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Method

An association test of the spatial distribution of rare missense variants within protein structures identifies Alzheimer's disease–related patterns

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More than 90% of genetic variants are rare in most modern sequencing studies, such as the Alzheimer's Disease Sequencing Project (ADSP) whole-exome sequencing (WES) data. Furthermore, 54% of the rare variants in ADSP WES are singletons. However, both single variant and unit-based tests are limited in their statistical power to detect an association between rare variants and phenotypes. To best use missense rare variants and investigate their biological effect, we examine their association with phenotypes in the context of protein structures. We developed a protein structure–based approach, protein optimized kernel evaluation of missense nucleotides (POKEMON), which evaluates rare missense variants based on their spatial distribution within a protein rather than their allele frequency. The hypothesis behind this test is that the three-dimensional spatial distribution of variants within a protein structure provides functional context to power an association test. POKEMON identified three candidate genes (*TREM2*, *SORL1*, and *EXOC3L4*) and another suggestive gene from the ADSP WES data. For *TREM2* and *SORL1*, two known Alzheimer's disease (AD) genes, the signal from the spatial cluster is stable even if we exclude known AD risk variants, indicating the presence of additional low-frequency risk variants within these genes. *EXOC3L4* is a novel AD risk gene that has a cluster of variants primarily shared by case subjects around the Sec6 domain. This cluster is also validated in an independent replication data set and a validation data set with a larger sample size.

[Supplemental material is available for this article.]

High-throughput DNA sequencing of diverse humans has identified millions of genetic variants, the vast majority of which are exceptionally rare. A survey of ~60,000 individuals from the Exome Aggregation Consortium (ExAC) found that out of ~7 million variants, 99% have a frequency <1% and 54% are singletons (Taliun et al. 2021). Similarly, in the Alzheimer's Disease Sequencing Project (ADSP) whole-exome sequencing (WES) of ~10,000 individuals, 97% of identified variants have a minor allele frequency

<1%, and 23% are singletons (Butkiewicz et al. 2018). However, the effect of most rare variants on diseases of interest remains unknown because of insufficient statistical power to detect the associations between these variants and phenotypes.

We hypothesized that rare missense variants contribute to common diseases by disrupting the protein function and are likely to form clustered or dispersed patterns within protein structures when examined in population-based studies. Therefore, incorporating spatial context will improve rare variant association tests. Prior studies have shown that missense variants show nonrandom

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patterns in protein structures, such as cancer-associated hotspot regions with a high density of missense somatic mutations (Tokheim et al. 2016). Our group (Sivley et al. 2018) also found that germline causal missense variants for Mendelian diseases show nonrandom patterns in three-dimensional (3D) space. These patterns include clusters that likely reflect disruption of a key functional region and dispersions that likely reflect depletion of variants within a sensitive protein core.

To test this hypothesis within sequencing studies of disease traits, we developed a kernel function to quantify genetic similarity among individuals by using protein structure information. When two individuals have different missense variants distal in genomic coordinates but close in 3D protein structure, these individuals will be assigned a high genetic similarity through our kernel function. When applied over an entire data set, our kernel function captures differences in the spatial patterns of rare missense variants among cases and controls or over continuous traits. Using a statistical framework similar to SKAT (Wu et al. 2011), we test the association of rare variants with quantitative and dichotomous phenotypes using this structure-based kernel. We call this approach protein optimized kernel evaluation of missense nucleotides (POKEMON). We validated that POKEMON can identify trait associations with spatial patterns formed by missense variants both in simulation studies and real-world data.

Results

POKEMON can detect associations with spatially clustered or dispersed rare variants

As a proof of concept, we evaluated the performance of POKEMON using simulations that mimic real-world case/control studies (Supplemental Fig. S1A–D). The simulation data varied in sample sizes, the odds ratio of the core variants, and the proportion of influential to neutral variants. We simulated a cluster pattern of influential variants by establishing a maximum odds ratio decaying over a fixed distance of 7 Å. We limit the number of variants within the genotype profile to 50, the mean number of variants mapped per protein in the ADSP WES discovery data set.

We test the structure kernel approach implemented in POKEMON and compare it to two other structure-informed association methods, PSCAN and POINT (Marceau West et al. 2019; Tang et al. 2020) and a frequency-based kernel analogous to a SKAT analysis of missense variants (Fig. 1). We evaluated the false positive rate (FPR) of POKEMON with the structure kernel and found that the averaged FPR is 0.0455 for all simulated configurations (Supplemental Fig. S2).

Additionally, to evaluate POKEMON's ability to identify a dispersed pattern, we simulated a scenario in which influential vari-

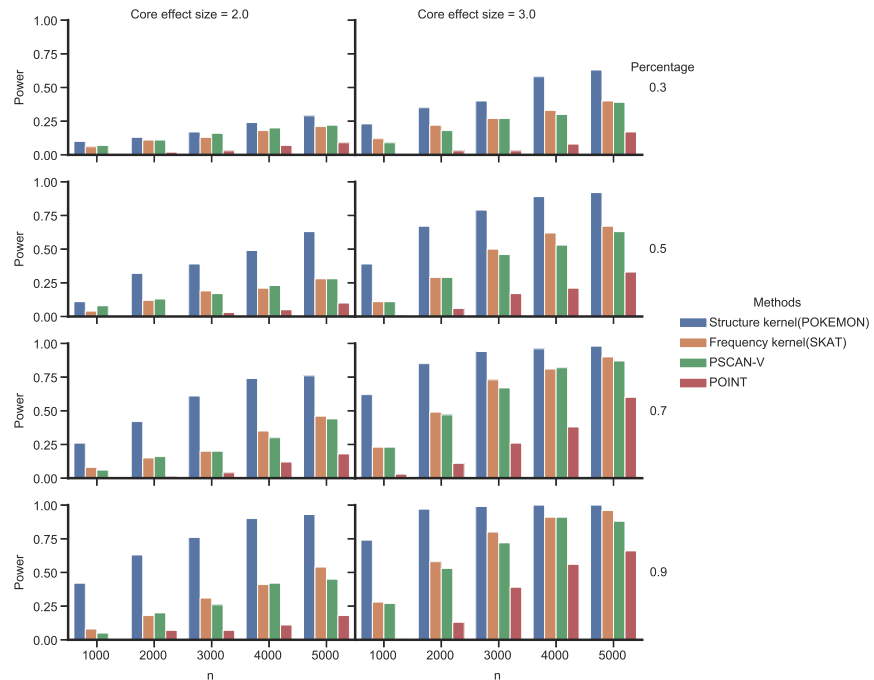


Figure 1. The empirical power for detecting the association between the phenotype and a core pattern on the protein among structure kernel (POKEMON), frequency kernel (SKAT), PSCAN with variance (PSCAN-V), and POINT. The core variant odds ratio is 2.0 or 3.0 (left to right). The percentage of pathological variants within the selected 50 variants ranges from 0.3 to 0.9 (top to bottom). The simulated phenotype is calculated based on the core variant odds ratio and the percentage of pathological variants. The empirical power is calculated by the percentage of tests with a *P*-value below the significance level out of 100 replicates.

ants are distributed on the protein's surface. None of the methods performed well when all influential variants on the surface had small odds ratios. When increasing the odds ratio to 1.5, POKEMON outperformed other methods in all scenarios (Supplemental Fig. S3).

We assessed POKEMON's power at a higher resolution for different core odds ratios and the proportion of influential to neutral variants. Figure 2 illustrates the dynamics of statistical power for the POKEMON test under the assumption of a spatial effect. POKEMON achieved a power of 0.8 with study designs commonly found in sequencing studies of complex disease: a population of 3000 cases/3000 controls, the core odds ratio of 3.0 (decaying to 1 within 7 Å), and 50% of the rare variants influential on the simulated phenotype with moderate effect. However, when the percentage of influential variants is low (<35%) and the core variant odds ratio is small (<1.8), POKEMON cannot reach 80% power. A small core odds ratio and a low percentage of influential variants are more challenging for POKEMON to assess because more control subjects will carry variants within the cluster region, making POKEMON less likely to identify associated patterns.

We further assessed if POKEMON can mitigate the confounding effect from population stratification typically seen in frequency-based tests. We simulated the scenarios from being highly correlated (with 95% subjects with ancestry-matched phenotype) to completely uncorrelated (with 50% subjects with ancestry-matched phenotype). When no covariates are included to adjust for population stratification, we found that tests with Protein Data Bank (PDB) or AlphaFold2 (Senior et al. 2020) structures have lower genomic inflation factors than the corresponding

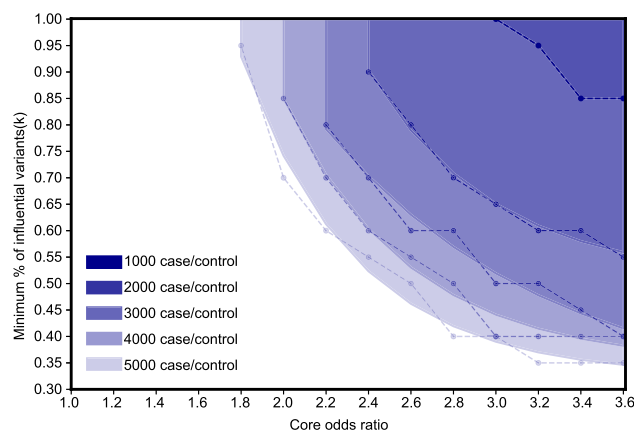


Figure 2. The power assessment for POKEMON with different configurations and the structure kernel. Each dashed line represents the minimum percentages of influential variants and minimum core variant odds ratios required to reach the power of 0.8 when the number of cases/controls is fixed. The empirical power is calculated by the percentage of tests with a P -value below the significance level out of 500 replicates. The edge of each shade is the inferred power boundary fit with an exponential function.

frequency tests (Fig. 3). Therefore, we conclude that although the POKEMON test is confounded by ancestry differences, it is less prone to population stratification than a frequency-based test.

POKEMON replicates the cancer-related spatial clusters from the TCGA data set

To show POKEMON's ability to identify spatial patterns from real-world data, we analyzed germline variants from The Cancer Genome Atlas (TCGA), which has previously been used to identify spatial clusters associated with cancer risk and metastasis (Huang et al. 2018). We constructed a case/control data set by combining 8647 subjects from TCGA across 33 cancer types with 4919 presumably cancer-free controls from the ADSP WES discovery data set. We restricted our POKEMON analysis to rare somatic and germline variants only and 31 proteins with functional assessment in the literature. Although the use of population-based controls is not ideal, this proof-of-concept analysis directly tested the hypothesis that cancer-related variants tend to cluster in a protein hotspot, whereas rare variants from cancer-free subjects are randomly distributed. We observed several highly significant associations within the 31 proteins evaluated and enriched significant results (20 with FDR corrected $P < 0.05$) (see Supplemental Table S1A).

From these results, we focus specifically on two genes highlighted in the literature—namely *RET* and *MET* (Table 1; Fig. 4A–F). We found similar clusters of variants for *RET* and *MET*, formed by somatic variants and pathological/likely pathological germline variants (Huang et al. 2018). For *MET*, POKEMON identified a cluster formed by V1088E, P1091L, C1009Y, V1110I, H1112Y, T1114S, F1142L, N1156K (case cluster 0 in Fig. 4B), which is around the pathological variant H1112R and overlap with the hotspot identified in Huang et al. (2018). For *RET*, POKEMON identified two clusters surrounding the pathological variants V804M and I852M (Fig. 4E,F).

POKEMON identified the clusters in *MET* via case/control analysis of rare germline and somatic variants while excluding known pathological variants. Apart from *MET*, we found seven genes (*BLM*, *MSH2*, *PMS2*, *POT1*, *PTPN11*, *TP53*, and *VHL*) with

pathological variants identified in Huang et al. (2018) showing significant association even after the pathological variants are excluded (Supplemental Table S1B). Thus, our significant association statistic is driven by additional rare variants within *MET* surrounding those with known pathological effects.

POKEMON identifies known AD risk genes (*TREM2* and *SORL1*) and a novel candidate gene (*EXOC3L4*)

Next, to seek any spatial rare variant patterns associated with AD, we applied POKEMON with structure kernel to the ADSP WES discovery data set with 5522 AD cases and 4919 controls. We performed the POKEMON test on 5969 genes with structures from the PDB and 17,450 with AlphaFold2 predicted structures. All the structures are with five or more rare missense variants mapped ($MAF < 0.05$). *APOE* $\epsilon 2$ dosages, *APOE* $\epsilon 4$ dosages, PC1, PC2, and sex are included as covariates (model 0). The overall results of our discovery analysis did not show large genomic inflation (GC) in terms of the POKEMON analysis ($GC = 1.205$ with 5969 PDB structures and $GC = 1.169$ with 17,450 structures), which is comparable to 1.11 with SKAT-O model in Bis et al. (2020).

We used two significance thresholds to identify candidate genes: a Bonferroni correction threshold and an FDR threshold < 0.2 . Overall, four genes meet our significance criteria. *TREM2* was identified with the Bonferroni correction, whereas *SORL1*, *EXOC3L4*, and *TAS2R39* were identified with the FDR threshold (Table 2). Full results with both model 0 and model 1 are in Supplemental Tables S2 and S3. We also note that *CSF1R*, a known dementia-associated gene, falls just below our FDR threshold (Supplemental Fig. S4A,B; Supplemental Table S8).

To determine if the cluster pattern we detected is stable even in the absence of the known associated variants within *SORL1* and *TREM2*, we excluded AD-related variants previously identified in GWAS studies and left only rare genetic variants with unknown effects on AD. A significant result from a POKEMON analysis of these

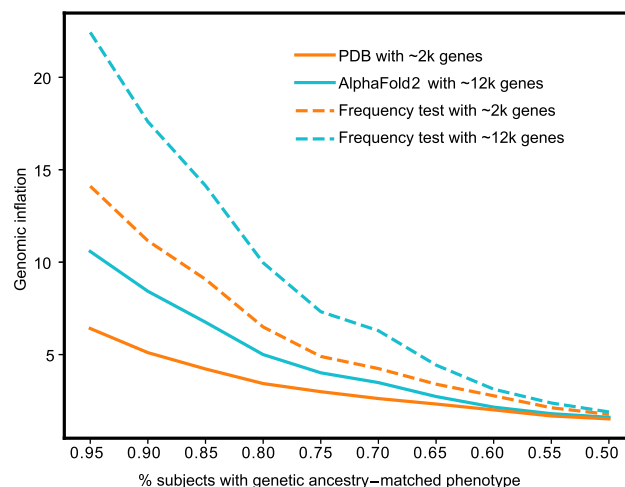


Figure 3. The genomic inflation assessment for POKEMON shows that POKEMON is less prone to population stratification than the frequency test. The genomic inflation is calculated for both approximately 2000 genes with available Protein Data Bank (PDB) structures and about 12,000 with available AlphaFold2 structures. The phenotype is simulated with varying percentages of subjects with genetic ancestry-matched phenotype. The results for frequency tests in the dashed lines are calculated with the same genes with available PDB structures or available AlphaFold2 structures.

Table 1. Results for genes from TCGA data set

Gene	Entry	Phenotype	Number of SNPs mapped	P-value	P-value (pathological variant excluded)
<i>MET</i>	PDB:1R0P	cancer	17	0.00488	0.00807
<i>RET</i>	PDB:2IVT	cancer	57	0.0174	0.0764

remaining variants indicates that additional rare variants within these genes contribute to AD risk.

Indeed, for *SORL1* (Ensembl: ENSG00000137642; PDB: 3WSY), although AD-related variants A528T (Overall MAC:439; MAF:0.0210), and E270K (Overall MAC:990; MAF:0.0474), respectively, were excluded (Vardarajan et al. 2015), the signals persist. The result indicates that the spatial pattern of variants within the 3WSY structure of *SORL1* is associated with AD (Table 3A). Similarly, for *TREM2* (Ensembl: ENSG00000095970; PDB: 6XDS), the signal persists after variant R47H (Guerreiro et al. 2013; Korvatska et al. 2015) is excluded (Table 3B).

We next tested the four significant genes (*TREM2*, *SORL1*, *EXOC3L4*, and *TAS2R39*) in two additional data sets, the ADSP WGS replication data set and the ADSP validation data set. The results for these four genes with model 0 are shown in Table 4. Additional results with Model0-10PCs, Model1-10PCs can be found in Supplemental Table S4. The ADSP WGS replication is in-

dependent of the ADSP WES discovery data set and contains non-Hispanic White, African American, and Hispanic individuals. The ADSP validation data set contains European descent subjects only, of which 9702 subjects are from the ADSP WES discovery data set and 5376 subjects are from the ADSP WGS replication data set. Furthermore, the joint genotype calling approach for the ADSP validation was updated; thus, the ADSP validation data set represents the largest consistently processed and ancestrally homogenous sequencing data set available for AD (Supplemental Fig. S5).

For *TREM2*, the signal regions are shown across three data sets (Fig. 5A–C; Supplemental Table S5). The replicated signal across three data sets contains a region from 16 to 66 amino acids (AA) with multiple variants, including Y38C, T66M, R47H, and R62H. These variants were found correlated with the loss of apo/lipoprotein binding (Yeh et al. 2016). For *SORL1*, we found that the signal regions are only identified in the ADSP WES discovery data set and replicated in the ADSP validation data set (Fig. 6A–C; Supplemental Table S6). One of the signal regions is case cluster 1 in Figure 6A and case cluster 6 in Figure 6C, which overlap with the 10CC-b subunit. The 10CC-b subunit has been found as a dynamic domain with large conformational change when pro-peptide binds (Kitago et al. 2015). Because the ADSP WES discovery data set and ADSP validation data set have European ancestry subjects only and the ADSP WGS replication data set includes multi-ancestry subjects, we infer that the signal region identified in *SORL1* is potentially population specific.

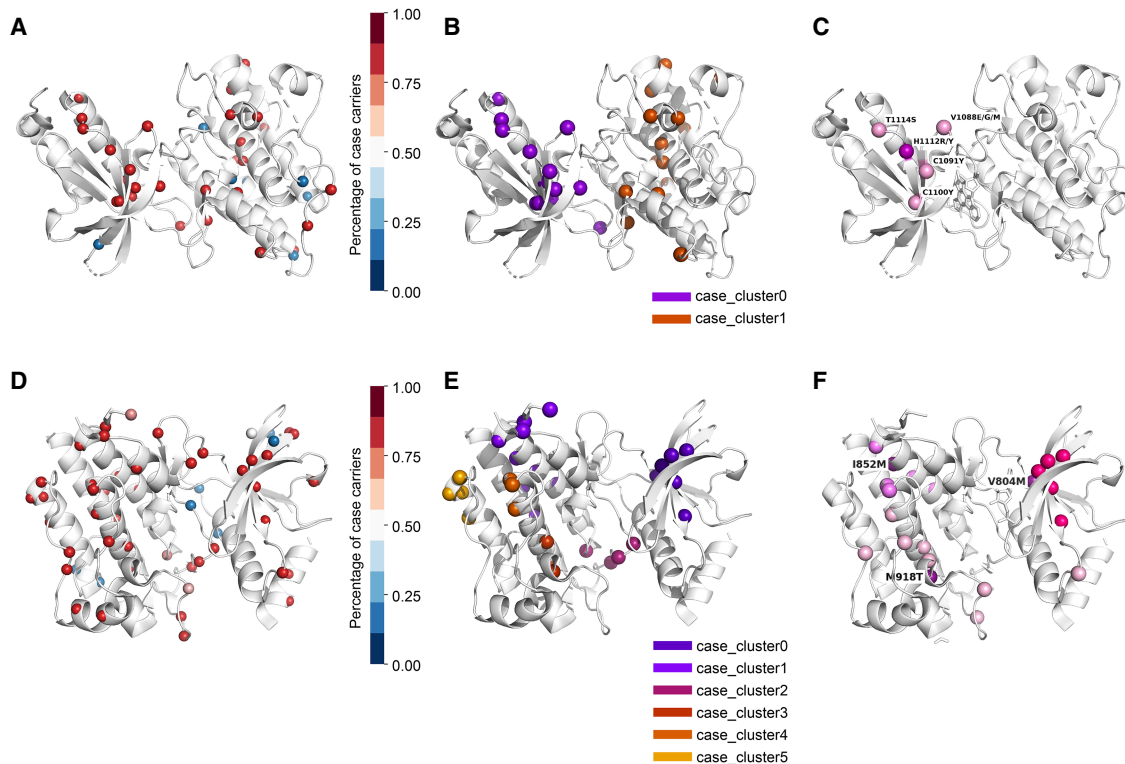


Figure 4. Spatial distribution of variants from TCGA data set within *MET* (PDB:1R0P) and *RET* (PDB:2IVT). (A) Rare missense variants mapped to the *MET*. The color scale indicates the percentage of case subjects that carry the variant of all subjects that have this variant. (B) Signal regions identified by POKEMON in *MET*. (C) A hotspot formed by germline and somatic variants is identified in Huang et al. (2018). Pathological variant H1112R/Y within the hotspot is highlighted with purple sphere models. (D) Rare missense variants mapped to the *RET*. (E) *RET* has signal regions identified by POKEMON. (F) Three hotspots formed by germline and somatic variants are identified in Huang et al. (2018). Three hotspots surrounding M918T, I852M, and V804M are colored pink, violet, and hot pink, respectively. M918T, I852M, and V804M are highlighted with purple sphere models.

Table 2. Genes associated with AD based on structure kernel

Gene	Entry	Number of SNPs mapped	P-value	FDR
PDB structures				
<i>TREM2</i>	PDB:6XDS	33	3.592×10^{-7}	3.351×10^{-5}
<i>SORL1</i>	PDB:3WSY	56	7.022×10^{-5}	6.701×10^{-5}
<i>CSF1R</i> ^a	PDB:4LIQ	38	5.186×10^{-4}	1.005×10^{-4}
AlphaFold2 structures				
<i>TREM2</i>	AlphaFold2: Q9NZC2	33	3.245×10^{-7}	1.146×10^{-5}
<i>EXOC3L4</i>	AlphaFold2: Q17RC7	68	2.504×10^{-5}	2.292×10^{-5}
<i>TAS2R39</i>	AlphaFold2: P59534	31	3.012×10^{-5}	3.438×10^{-5}

^a*CSF1R* falls just below the FDR threshold.

EXOC3L4 has a case cluster 0 range within 581-670AA in the ADSP WES discovery data set, also shown in the ADSP WGS replication data set as case cluster 3 (577-714AA) and in the ADSP validation data set as case cluster 2 (555-714 AA), as shown in Figure 7A–C and Supplemental Table S7. Although the study of *EXOC3L4* function is limited, *EXOC3L4* belongs to the Sec6 protein family, and its C-terminal region is structurally and topologically similar to the Sec6 domain. The case cluster identified above overlaps with Sec6 domain, specifically the D and E regions (Fig. 8A–C), forming the exocyst complex. The exocyst complex involves multiple cellular processes, including exocytosis and cell growth cytokinesis, cell migration, and tumorigenesis (Miller et al. 2018). Miller et al. (2018) found rare variants in the splicing regulatory elements of *EXOC3L4* are associated with brain glucose metabolism measured by FDG PET-scans. Although the splice variant found by Miller et al. (2018) helps skip the second exon of *EXOC3L4*, which is the N terminal of the Sec6 domain, our finding is a cluster of case variants located in the C terminal of the Sec6 domain. Our results suggest alterations to the Sec6 domain of *EXOC3L4* may increase AD risk.

Discussion

We have shown that POKEMON improves the power to detect rare variant gene association in the context of protein structure. We found POKEMON outperforms other structure-based methods through simulation studies except in a small number of cases in which all existing methods have insufficient power. We applied POKEMON to the ADSP Discovery WES data set and identified spatial patterns of rare variants related to AD risk in two known AD genes: *SORL1* and *TREM2*. We also identified a potentially novel AD-associated cluster of variants within *EXOC3L4*, located around the C-terminal end of the Sec6 domain. Specifically, the cluster within *EXOC3L4* is validated both in the ADSP WGS replication and ADSP validation data sets.

An advantage for POKEMON over other rare variant analysis methods is that statistical power increases with the observation of any new variant, including singletons, assuming the existence of spatial patterns. In most rare variant association tests, increasing sample size only increases the power for nonsingleton variants in the resulting data. Even for those nonsingleton variants, the improvement in power is not necessarily proportional to the increased sample size. Moreover, additional neutral variants will be introduced, negatively impacting the statistical power when the sample size increases. In contrast, POKEMON can use rare variants and even singletons with its structure kernel, regardless of their low allele frequency. The increasing number of rare variants helps form

the spatial pattern, which can be identified by POKEMON with a higher power (Supplemental Fig. S1D). We also showed that the spatial patterns are not driven by a single variant but rather a collection of variants with modest effects by excluding variants with known effects for *TREM2* and *SORL1* in the ADSP WES discovery data set.

POKEMON is designed to leverage preexisting biological information for sequencing data sets in which only variant counts or frequencies are typically considered. Although protein structure information of variants has been incorporated into association tests like POINT and PSCAN (Marceau West et al. 2019; Tang et al. 2020), they serve as guiding information for more traditional association tests ultimately based on allele frequency. Therefore, these approaches are still potentially subject to the limitations in unit-based or single variant tests. With the structure kernel, POKEMON uses the spatial information of a missense variant, which is independent of allele frequency. Assuming the rare variants form spatial patterns, POKEMON mitigates the power issue induced by increasing numbers of singleton variants as the sample size of sequencing studies increases.

We anticipate POKEMON will be helpful as a large-scale screening method to detect potentially disease-associated proteins in a proteome-wide fashion under the hypothesis that influential rare variants have a spatial pattern within protein structures. Currently, available protein structures deposited in the PDB only cover a small portion of the identified molecular functions in the human genome (Somody et al. 2017). We expect that the improvement in cryo-EM and advances in protein prediction methods like AlphaFold2 (Senior et al. 2020) will massively increase the availability and quality of structural information for proteins

Table 3A. Results for *SORL1* with and without known loci in ADSP WES discovery

Gene	PDB Entry	P-value
<i>SORL1</i>	PDB:3WSY	7.022×10^{-5}
<i>SORL1</i> (exclude A528T)		1.481×10^{-2}
<i>SORL1</i>		7.022×10^{-5}
<i>SORL1</i> (exclude E270K)		6.34×10^{-5}

Table 3B. Results for *TREM2* with and without known locus in ADSP WES discovery

Gene	PDB Entry	P-value
<i>TREM2</i>	PDB: 6XDS	3.592×10^{-7}
<i>TREM2</i> (exclude R47H)		4.535×10^{-4}

Table 4. Results for candidate genes from the replication data sets

Gene	Entry	ADSP WES discovery Overall: 10,441 Case/control: 5522/4919		ADSP WGS replication Overall: 7762 Case/control: 3757/4005		ADSP validation Overall: 15,078 Case/control: 8294/6784	
		Number of SNPs mapped	P-value	Number of SNPs mapped	P-value	Number of SNPs mapped	P-value
<i>TREM2</i>	PDB:6XDS	33	3.592×10^{-7}	21	3.027×10^{-2}	33	1.484×10^{-8}
<i>SORL1</i>	PDB:3WSY	56	7.022×10^{-5}	53	0.16	82	0.00361
<i>CSF1R</i> ^a	PDB:4LIQ	38	5.186×10^{-4}	38	0.108	46	5.594×10^{-3}
<i>TREM2</i>	AlphaFold2: Q9NZC2	33	3.245×10^{-7}	21	1.194×10^{-2}	33	2.594×10^{-7}
<i>EXOC3L4</i>	AlphaFold2: Q17RC7	68	2.504×10^{-5}	83	5.104×10^{-3}	90	9.221×10^{-5}
<i>TAS2R39</i>	AlphaFold2: P59534	31	3.012×10^{-5}	27	0.522	37	0.0247

^a*CSF1R* falls just below the FDR threshold.

and complexes. A key feature of POKEMON is to test if the structure kernel explains part of the variance of the phenotype; therefore, POKEMON only provides a single association statistic for the influence of missense variants within the protein on the phenotype. Follow-up analyses to assess specific variants or refine variant subsets may provide more detailed quantitative assessments of specific variant spatial patterns.

Methods

Derivation of the POKEMON method

We briefly review the linear mixed model used in association tests and then introduce the construction of a structure kernel for POKEMON. Assume we have n individuals for whom we have p nongenetic covariates, genotypes for m SNPs, and the

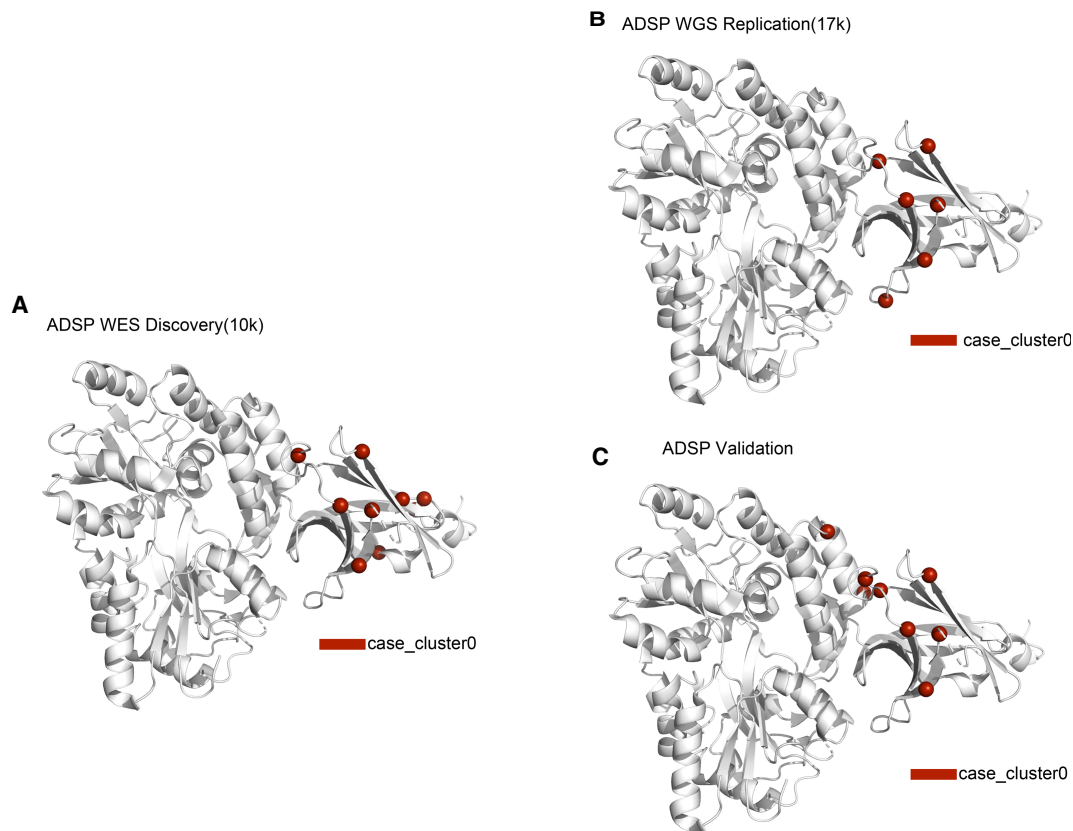


Figure 5. *TREM2* has the signal region identified in the ADSP WES discovery data set (A) and replicated both in the ADSP WGS replication (B) and the ADSP validation (C) data sets. The signal cluster is identified in the POKEMON test with the DBSCAN algorithm. All variants within the clusters are rare variants with $MAF < 0.05$. Clusters classified as case clusters are formed by variants carried primarily by AD subjects, and clusters classified as control clusters are formed by variants carried primarily by cognitively normal subjects. Variants assigned with a cluster label are shown, but all the other variants are not shown in the figure.

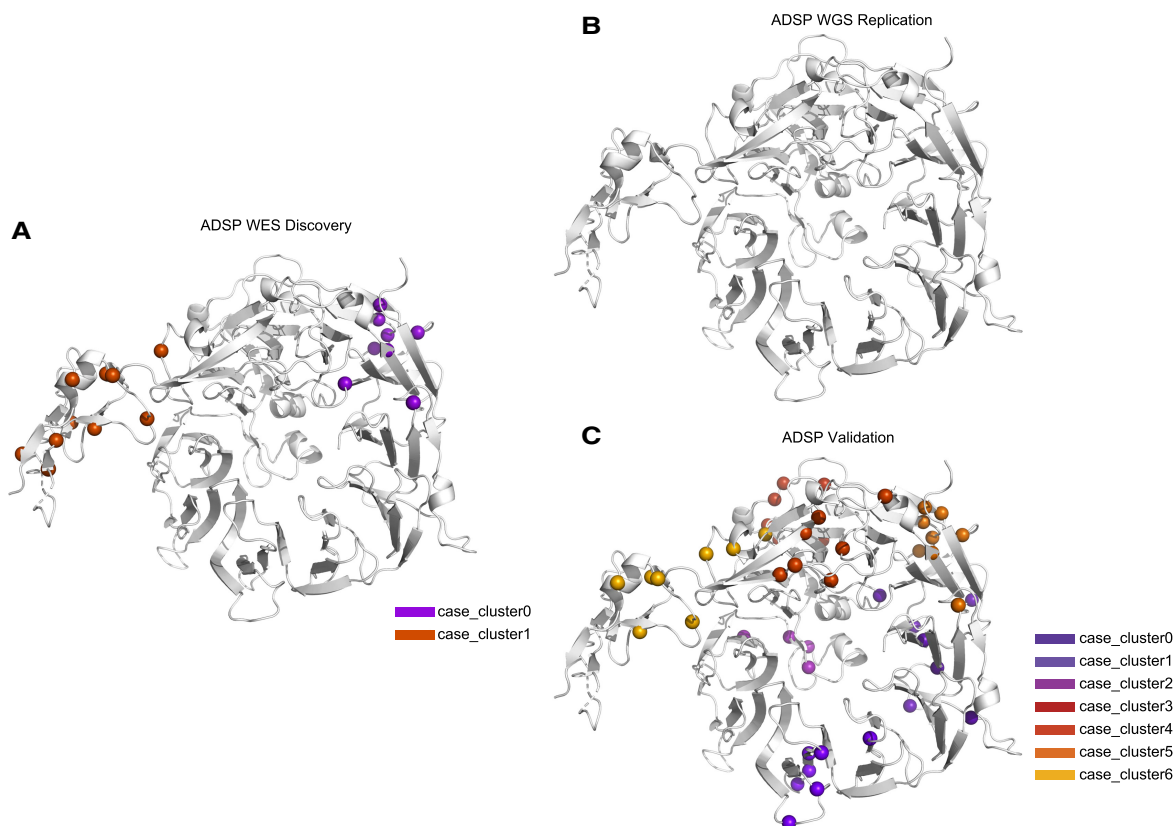


Figure 6. *SORL1* has a signal region identified in the ADSP WES discovery data set (A) and replicated in the ADSP validation data set (C) but not in the ADSP WGS replication data set (B). The signal cluster is identified in the POKEMON test with the DBSCAN algorithm. All variants within the clusters are rare variants with MAF < 0.05. Clusters classified as case clusters are formed by variants carried primarily by AD subjects and clusters classified as control clusters are formed by variants carried primarily by cognitively normal subjects. Variants assigned with a cluster label are shown, but all the other variants are not shown in the figure.

phenotype. Phenotype \mathbf{y} is a $n \times 1$ vector. Genotype \mathbf{G} is a $n \times m$ matrix. Covariate \mathbf{X} is a $n \times p$ matrix.

A linear mixed model contains a fixed effect from covariates $\mathbf{X}\boldsymbol{\beta}$, a random effect annotated by $\mathbf{G}\mathbf{u}$ with \mathbf{u} being the unknown vector of random effects, and an unknown vector of random errors $\boldsymbol{\epsilon}$ (Equation 1a). The \mathbf{y} is fit with a high-dimension normal distribution (Equation 1b). The random effect contains two parts—namely, an environmental effect $\sigma_e^2 \mathbf{I}$ and a genetic effect $\sigma_g^2 \mathbf{K}_g$. \mathbf{K}_g is the kernel containing the genetic similarity between individuals, and σ_g^2 is the amount of variance of \mathbf{y} explained by \mathbf{K}_g . The null hypothesis $\sigma_g^2 = 0$ indicates that the \mathbf{K}_g does not explain any variance of \mathbf{y} .

$$\mathbf{y}_i = \mathbf{X}_i \boldsymbol{\beta} + \mathbf{G}_i \mathbf{u} + \boldsymbol{\epsilon}_i \quad (1a)$$

$$\mathbf{y} \sim N(\mathbf{X}\boldsymbol{\beta}, \sigma_e^2 \mathbf{I} + \sigma_g^2 \mathbf{K}_g) \quad (1b)$$

For continuous traits, the null model is a linear regression with covariates only:

$$\hat{\mathbf{y}}_i = \mathbf{X}_i \boldsymbol{\beta} + \boldsymbol{\epsilon}_i \quad (2)$$

$\hat{\mathbf{y}}$ is the vector with the i th value equivalent to \hat{y}_i , so the score statistic Q is defined as:

$$\frac{Q}{\sigma_e^2} = \mathbf{y}^T \mathbf{S} \mathbf{K}_g \mathbf{S} \mathbf{y} = (\mathbf{y} - \hat{\mathbf{y}})^T \mathbf{K}_g (\mathbf{y} - \hat{\mathbf{y}}) \quad (3)$$

Similarly, for dichotomous traits, the null model is a logistic regression with covariates only. $\hat{\pi}_i$ is the estimated probability for $\mathbf{y}_i = 1$ under the null model.

$$\hat{\pi}_i = \text{logit}^{-1}(\mathbf{X}_i \boldsymbol{\beta}) \quad (4)$$

$\hat{\mathbf{y}}$ is the vector with the i th value equivalent to \hat{y}_i , so the score statistic Q is defined as:

$$\frac{Q}{\sigma_e^2} = \mathbf{y}^T \mathbf{S} \mathbf{K}_g \mathbf{S} \mathbf{y} = (\mathbf{y} - \hat{\boldsymbol{\pi}})^T \mathbf{K}_g (\mathbf{y} - \hat{\boldsymbol{\pi}}) \quad (5)$$

Under the null hypothesis, Q follows a mixed χ^2 distribution (Equation 6), where \mathbf{S} projects \mathbf{y} into a space orthogonal to covariates, and λ_i are the eigenvalues of $\mathbf{S} \mathbf{K}_g \mathbf{S}$.

$$\frac{Q}{\sigma_e^2} \sim \sum_{i=1}^n \lambda_i \chi_1^2 \quad (6)$$

For POKEMON, we construct the $n \times n$ kernel \mathbf{K}_g in the context of protein as follows: For \mathbf{K}_g , each entry is the genetic similarity between individuals based on the variants they carry, which is weighted by the variant's distance in the protein structure (Equation 7), where d_{kl} is the distance of pairwise single-nucleotide variants (SNVs) in angstroms (Å) within the protein, and k and l represent the k th variant from individual i and the l th variants from individual j .

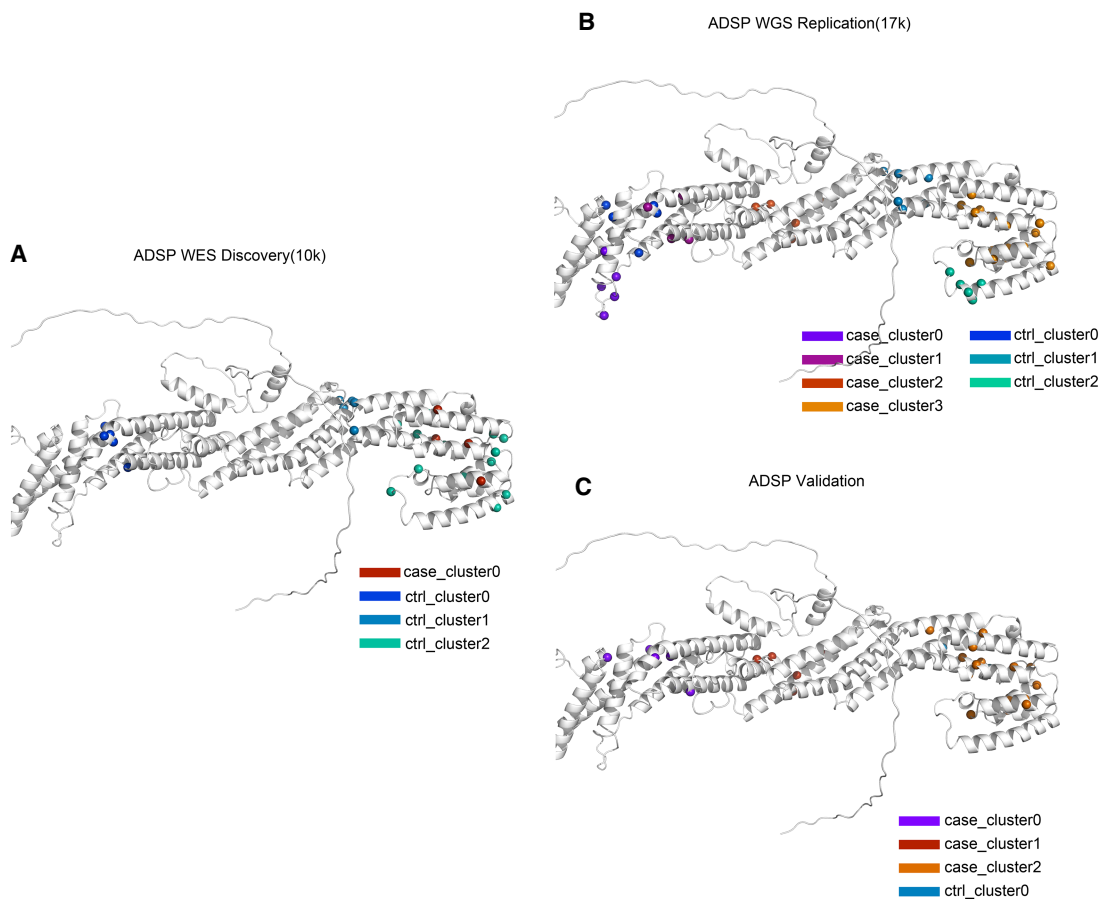


Figure 7. Signal regions on *EXOC3L4* (AlphaFold2: Q17RC7.A) are identified by POKEMON from the ADSP WES discovery data set (A) and validated in both the ADSP WGS replication (B) and the ADSP validation (C) data sets. The signal regions are identified in the POKEMON test with the DBSCAN algorithm. All variants within the clusters are rare variants with MAF < 0.05. Clusters classified as case clusters are formed by variants carried primarily by AD subjects and clusters classified as control clusters are formed by variants carried primarily by cognitively normal subjects.

$$K_{ij} = \sum_{k|l} A_k A_l \min\{f(d_{kl})\}. \quad (7)$$

Some protein structures are formed by identical subunits (homo-multimer), which introduces redundancy in the variant-to-amino acid projection (i.e., one variant can map to multiple amino acids located in different subunits). To eliminate the spatial similarity induced by multiple mapping locations of a single variant in a homo-multimer, we took d_{kl} to be the minimum distance among all pairwise distances. Function $f(d)$ converts a Euclidean distance to the similarity score for a pair of variants.

$$f(d_{kl}) = e^{-\frac{d_{kl}^2}{2t^2}}. \quad (8)$$

As a default, the exponential function for f is in Equation 8 with t set to a value of 14 Å; and 14 Å is a commonly adopted short-range nonbonded cutoff in molecular dynamic simulation (Monticelli et al. 2008).

Apart from spatial patterns, we also account for the magnitude of the protein change resulting from the different amino acid substitutions. We scaled the pairwise variants by their amino acid substitution, which is defined as A_k and A_l . A_k and A_l are the weights for amino acid substitution for variant k and variant l according to the BLOSUM62 matrix (Henikoff and Henikoff 1992), respectively. For a less conservative amino acid substitution, the

score s_k in BLOSUM62 matrix will be negative; consequently, A_k will be greater than 1. In contrast, for a neutral or conservative amino acid substitution, s_k will be positive and A_k will be less than 1.

$$A_k = -\sqrt{e^{s_k}}. \quad (9)$$

The structure kernel is nonlinear in contrast to the SKAT tests (Wu et al. 2011), which uses a linear kernel (e.g., $\mathbf{K} = \mathbf{G}\mathbf{W}\mathbf{W}'\mathbf{G}'$) to calculate the genetic similarity between individuals. The genetic similarity in a linear kernel between individuals is the sum of weighted SNVs being shared. However, singletons are carried by only a single individual and thus fail to be included in calculating genetic similarity. With the structure kernel, a pair of singleton variants will be assigned non-zero weights if they are spatially proximate in the protein structure. The interpretation of the structure kernel is that case individuals are genetically similar because they share more spatially clustered or dispersed rare variants than the control individuals.

We also allow for incorporating allele frequency in the POKEMON test by a combined kernel function. One can consider that variants clustered in protein structure already contribute to a high genetic similarity based on structure kernel. With the combined kernel, those variants will be further up-weighted if they are rare in allele frequency and vice versa. The combined kernel function is based on the \mathbf{K}_s and extended by further scaling variants by weights derived from the allele frequency. $w_k = \text{Beta}$

$(MAF_k; a, b)$ is the weight for the k th variant characterized by beta density with $a = 1$ and $b = 25$ as default.

$$K_{ij} = \sum_{i_k, j_l, k \neq l} A_k A_l \min\{f(d_{kl})\} + \sum_{i_k} w_k A_k^2 \quad (10)$$

The power of the frequency-based SKAT test is sensitive to the choice of beta weights. Therefore, although the default beta weights are generally acceptable, we suggest evaluating the beta weights based on the frequency distribution in the data of interest and selecting the optimal beta weights for a combined kernel (Chen et al. 2018).

POKEMON workflow

An overview of the POKEMON workflow is shown in Supplemental Figure S6. POKEMON requires a genotype matrix and consequence profile containing variant-to-amino acid mapping information as inputs. Additional covariate files are optional to adjust for covariates. POKEMON first maps the variants by the coordinates into the protein, which is accomplished with the consequence profile generated by Ensembl Variant Effect Predictor (VEP v95) and the reference from SIFTS mapping PDB entry to UniProt residue level (Dana et al. 2019). A single variant may be mapped to multiple amino acids for multimers with identical subunits. The protein structures are fetched from PDB during the analysis. If multiple protein structures are available for a single gene, the structure with the most variants mapped will be selected. However, if a PDB entry is given, POKEMON also allows the analysis of a specified protein structure. After mapping, the score between a pair of variants is calculated based on the minimum distance between them, which is further scaled by the amino acid substitution weight from the BLOSUM62 matrix by default. The pairwise genetic similarity between individuals is the summation of all pairwise scores of variants. The genetic similarity kernel K_g will be evaluated in the variance component test.

Data simulation

Simulation strategy for power assessment

We conducted simulation studies to assess POKEMON's power in detecting disease-associated protein variant patterns. We hypothesized that variants with moderate effects on a phenotype form spatial patterns within a protein structure and alter the protein's function. To test the hypothesis, we established two patterns. The first pattern entails an embedded core within the protein disrupted by rare variants (i.e., variant clustering), whereas the other represents the localization of influential variants to the protein's surface (i.e., variant dispersion). Both patterns are shown in Supplemental Figure S1A,B. We randomly selected a protein PDB:2OGV to carry out simulations because the structural information for PDB:2OGV is available for both PSCAN and SKAT.

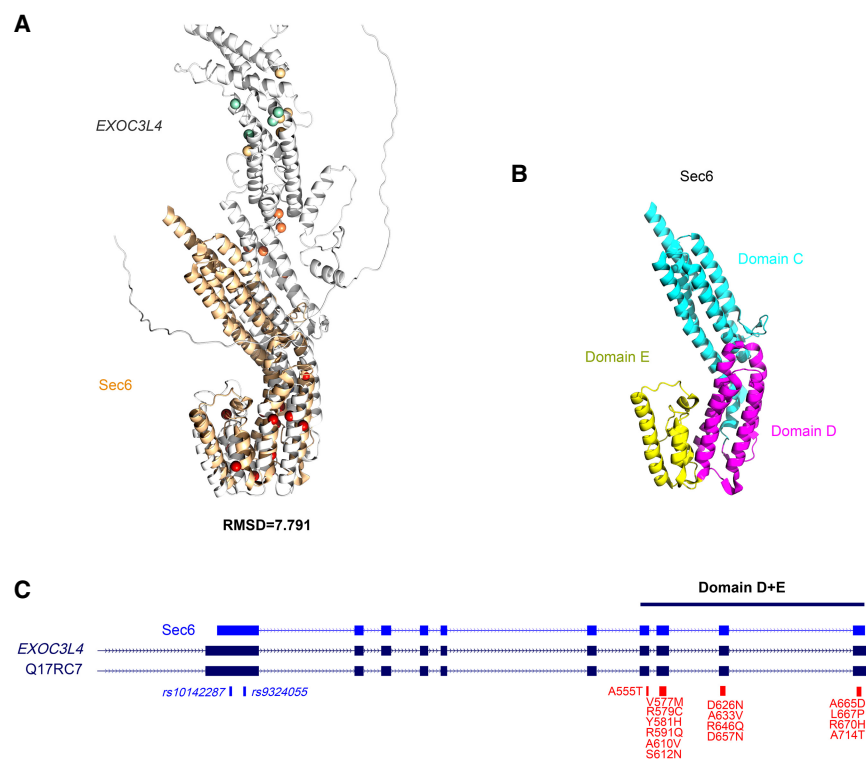


Figure 8. Sec6 domain in *EXOC3L4* contains a cluster of variants primarily carried by AD case individuals. (A) Alignment of the *EXOC3L4* and Sec6. The structure for *EXOC3L4* is from AlphaFold2 with entry Q17RC7, and the structure for Sec6 is PDB:2FJI. The alignment is performed with PyMOL. (B) The structure for the C-terminal domain of Sec6 is formed by three domains C, D, and E. (C) The genomic coordinate of Sec6, *EXOC3L4*, the splicing variants from Miller et al. (2018), and variants from case cluster 2 in Figure 7C. The splicing variants are colored blue and labeled with dbSNP Reference SNP number. The variants from case cluster 2 in Figure 7C are colored in red and labeled with the amino acid change.

We simulated a clustering pattern by distributing influential variants within the core of the protein structure and scaling the variant odds ratios proportionally to their distance from the core. We then randomly sampled 50 variants from the protein. The minor allele frequencies for all the variants were randomly sampled from a log-transformed uniform distribution within an interval $(-4, -2.3)$. This variant sampling strategy restricted the selected minor allele frequencies within the range $(0.0001, 0.005)$ and generated singletons, which is consistent with ADSP WES studies (Supplemental Fig. S7). To investigate how neutral variants influence the power, we varied the percentage of influential variants out of all sampled variants (Supplemental Fig. S1D). For each set of parameters (e.g., sample size, core variant odds ratio, etc.), the empirical power was estimated by the percentage of successful tests out of 100 independent tests with a significance level of 0.05. We compared the empirical power of POKEMON with three other methods: SKAT, PSCAN-V, and POINT. The number of case and control subjects sampled is from 1000 to 5000. Additional details for the simulation can be found in Supplemental Figure S1A–D and Supplemental Methods.

We also simulated a dispersion pattern by distributing influential variants on the protein's surface. Considering the selected protein PDB:2OGV is about 40 Å in diameter, we defined the surface variants as those >21 Å away from the core, which yielded 33 variants. All the surface variants were assigned with the same odds ratio (e.g., 1.1), whereas the rest were considered neutral with an odds ratio of 1. The simulation settings were similar to the

clustering pattern, with the only difference that we sampled 30 variants from the protein, which allowed us to tune the percentage of influential variants to as large as 90%.

Simulation strategy for genomic inflation assessment

We selected 671 subjects identified as African ancestry and 522 as European ancestry from the 1000 Genomes Project. When we chose different percentages of subjects with genetic ancestry-matched phenotype r , $r\%$ of the European ancestry subjects will be assigned a phenotype equivalent to 1. In contrast, the rest within European ancestry subjects will be assigned a phenotype equivalent to 0. Similarly, $r\%$ of the African ancestry subjects will be assigned a phenotype equivalent to 0, and the rest within African ancestry subjects will be assigned a phenotype equivalent to 1. Then we will test this phenotype with 2719 available protein structures from PDB and 13,691 structures from AlphaFold2. The genomic inflation is calculated for PDB structures and AlphaFold2 structures, respectively.

Applying POKEMON to ADSP data

The ADSP WES discovery data set, ADSP WGS replication data set used in this study is available at ADSP (<https://www.niagads.org/adsp/content/home>). An application to the NIAGADS Data Sharing Service is needed to access the data.

The model we used for all the results in Tables 1–4 is model 0, which adjusted for *APOE* $\epsilon 2$ and $\epsilon 4$ dosages, PC1, PC2, and sex. In model 0, *APOE* $\epsilon 2$ and $\epsilon 4$ dosages are to exclude signals induced by the well-known *APOE* association. PC1 and PC2 are included to avoid false positive signals owing to population structure.

We also evaluated other models that included additional covariates and all the results are in the [Supplemental Tables](#). Model 1 adjusted for *APOE* $\epsilon 2$ and $\epsilon 4$ dosages, PC1, PC2, sex, and age at diagnosis or last follow-up. Model 0-10PCs adjusted for *APOE* $\epsilon 2$ and $\epsilon 4$ dosages, PC1-10, and sex. Model 1-10PCs adjusted for *APOE* $\epsilon 2$ and $\epsilon 4$ dosages, PC1-10, sex, and age.

ADSP WES discovery data set

We used the whole-exome sequencing (WES) data from the discovery phase case-control study under the Alzheimer's Disease Sequencing Project (ADSP). ADSP WES data contains 5740 late-onset AD cases and 5096 cognitively normal controls primarily of European ancestry, with 218 cases and 177 controls of Caribbean Hispanic ancestry. Cases were determined based on diagnosis using cognitive testing data and medical records, and controls were determined on their low risk of developing AD by age 85 yr (Beecham et al. 2017; Bis et al. 2020).

We selected 10,441 subjects of European ancestry from the ADSP as the study group (5522 late-onset AD cases and 4919 cognitively normal controls) and shown in [Supplemental Figure S5](#). We retained the missense variants with minor allele frequency < 0.05 for our assessment. Overall, we selected 5969 genes with experimentally determined protein structures and 17,450 with AlphaFold2 predicted structures, all of which have five or more rare missense variants mapped to the structure. The mean number of rare missense variants mapped to the PDB structure per gene was approximately 50.

ADSP WGS replication data set

We used the whole-genome sequencing (WGS) data from the Alzheimer's Disease Sequencing Project (ADSP) as the replication data set. ADSP WGS contains 3757 AD cases and 4005 cognitively normal controls. Within these 7762 samples, 5375 are non-

Hispanic White, 1571 are African American, and 803 are of Hispanic, Asian, or Native American ancestry ([Supplemental Fig. S5](#)). All the subjects in the ADSP WGS replication data set are independent of those in the ADSP WES discovery data set.

ADSP validation data set

The validation data set contains the 9702 subjects from the discovery phase case-control study plus an additional 5375 subjects from the replication data set for a total of 15,078 non-Hispanic White subjects ([Supplemental Fig. S5](#)). The WES data for the 9702 subjects were reprocessed using joint genotype calling approaches implemented in the VCPA pipeline (Leung et al. 2019), which were updated from the ATLAS genotype calling process implemented for the ADSP WES discovery data set. Therefore, we consider that this validation data set is valuable by expanding the sample size for a genetically homogenous population group and accounting for the variability in the variant calling process.

Applying POKEMON to TCGA data

The TCGA data is a real-world, true-positive example of spatial patterns of missense variants associated with phenotypes (Kamburov et al. 2015). To create a data set in the form of a case-control study, we combined 4919 control subjects from the ADSP WES discovery data set and 8647 subjects from TCGA data diagnosed with 33 cancer types (Huang et al. 2018). We assumed that 4919 cognitively normal control subjects from the ADSP WES discovery data set are cancer-free controls. Although this is not an ideal study design, any violation of this assumption would reduce statistical power rather than identifying spurious associations. The combined case/control data set provided a real-world assessment of our hypothesis that rare variants from cancer tissues would form spatial patterns. In contrast, those from control subjects would be randomly distributed within the protein.

Both germline and somatic variants from the TCGA are included. Moreover, we set a stringent MAF threshold as <0.01 to retain rare variants. In summary, we performed POKEMON tests on 31 genes with potential hotspots (Huang et al. 2018) and available protein structures with no covariate included.

Software availability

The code for this study is available as [Supplemental Code](#) and at GitHub (<https://github.com/bushlab-genomics/POKEMON>).

Competing interest statement

The authors declare no competing interests.

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AG005134, P50 AG016574, P50 AG005138, P30 AG008051, P30 AG013854, P30 AG008017, P30 AG010161, P50 AG047366, P30 AG010129, P50 AG016573, P50 AG016570, P50 AG005131, P50 AG023501, P30 AG035982, P30 AG028383, P30 AG010124, P50 AG005133, P50 AG005142, P30 AG012300, P50 AG005136, P50 AG033514, P50 AG005681, and P50 AG047270), Alzheimer's Disease Neuroimaging Initiative (ADNI) (U19AG024904), Amish Protective Variant Study (RF1AG058066), Cache County Study (R01AG11380, R01AG031272, R01AG21136, RF1AG054052), Case Western Reserve University Brain Bank (CWRUBB) (P50AG008012), Case Western Reserve University Rapid Decline (CWRURD) (RF1AG058267, NU38CK000480), CubanAmerican Alzheimer's Disease Initiative (CuAADI) (3U01AG052410), Estudio Familiar de Influencia Genética en Alzheimer (EFIGA) (5R37AG015473, RF1AG015473, R56AG051876), Genetic and Environmental Risk Factors for Alzheimer Disease Among African Americans Study (GenerAAtions) (2R01AG09029, R01AG025259, 2R01AG048927), Gwangju Alzheimer and Related Dementias Study (GARD) (U01AG062602), Hussman Institute for Human Genomics Brain Bank (HIHGBB) (R01AG027944, Alzheimer's Association "Identification of Rare Variants in Alzheimer Disease"), Ibadan Study of Aging (IBADAN) (5R01AG009956), Mexican Health and Aging Study (MHAS) (R01AG018016), Multi-Institutional Research in Alzheimer's Genetic Epidemiology (MIRAGE) (2R01AG09029, R01AG025259, 2R01AG048927), Northern Manhattan Study (NOMAS) (R01NS29993), Peru Alzheimer's Disease Initiative (PeADI) (RF1AG054074), Puerto Rican 1066 (PR1066) (Wellcome Trust [GR066133/GR080002], European Research Council [340755]), Puerto Rican Alzheimer Disease Initiative (PRADI) (RF1AG054074), Reasons for Geographic and Racial Differences in Stroke (REGARDS) (U01NS041588), Research in African American Alzheimer Disease Initiative (REAAADI) (U01AG052410), Rush Alzheimer's Disease Center (ROSMAP) (P30AG10161, R01AG15819, R01AG17919), University of Miami Brain Endowment Bank (MBB), and University of Miami/Case Western/North Carolina A&T African American (UM/CASE/NCAT) (U01AG052410, R01AG028786).

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