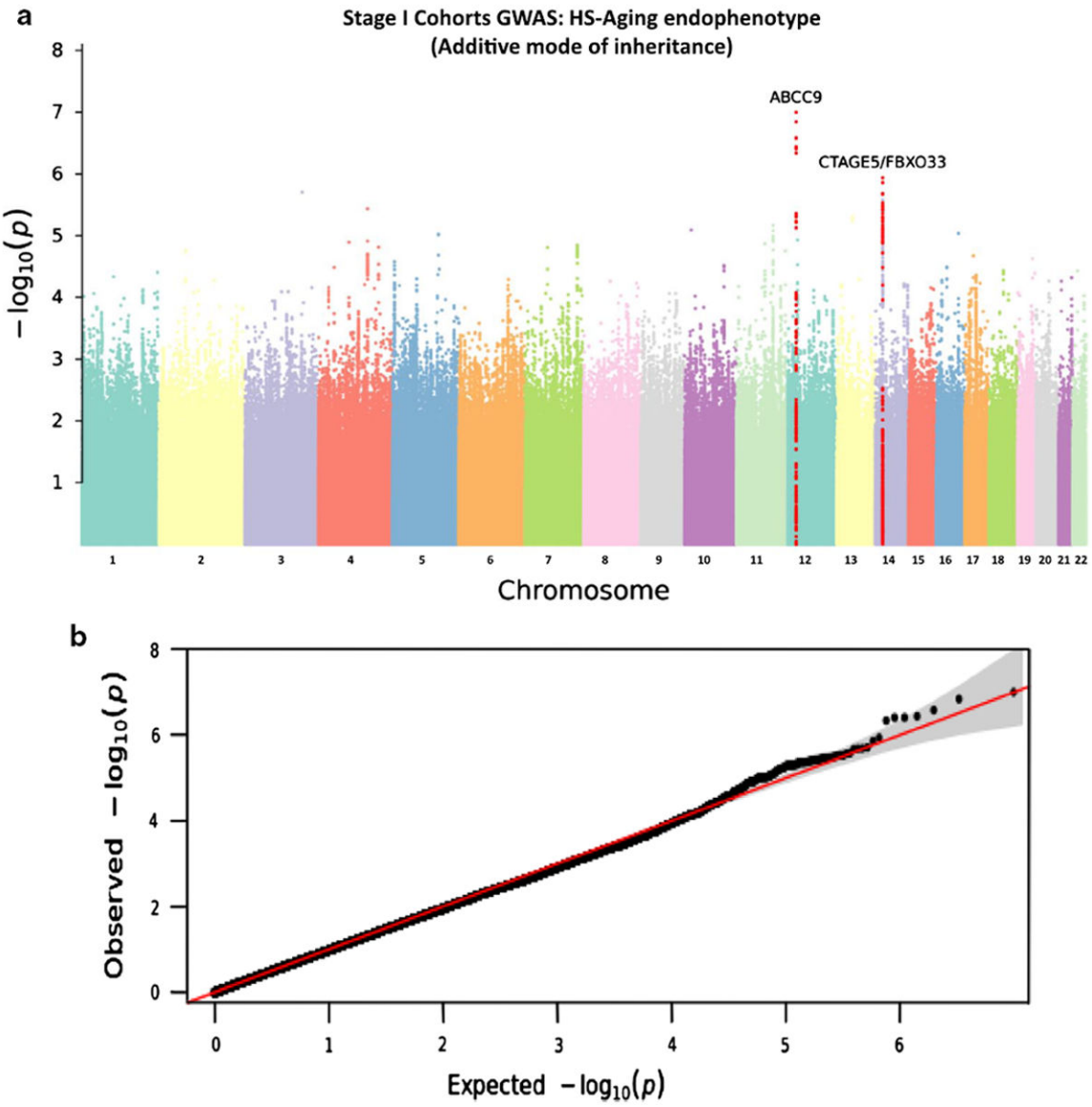

Fig. 5.

HS-Aging GWAS using data meta-analyzed from the three Stage I cohorts. **a** Manhattan plot showing *p* values for HS-Aging GWAS with meta-analyzed data from the three Stage I cohorts. No SNP was found to reach genome-wide statistical significance ($p < 5 \times 10^{-8}$ to correct for multiple comparisons) in this GWAS. Two loci with the lowest *p* values SNPs in the Stage I GWAS are highlighted in *red*: *ABCC9* (Chr 12p) and *CTAGE5/FBXO33* (Chr 14q). **b** QQ Plot for HS-Aging GWAS using ADGC data. Observed (log-transformed) *p* values for each SNP are plotted against their corresponding expected values under the null hypothesis of no association with HS-Aging

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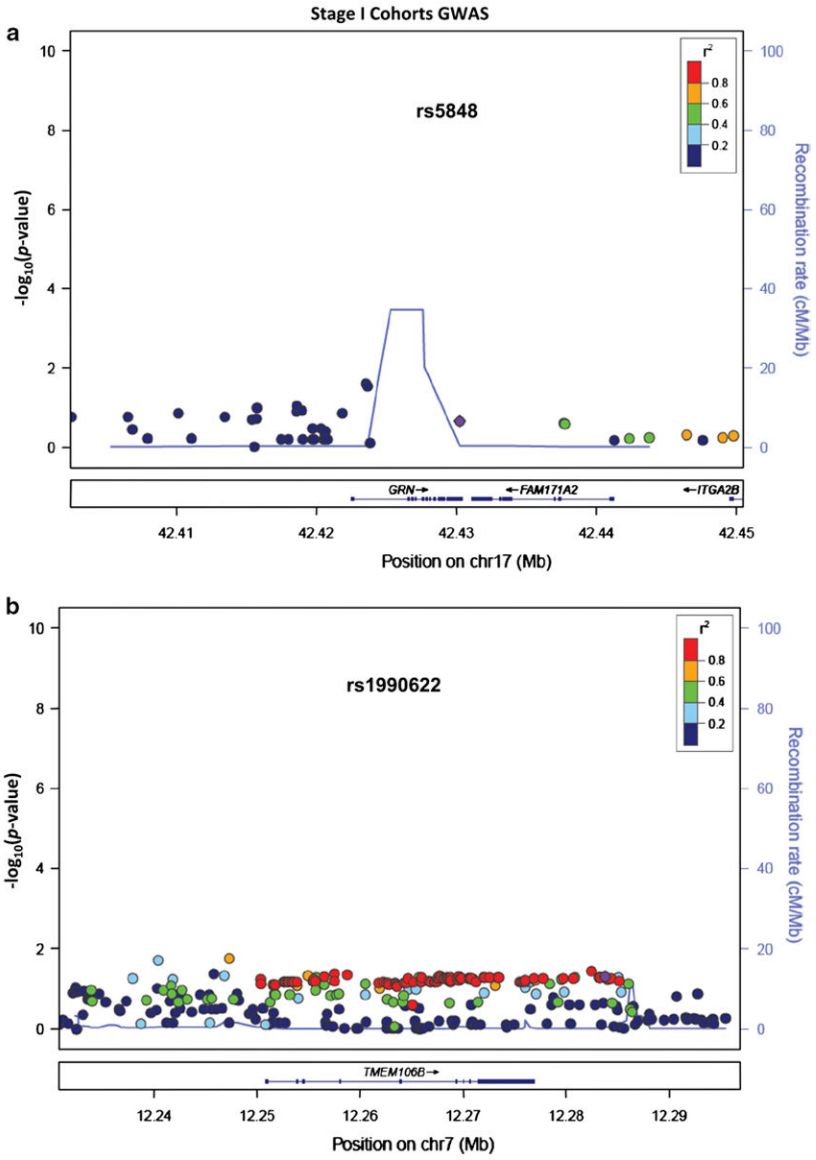

Fig. 6.

LocusZoom plots of HS-Aging GWAS data. Results of HS-Aging GWAS meta-analyzed data from Stage I datasets are shown. The *y* axis shows *p* values describing the results of association study with HS-Aging phenotype centered on **a** *GRN* locus (rs5848) on Chromosome 17 and **b** *TMEM106B* locus (rs1990622) on Chromosome 7. Note that the SNPs being evaluated have been published previously [17, 63] and this is a sample of cases and controls that is independent from prior published studies of these alleles. Since this part of our study is a replication experiment, and focused on the two particular alleles, $p < 0.05$ findings would constitute a successful replication

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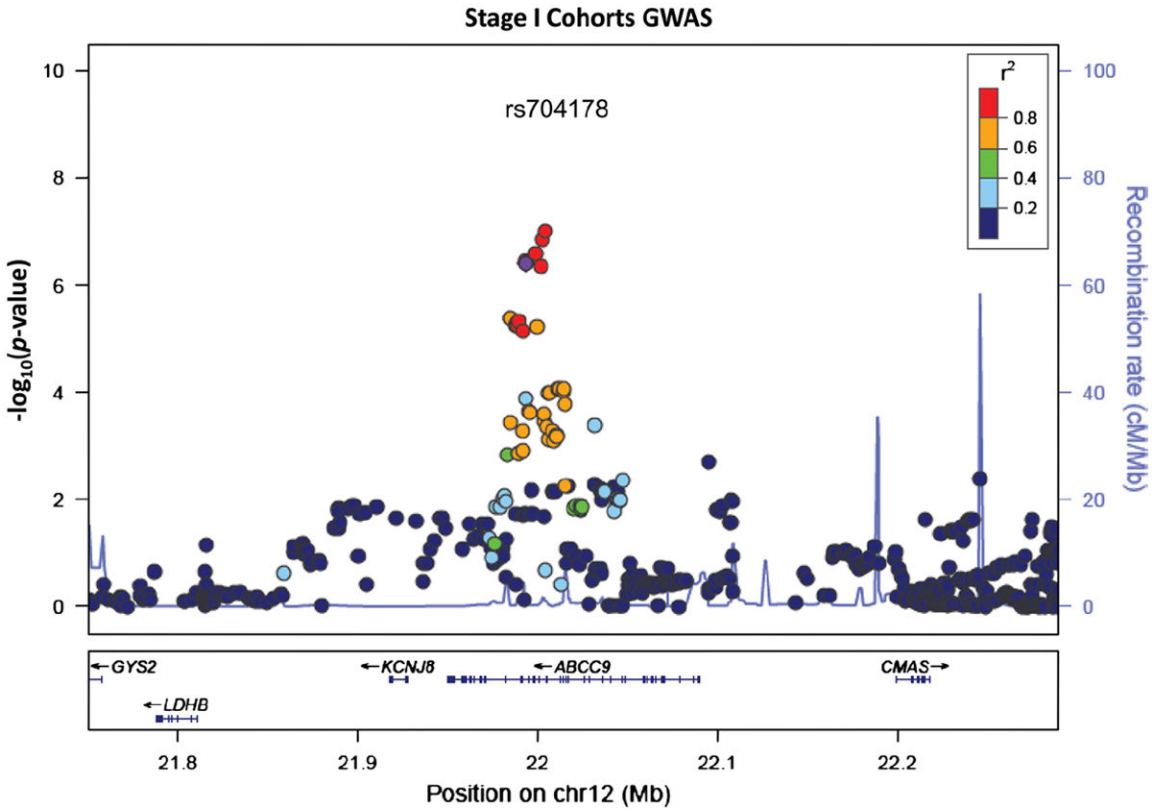

Fig. 7.

LocusZoom plots of HS-Aging GWAS data. Results of HS-Aging GWAS meta-analyzed data from Stage I datasets are shown. The *y* axis shows *p* values describing the results of association study with HS-Aging phenotype centered on the *ABCC9* rs704178 risk allele that was the subject of further study. The data shown here are independent of the subsequent studies in two additional Stage II cohorts

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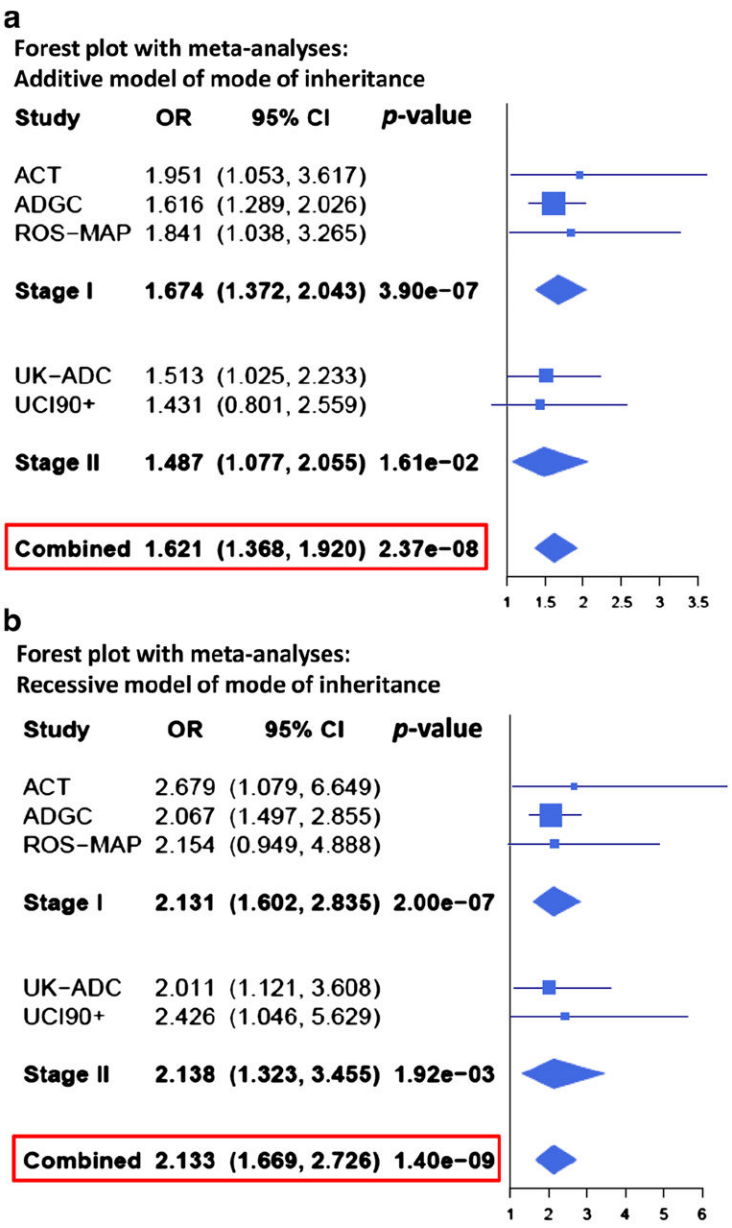

Fig. 8.

The *ABCC9* risk allele (rs704178) association with HS-Aging pathology. Analyses were performed for each individual autopsy cohort, and then combined using meta-analyses across all five Stage I and Stage II cohorts, using inverse-variance-based statistic [70]. Forest plots displaying cohort-specific and aggregated odds ratios (OR) and 95 % confidence intervals (CIs) were created reflecting these meta-analyzed results. *Lines* represent 95 % CIs for the ORs, and the size of the *square* (cohort) or *trapezoid* (meta-analyzed group) reflects the cohort size. Shown here are the results when assuming an additive mode of inheritance (**a**) or recessive mode of inheritance (**b**)

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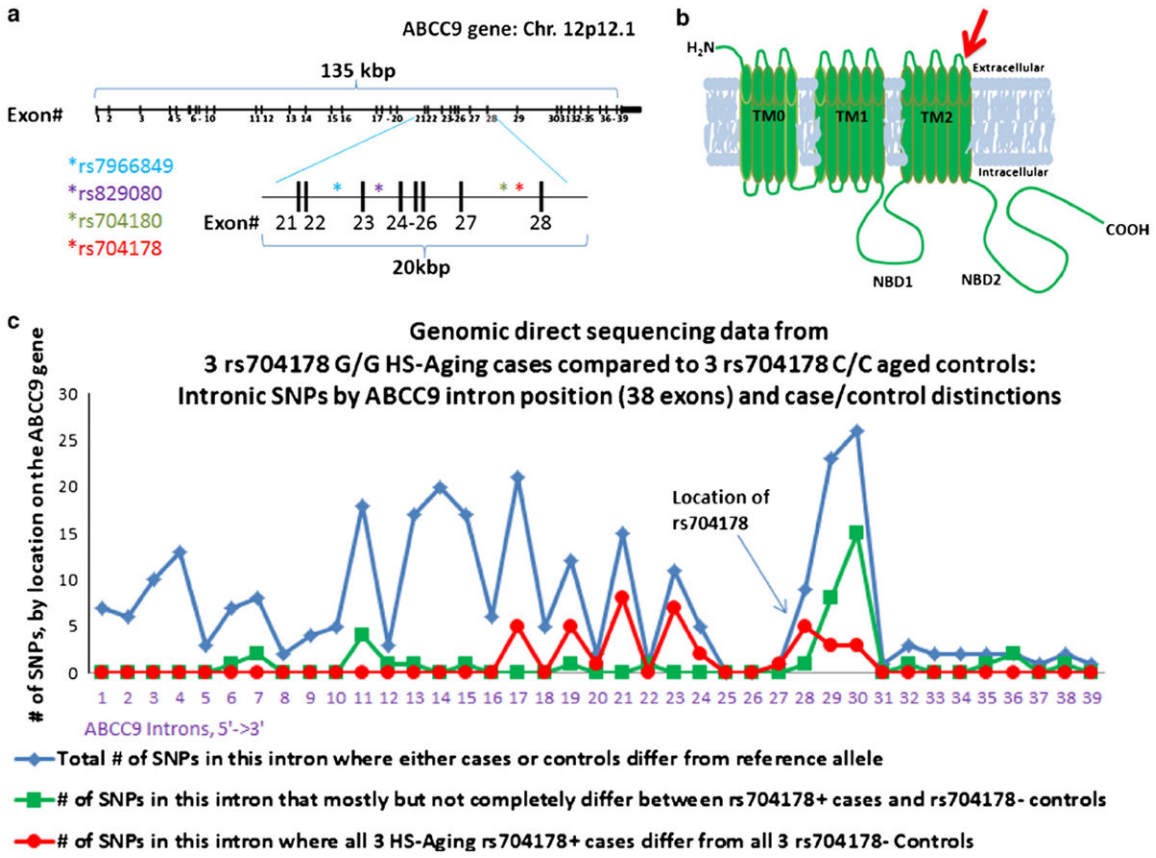

Fig. 9.

ABCC9 gene and protein organization in relation to the HS-Aging risk alleles. **a** There are at least 38 exons in the *ABCC9* gene, and the SNPs that are most strongly linked to HS-Aging are present within a 20-kilobase region. **b** The approximate location of the exons on the corresponding ABCC9 polypeptide is shown with a *red arrow*. *TM* transmembrane, *NBD* nucleotide-binding domains. **c** Direct genomic sequencing of the *ABCC9* gene was performed for six cases (3 rs704178 G/G HS-Aging cases, 3 elderly controls with rs704178 C/C genotype). Intron-by-intron analyses indicate that the intronic polymorphisms linked to HS-Aging pathology span more than ten exons

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Table 1

Research subjects (n = 2,666 research subjects total) and the five autopsy cohorts analyzed in the current study

Autopsy cohort	Pathologic phenotype evaluated	HS cases	Avg age (years ± st dev)	Non-HS controls	Avg age (years ± st dev)	Total	Role in study
ADGC	HS pathology	186	84.1 ± 8.2	1,450	81.5 ± 8.7	1,636	Stage I (GWAS)
ROS-MAP	HS pathology	29	93.3 ± 5.4	398	90.8 ± 4.1	427	
ACT	HS pathology	26	87.6 ± 4.2	150	88.4 ± 2.2	176	
UK-ADC	Hippocampal TDP-43 pathology	94	92.3 ± 6.9	161	93.3 ± 6.4	255	Stage II #1
UCI90+	HS pathology	28	94.1 ± 2.3	144	92.3 ± 4.5	172	Stage II #2
	Total	363		2,303		2,666	

GWAS was performed on the three Stage I datasets, from Alzheimer's Disease Genetics Consortium (ADGC, which are based mostly on dementia research clinics), Rush Religious Order Study and Memory and Aging Project (ROS-MAP, which is an epidemiologic cohort), and Adult Changes in Though (ACT, which is a community-based autopsy cohort). First Stage II was performed at University of Kentucky Alzheimer's Disease Center (UK-ADC), and second Stage II at the University of California Irvine 90+ Study (UCI90+). None of the cases used in the assessment of HS-Aging overlapped between cohorts

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Table 2

Genotyping quality control (QC) for GWAS: Stage I datasets

Cohort (ADCs, via ADGC)	**Level 0 QC**	**Level 1 QC**				
	Number of SNPs above imputation threshold	**Number of SNPs be low initial call rate threshold (0.85)**	**Number of SNPs be low initial MAF threshold (0.01)**	**Number of SNPs out of Hardy–Weinberg equilibrium (p < 1E–5)**	**Number of SNPs post-level 1 QC**	**Number of subjects with NACC and ADGC data**
ADC1	36,642,556	832,579	28,964,437	75,832	6,886,357	1,484
ADC2	36,274,850	643,766	28,678,144	36,123	6,964,483	210
ADC3	36,552,919	540,092	28,804,571	48,516	7,222,900	529
ADC4	36,644,873	848,668	29,326,492	119,205	6,594,201	127
ADC5	36,644,873	893,321	29,350,835	30,593	6,581,733	103
ADC6	36,644,873	849,334	29,616,694	24,304	6,345,693	56
	Level 2 QC					
Cohort	Union of SNPs passing level 1 QC	Number of SNPs below call rate threshold (0.95)	Number of SNPs below MAF threshold (0.05)	Number of SNPs out of Hardy–Weinberg equilibrium (p < 1E–5)	Number of SNPs post-level 2 QC	Number of subjects
ADCs	7,948,061	2,771,882	2,604,515	3,142	4,271,710	2,509
Cohort	Number of SNPs above imputation threshold	Number of SNPs below call rate threshold (0.95)	Number of SNPs below MAF threshold (0.05)	Number of SNPs out of Hardy–Weinberg equilibrium (p < 1E–5)	Number of SNPs post-QC	Number of subjects
ACT	35,550,922	1,831,347	30,043,290	24,473	4,407,882	176
ROS-MAP	35,606,835	2,242,313	30,159,840	36,529	4,059,683	427

Genotypes were imputed to the Phase I v.3 1000 Genomes Project March 2012 release by the ADGC. ADCs were imputed in six iterations (ADC 1–6). Resulting SNPs within each dataset were screened for call rate, minor allele frequency (MAF) and Hardy–Weinberg equilibrium. QC filters were applied in two separate stages for the ADGC dataset which comprises six subsets (ADC 1–6). Note that a SNP can fail more than one of the QC criteria. Overall the number of SNPs that passed QC and were evaluated in the GWAS, i.e., the union of SNPs passing QC across the ADC, ACT and ROS-MAP cohorts, was 4,913,579

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Table 3

Results from Stage I datasets for polymorphisms (rs5848 and rs1990622) linked previously to hippocampal sclerosis pathology in aged individuals

Dataset	Major allele	Minor allele	Odds ratio	95 % Confidence interval	Risk allele	*p* value*
rs5848 (*GRN*)						
ADGC	C	T	1.08	0.84–1.40	T	0.541
ACT	C	T	1.90	0.95–3.83	T	0.073
ROS-MAP	C	T	1.12	0.53–2.37	T	0.774
Meta-analysis results			1.16	0.92–1.46	T	0.218
rs1990622 (*TMEM106B*)						
ADGC	T	C	1.19	0.95–1.49	T	0.132
ACT	T	C	1.24	0.63–2.44	T	0.528
ROS-MAP	T	C	1.43	0.82–2.50	T	0.198
Meta-analysis results			1.22	1.00–1.49	T	0.049

* *p* values shown here are assuming a two-tailed test

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Table 4

GWAS from Stage I cohorts with $p < 10^{-5}$: genomic loci, including the genes (if any) and individual SNP at each locus with the lowest p value

Gene/locus area	# SNPS at locus with $p < 10^{-5}$	SNP at locus with lowest p value	Chromosome: genomic position[a]	Minor allele	Major allele	MAF cases	MAF controls	p value (additive MOI)
ABCC9	15	rs7966849	12: 22004199	G	A	0.38	0.51	1.01E–07
CTAGE5/FBXO 33	50	rs8018486	14: 39818617	G	A	0.11	0.21	1.15E–06
Intergenic	1	rs114816986	3: 155035752	A	G	0.30	0.08	1.97E–06
Intergenic	1	rs62324179	4: 127661386	T	C	0.26	0.18	3.65E–06
Intergenic	3	rs80155385	13: 59845469	C	G	0.22	0.05	4.95E–06
Intergenic	2	rs3018573	11: 98516757	G	A	0.52	0.41	6.81E–06
FRMD4A	1	rs72769570	10: 13810428	A	G	0.19	0.05	8.09E–06
WWOX	1	rs55751884	16: 78231495	C	T	0.26	0.12	9.19E–06
Intergenic	12	rs12514988	5: 123179750	T	C	0.16	0.04	9.48E–06

GWAS from Stage I cohorts yielded 86 different nominal SNPs with $p < 10^{-5}$. These all derived from nine genomic regions or loci, which are shown here with each genomic locus represented by the SNP with the lowest p value from each

[a]Genomic position refers to GRCh37/hg19 assembly

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Table 5

Genotype frequencies by HS-Aging status from Stage I cohorts

HS-Aging	rs704178 genotypes		
	CC	GC	GG
Cases	0.164	0.441	0.395
Controls	0.259	0.500	0.242
Total	0.249	0.493	0.258

The three Stage I cohorts (ADGC/NACC, ROS-MAP, and ACT) are aggregated to compute genotype frequencies for the ABCC9 putative risk SNP, rs704178

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Table 6

Number of research subjects in each Stage II cohort arranged by *ABCC9* genotype status (risk allele is G) and neuropathology [P-TDP-43 or hippocampal sclerosis (HS)]

Cohort	Neuropathology	rs704178 genotypes		
		CC	GC	GG
UK-ADC	0[a]	35	75	35
	1[a]	7	11	7
	2[a]	8	24	25
UCI90+	HS−	34	76	34
	HS+	7	9	12

Stage II cohorts used autopsy series independent of the Stage I datasets, with phospho-TDP-43 (P-TDP43) pathology in the UK-ADC as the endophenotype. Different strategies and models could be applied for statistical testing; for results of statistical analyses, see Fig. 8 and Supplemental Fig. 5

[a]In the UK-ADC, severity of aberrant P-TDP-43 (0–2 scale) was the pathologic readout

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Table 7

Sulfonylurea use and HS-Aging risk in persons age 85 years and older at death: data from the National Alzheimer's Coordinating Center (NACC) database

	Sulfonylurea use (*n* = 36)		No sulfonylurea use (*n* = 588)	
	HS-Aging pathology (*n* = 11)	**No HS-Aging pathology (*n* = 25)**	**HS-Aging Pathology (*n* = 97)**	**No HS-Aging pathology (*n* = 491)**
Age at death, years	88.7 ± 4.8	90.8 ± 4.3	91.2 ± 4.3	90.9 ± 4.4
Number of longitudinal evaluations	4.5 ± 1.1	4.4 ± 1.7	3.9 ± 1.8	3.9 ± 1.7
Last evaluation to death, months	13.8 ± 10.0	15.3 ± 15.3	18.6 ± 19.7	15.1 ± 15.1
Estimated sulfonylurea exposure, years	3.4 (range 0.5–6.2)	3.5 (range 0.5–6.8)	N/A	N/A
Taking sulfonylurea at last evaluation, %	81.8 %	72.0 %	N/A	N/A

Summary statistics for data used to test of the hypothesis that sulfonylurea use is correlated with increased risk for HS-Aging pathology in the NACC database. Results presented are mean ± SD unless otherwise stated. These analyses involved 624 patients who died after age 85, between 2010 and 2013, with detailed longitudinal drug history and autopsy data. "Sulfonylurea use" indicated self-reported use of any sulfonylurea drug at any longitudinal visit. Crude OR for use of any sulfonylurea was 2.23 [95 % CI: 1.06–4.68, $p = 0.03$; age-adjusted OR = 2.19 (95 % CI: 1.04–4.63, $p = 0.04$)]. Mean age at death was not different for subjects stratified by the presence of HS-Aging and use of any sulfonylurea ($F_{3,620} = 1.10$, $p = 0.35$) or was mean time between last evaluation and date of death ($F_{3,620} = 1.33$, $p = 0.26$). The average estimated drug exposure and % of patients taking a sulfonylurea at last visit was not different in persons with and without eventual HS-Aging pathology

N/A not applicable