




ARTICLE

Genetic association of the cytochrome c oxidase-related genes with Alzheimer's disease in Han Chinese

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Alzheimer's disease (AD) is the most common cause of dementia. Mitochondrial dysfunction has been widely reported in AD due to its important role in cellular metabolism and energy production. Complex IV (cytochrome c oxidase, COX) of mitochondrial electron transport chain, is particularly vulnerable in AD. Defects of COX in AD have been well documented, but there is little evidence to support the genetic association of the COX-related genes with AD. In this study, we investigated the genetic association between 17 nuclear-encoded COX-related genes and AD in 1572 Han Chinese. The whole exons of these genes were also screened in 107 unrelated AD patients with a high probability of hereditarily transmitted AD. Variants in *COX6B1*, *NDUFA4*, *SURF1*, and *COX10* were identified to be associated with AD. An integrative analysis with data of eQTL, expression and pathology revealed that most of the COX-related genes were significantly downregulated in AD patients and mouse models, and the AD-associated variants in *COX6B1*, *SURF1*, and *COX10* were linked to altered mRNA levels in brain tissues. Furthermore, mRNA levels of *Ndufa4*, *Cox5a*, *Cox10*, *Cox6b2*, *Cox7a2*, and *Lrrprc* were significantly correlated with A β plaque burden in hippocampus of AD mice. Convergent functional genomics analysis revealed strong supportive evidence for the roles of *COX6B1*, *COX10*, *NDUFA4*, and *SURF1* in AD. As the result of our comprehensive analysis of the COX-related genes at the genetic, expression, and pathology levels, we have been able to provide a systematic view for understanding the relationships of the COX-related genes in the pathology of AD.

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INTRODUCTION

Alzheimer's disease (AD) is the most prevalent age-related neurodegenerative disease, and is characterized by progressive brain atrophy and neuronal cell loss leading to cognitive impairment [1–3]. The deposition of β -amyloid (A β) plaques and the accumulation of neurofibrillary tangles formed by hyperphosphorylated tau in the brain were recognized as the main neuropathological hallmarks of AD [3, 4]. It is widely accepted that genetic factors play an important role in the pathogenesis of AD [5, 6]. Over 100 mutations in three main genes, amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*), and presenilin 2 (*PSEN2*) have been reported to cause familial AD [5, 6]. However, familial AD constitutes only 5% of total AD incidence. For the remaining 95%, genome-wide association studies (GWASs) in recent years have identified several AD susceptibility genes in populations of European ancestry [7], and the associations of part of these genes with AD were validated in Chinese populations in our previous study (including *APOE*, *PICALM*, *BIN1*, *CLU*, and *MS4A4E* [8]) and others [9]. However, the genes identified by GWAS can only explain about 16% of the total phenotypic variance, indicating

there is missing heritability for AD [10]. Therefore, additional AD-related genes and variants are yet to be identified.

The mitochondrion is the center of cellular energy production, and its dysfunction contributes to the pathogenesis of neurodegenerative diseases [11–13]. An increasing amount of evidence has demonstrated that mitochondrial defects, including deficiency in oxidative phosphorylation (OXPHOS), increased reactive oxygen species (ROS) level, reduced ATP production, impaired balance of mitochondrial fission and fusion, are early events occurring in the brain of AD patients and animal models [11, 14–24]. On the other hand, the proteins of AD pathological pathways, including APP and A β , have been found to target mitochondria and disrupt mitochondrial function [11, 23, 25–27]. One recent study has found the enhancement of mitochondrial proteostasis could be helpful in reducing the toxic effect of A β [28].

Defects in the complexes of the mitochondrial respiratory chain, particularly those decreasing the efficiency of complex IV (also called cytochrome c oxidase (COX)), have been widely reported in AD patients and animal models [19, 20, 29–32]. In mammals, the COX is composed of 13 subunits [33, 34], and of

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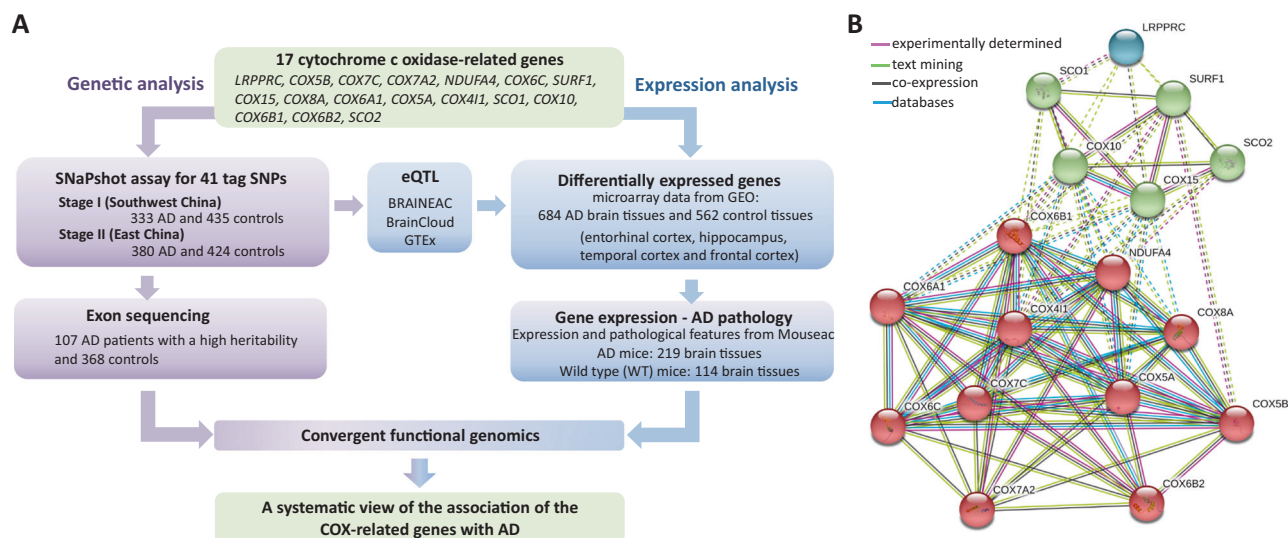


Fig. 1 The strategy for investigating the association between the COX-related genes and AD. **a** The workflow in this study. **b** Protein–protein interactions (PPI) of 17 proteins encoded by the COX-related genes. The PPI analysis was performed using the STRING database (<https://string-db.org/cgi/input.pl>), which presents known and predicted PPI [94]. Two clusters were obtained, one cluster (red nodes) included 11 subunits of COX; the other cluster (blue and green nodes) contained 6 assembly factors of COX

these, 3 subunits are encoded by the mitochondrial genome (COX1, COX2, and COX3) and are the catalytic core of COX, while the other 10 subunits (COX4, COX5a, COX5b, COX6a, COX6b, COX6c, COX7a, COX7b, COX7c, and COX8) are nuclear-encoded and are structural subunits surrounding the catalytic core [33, 34]. Furthermore, there is another COX subunit NDUFA4, albeit with some controversy [35], newly identified in a recent study [36]. The expression levels of the nuclear-encoded COX subunits were found to be significantly reduced in brains and platelets of AD patients [20, 30, 37–43]; whereas, the mitochondrial-encoded 3 subunits were not significantly changed or even increased in AD patients [20, 30]. In addition to the subunits, there are many proteins participating in the assembling of COX, which are all nuclear-encoded proteins and are important regulators for the COX structure and function [33, 34, 44]. Among these assembly factors, mutations in *COX10*, *COX15*, *SURF1*, *LRPPRC*, *SCO1*, and *SCO2* were identified as contributing to disorders of COX deficiency [44, 45]. One study in an Italian population, albeit with a limited sample size, has identified variants in the *COX10* and *COX15* genes to be associated with AD [46]. In general, the genetic association between the COX-related genes and AD has seldom been observed in human populations.

Considering the fact that decreased COX activity has been regarded as a characteristic of AD, we hypothesize that variants in the COX-related genes may confer genetic risk to AD. However, as yet there is no clear genetic evidence to support the association between the COX-related genes and AD. In this study, we analyzed variants in 17 COX-related genes (11 subunits and 6 assembly factors) in 713 AD patients and 859 controls from Han Chinese populations to look for their potential association with AD. We also performed an integrative analysis using publically available genetic data, dealing with gene expression and AD pathology, to clarify the potential role of the COX-related genes in AD. We were able to provide evidence supporting an active involvement of the COX-related genes in the development of AD.

MATERIALS AND METHODS

Subjects

A two-stage case-control study was performed to analyze the association between common variants in the COX-related genes and AD in this study (Fig. 1a). All the samples were of Han Chinese origin

and were analyzed for the *APOE* and other AD-related genes in our previous studies [8, 47–50]. In brief, the discovery stage (stage I) was based on samples collected in Southwest China from 333 AD cases (62.8% females, age 74.08 ± 8.89 years, 24.4% were *APOE* $\epsilon 4$ carriers) and 435 healthy individuals (50.6% females, 17.7% were *APOE* $\epsilon 4$ carriers). The validation stage (stage II) was based on samples collected in East China from 380 AD cases (54.2% females, age 76.18 ± 9.63 years, 38.8% were *APOE* $\epsilon 4$ carriers) and 424 healthy controls (71.0% females, 17.8% were *APOE* $\epsilon 4$ carriers).

In addition, we took advantage of the exome sequencing data of 107 genetically unrelated AD patients (authors' unpublished data) that were selected using an extreme phenotype sampling strategy [51], to identify potential disease-causal/rare/functional variants in the exonic regions of the COX-related genes by comparing to 368 control subjects (including 160 in-house non-dementia controls [52], Han Chinese in Beijing (CHB, $N = 103$) and Southern Han Chinese (CHS, $N = 105$) from the 1000 Genomes Project phase 3 [53, 54]). These genetically unrelated AD patients (46.7% females, age 64.6 ± 10.29 years, 37.4% were *APOE* $\epsilon 4$ carriers) either had an early onset age ≤ 55 years old and/or a positive family history (presence of disease in one or more first-degree family members). Several patients were identified to carry potentially pathogenic mutations in *PSEN1* (one patient), *PSEN2* (three patients), and *GRN* (one patient), but no patient had potentially pathogenic mutation in *APP* and *MAPT* (authors' unpublished data). We focused on these patients with early onset AD and/or family history of the disease, instead of sporadic or late-onset AD, as these patients might be more susceptible to genetic influence and be easier to show the potential consequence of the COX-related gene mutations. This sampling strategy was different from that of the IGAP dataset where all cases with age-at-onset < 65 years of age were excluded [7], but it could be used as an initial study for identifying potentially pathogenic variants to be validated in sporadic AD patients.

The AD patients were diagnosed by at least two clinical psychiatrists according to the revised National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria [55–57], and Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV). Sample collection complied with the declaration of Helsinki, with informed consent being obtained from all the subjects. This study was approved by

the institutional review board of the Kunming Institute of Zoology, Chinese Academy of Sciences.

Genotyping and sequencing of genes encoding subunits and assembly factors of COX

Genomic DNA was extracted from peripheral blood by using AxyPrep Blood Genomic DNA Miniprep Kit (Axygen, USA). Seventeen genes encoding the subunits (11 genes) and assembly factors (6 genes) of the COX were selected to analyze the common variants and variations in the exonic regions (Fig. 1b and Supplementary Table S1). The linkage disequilibrium (LD) pattern of the $-10 \sim +10$ kb region of the targeted genes were analyzed by using Haploview software [58] to pick up SNPs that are capable of tagging more SNPs in the targeted region. A total of 41 SNPs each with a minor allele frequency (MAF) $>10\%$ in the CHB population in the International HapMap Project database (HapMap, <http://hapmap.ncbi.nlm.nih.gov/> [59]) were selected (Supplementary Table S1). According to the LD pattern of Asian population in the 1000 Genomes Project phase 3 (<http://www.1000genomes.org>) [53, 54], these 41 SNPs are capable of tagging a total of 815 SNPs ($r^2 > 0.8$) in the gene region (Supplementary Table S1). The genotyping analysis was performed by using the SNaPshot assay, as described in our previous studies [8, 47, 49, 50]. In brief, multiplex PCR was performed by using 41 pairs of primers (Supplementary Table S2). After cleaning up with SAP and ExoI (TaKaRa Biotechnology Co. Ltd., Dalian, China), the PCR products were subjected to single-base extension reaction by using respective extension primers (Supplementary Table S2) in a total volume of 10 μ L containing 4 μ L multiplex PCR products, 5 μ L SNaPshot Multiplex Ready Reaction Mix and 0.4–0.8 μ M pooled SNP-specific oligonucleotide primers (Supplementary Table S2). After purification, a mixture of 4 μ L purified products and 6 μ L ddH₂O was analyzed by the 3730xl DNA analyzer (Applied Biosystems) at the Kunming Biodiversity Large-Apparatus Regional Center, Kunming Institute of Zoology. We used GeneMarker software (<http://www.softgenetics.com/GeneMarker.html>) to read the genotyping results.

For sequencing the exon and flanking regions of the COX-related genes, we used the next generation sequencing technologies as described in our previous studies [47, 52, 60]. In brief, the paired-end reads were generated on the HiSeq 4000 sequencer (Illumina). High-quality sequence reads were aligned with the reference genome hg19 by Burrows-Wheeler Aligner (BWA, <http://bio-bwa.sourceforge.net/>) [61] and Samtools (<http://samtools.sourceforge.net/>) [62, 63]. The Genome Analysis Toolkit (GATK, <https://www.broadinstitute.org/gatk/>) [64] was applied for SNP calling.

Association analyses of the variants

The frequency of variants was searched in publically available databases: the 1000 Genomes Project phase 3 (<http://www.1000genomes.org>) [53, 54] and the Asian samples in the Exome Aggregation Consortium (ExAC: <http://exac.broadinstitute.org>) [65]. Data from the International Genomics of Alzheimer's Project (IGAP) [7] was retrieved to investigate whether the variants identified in this study are associated with AD in populations of European ancestry. The potential pathogenicity of each variant was estimated by using the Combined Annotation Dependent Depletion (CADD) database [66]. Quanto software [67] was used to evaluate the statistical power of the studied sample. The deviation from the Hardy-Weinberg equilibrium (HWE) for each SNP was calculated using the PLINK software [68]. The LD plot of the identified variants was checked in LocusZoom (<http://locuszoom.org/>) [69]. The allele and genotype frequencies of all the SNPs in AD patients and controls were analyzed by using the two-tailed Fisher's exact test.

The association between the genotype and mRNA expression was analyzed by using the available brain expression quantitative trait loci (eQTL) database: BRAINEAC (<http://www.braineac.org/>) [70], Brain-Cloud (<http://braincloud.jhmi.edu/>) [71] and the Genotype-Tissue Expression project (GTEx: <http://www.gtexportal.org/home/>) [72].

Data-mining of mRNA expression in AD patients

The expression level of the COX-related genes in AD was retrieved from the AlzData database (www.alzdata.org) [73], in which we have integrated and normalized the original microarray data of 684 AD brain tissues and 562 control tissues from Gene Expression Omnibus (GEO: <http://www.ncbi.nlm.nih.gov/sites/GDSbrowser>) [19, 74–86]. We used the stage I data as defined in Xu et al. [73] that contains expression data of 269 AD brain tissues and 271 control brain tissues from four brain regions (entorhinal cortex, hippocampus, temporal cortex, and frontal cortex) to investigate the alteration of mRNA levels of the COX-related genes in AD, by using a two-tailed Student's *t*-test with the GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). The expression levels of the COX-related genes were further investigated in the stage II data as defined in Xu et al. [73], which contained the microarray data of 415 AD brain tissues and 291 control tissues from temporal cortex and frontal cortex.

Correlation between mRNA levels of the COX-related genes and pathology level in AD mouse models

The spatial-temporal expression data and pathological features of AD transgenic and wide-type (WT) mice were retrieved from Mouseac (www.mouseac.org) [87], including 219 brain tissues from five kinds of AD mouse models with familial AD mutations and 114 brain tissues of age-matched WT mice. The expression levels of the COX-related genes in the hippocampus and cortex tissues from mice at four life stages (2, 4, 8, and 18 months) were compared between AD mouse models and WT mice. The pathology level of A β plaques was quantified. The correlation between mRNA levels of the COX-related genes and pathology level was measured using the Pearson correlation test with the GraphPad Prism software (GraphPad Software, La Jolla, CA, USA).

Convergent functional genomics (CFG) for the COX-related genes in AD

A CFG approach was applied to integrate multiple lines of evidence for the association between the COX-related genes and AD [73, 88, 89]. The CFG score for each gene was estimated based on the following evidence: (1) variant(s) in the gene was associated with AD in the combined Han Chinese population ($P < 0.05$); (2) variant(s) in the gene was associated with AD in the IGAP population ($P < 0.05$) [7]; (3) the AD-associated SNP(s) was eQTLs that related to altered gene expression in brain ($P < 0.05$) [70–72]; (4) the gene showed a differential expression in AD patients ($P < 0.05$) [73]; (5) the gene showed a differential expression in AD mice ($P < 0.05$) [87]; (6) the mRNA level was correlated with AD pathology in AD mice ($P < 0.05$) [87]. When any of the above mentioned features were present, one point was assigned, leading to a CFG score ranging from 0 to 6 points. Note that the actual inclusion criteria for the above evidence for the CFG analysis were somewhat arbitrary, as the cutoff of significant *P*-value was set based on the manner of *P*-value distribution for each line of evidence [73]. For instance, evidence from the current genetic association analysis and from IGAP was given equal weight despite a much higher likelihood of false-positive association in the IGAP data without correction for multiple hypothesis testing [7]. Therefore, some targets with equally weighted significance at single dimension could be false positive. Nonetheless, the truly disease-related gene would be expected to be cross-validated by multi-dimension of evidence, regardless of the level of significance at single-dimension [73].

RESULTS

Genetic variants in the COX-related genes were associated with AD in Han Chinese

In order to perform a comprehensive analysis of the genetic association between the COX-related genes and AD in Chinese, we

Table 1. SNPs in the COX-related genes that were associated with AD in the discovery (I) and validation (II) stages

SNP ID ^a	Gene	Allele		P-Value ^b	OR	Genotype		P-Value ^b
		Case	Control			Case	Control	
Stage I								
rs1800649	COX5B	201/465	310/560	0.025	0.781	30/141/162	50/210/175	0.060
rs13177204	COX7C	153/507	234/636	0.098	0.820	27/99/204	32/170/233	0.033
rs9360898	COX7A2	182/484	207/663	0.115	1.204	26/130/177	16/175/244	0.044
rs12671052	NDUFA4	70/596	124/746	0.029	0.707	2/66/265	5/114/316	0.078
rs12667441	NDUFA4	68/592	124/746	0.021	0.691	2/64/264	5/114/316	0.058
rs72619327	SURF1	86/580	149/721	0.023	0.718	7/72/254	9/131/295	0.030
rs571549	SURF1	258/406	274/596	0.003	1.382	52/154/126	50/174/211	0.011
rs11078233	COX10	288/378	338/532	0.083	1.200	57/174/102	77/184/174	0.013
rs10420252	COX6B1	66/600	109/761	0.109	0.768	7/52/274	4/101/330	0.016
Stage II								
rs1800649	COX5B	266/494	286/562	0.591	1.058	47/172/161	51/184/189	0.818
rs13177204	COX7C	162/598	180/664	0.996	0.999	19/124/237	18/144/260	0.825
rs9360898	COX7A2	172/588	181/667	0.534	1.078	19/134/227	17/147/260	0.763
rs12671052	NDUFA4	94/666	131/717	0.076	0.773	7/80/293	8/115/301	0.131
rs12667441	NDUFA4	94/662	132/716	0.072	0.770	7/80/291	9/114/301	0.153
rs4626565	COX6C	91/669	134/714	0.027	0.725	4/83/293	10/114/300	0.077
rs1130569	COX6C	92/668	136/712	0.024	0.721	4/84/292	11/114/299	0.064
rs72619327	SURF1	135/625	124/724	0.087	1.261	9/117/254	7/110/307	0.215
rs571549	SURF1	242/518	251/597	0.330	1.111	33/176/171	28/195/201	0.497
rs9891988	COX10	297/459	375/473	0.045	0.816	55/187/136	82/211/131	0.120
rs2530377	COX10	243/513	318/530	0.025	0.790	48/147/183	62/194/168	0.043
rs11078233	COX10	322/434	330/518	0.134	1.165	73/176/129	68/194/162	0.339
rs10420252	COX6B1	97/663	128/712	0.155	0.814	10/77/293	11/106/303	0.244

^aSNPs showed significantly different allele frequencies between AD cases and controls in stage II analysis were marked in bold

^bP-Value, Fisher's exact test

selected 17 COX-related genes (11 subunit genes and 6 assembly factors) and screened for common variants in a two-stage case-control study of 1572 Han Chinese subjects (Supplementary Table S1). All of the 41 SNPs were in Hardy-Weinberg equilibrium (Supplementary Table S1). The MAF values of these SNPs ranged from 9.2 to 49.3%. The power to detect the odds ratio (OR) value as 1.25 for risk allele was expected to range from 46.22 to 84.33% under a log additive model, with a false positive rate being under 5%.

In the Stage I study of the cohort from Southwest China, SNPs in *NDUFA4*, *SURF1*, *COX10*, *COX6B1*, *COX5B*, *COX7C*, and *COX7A2* were associated with genetic risk of AD (Table 1 and Supplementary Table S3). In the Stage II study of an independent Han Chinese population from East China, the trends of the SNPs in *NDUFA4*, *SURF1*, *COX10*, and *COX6B1* were validated, although they did not reach a statistically significant level (Table 1 and Supplementary Table S4). As the sample size of both study cohorts were relatively small, we pooled these samples together to increase the statistical power. In the combined Chinese AD cohorts, SNPs in *NDUFA4* (rs12671052, OR = 0.746, $P = 0.006$; rs12667441, OR = 0.738, $P = 0.005$), *SURF1* (rs571549, OR = 1.230, $P = 0.007$), *COX10* (rs9891988, OR = 0.851, $P = 0.026$; rs2530377, OR = 0.836, $P = 0.018$; rs11078233, OR = 1.181, $P = 0.023$) and *COX6B1* (rs10420252, OR = 0.802, $P = 0.042$) remained to be associated with AD (Table 2 and Supplementary Table S5). Considering the fact that the potential population stratification of the Han regional samples [90] might affect this result, the data of the Han Chinese in Beijing (CHB, $N = 103$) and Southern Han Chinese (CHS, $N = 105$) were retrieved from 1000 Genomes Project phase 3

[53, 54] and were combined with the Han Chinese controls in this study as a control of the general populations. We found that the association between SNPs in *NDUFA4*, *SURF1*, *COX10*, *COX6B1*, and AD remained to be significant in the combined population (Table 2). Note that the significance did not survive the Bonferroni test, in part due to the relatively small sample size.

Exons sequencing of the COX-related genes identified variants associated with AD risk

In order to further explore whether there are potential mutations or undetected rare variants in the COX-related genes to be associated with AD, the coding and flanking regions of the COX-related genes were sequenced in 107 AD cases with extreme phenotypes (onset age ≤ 55 years old and/or with family history of AD), and were compared with a combined control population containing 368 individuals [52–54]. The genotyping data from 4327 East Asians from the ExAC (<http://exac.broadinstitute.org>) [65] was used as another control. A total of 72 variants were identified in the exons and flanking regions of these genes (Table 3 and Supplementary Table S6). Among them, 9 variants in *COX6B1*, *COX6B2*, *COX10*, *COX4I1*, *COX15*, and *SURF1* showed significantly different frequencies between the cases and the controls (Table 3). Analysis of the LD pattern revealed that these variants were not linked with those AD-associated SNPs identified in the SNaPshot assay (Supplementary Figure S1). Among these variants, rs7991 in the *COX6B1* gene was associated with AD risk (Table 3). Interestingly, the association between rs7991 and AD was also observed in the IGAP dataset [7], though the association was not significant after multiple testing correction, suggesting a

Table 2. SNPs in the COX-related genes found to be associated with AD in the combined Han Chinese population

SNP ID	Gene	Case	Control-1 ^a	P-Value ^b	OR	Control-2 ^c	P-Value ^b	OR	IGAP P-Value ^d	PHRED ^e	eQTL P-Value ^f	eQTL ID ^f
rs12671052	<i>NDUFA4</i>	164/1262	255/1463	0.006	0.746	318/1816	0.004	0.742	—	1.911	—	—
rs12667441	<i>NDUFA4</i>	162/1254	256/1462	0.005	0.738	318/1816	0.003	0.738	—	4.419	—	—
rs571549	<i>SURF1</i>	500/924	525/1193	0.007	1.230	650/1484	0.004	1.235	0.56	2.336	—	—
rs2076022	<i>COX6A1</i>	426/1000	455/1263	0.035	1.183	585/1549	0.111	1.128	0.15	9.205	—	—
rs9891988	<i>COX10</i>	572/850	758/958	0.026	0.851	948/1184	0.013	0.840	0.52	7.149	0.16	t3711165
rs2530377	<i>COX10</i>	463/959	629/1089	0.018	0.836	771/1363	0.031	0.854	0.23	1.426	0.09	t3711165
rs11078233	<i>COX10</i>	610/812	668/1050	0.023	1.181	844/1290	0.047	1.148	0.12	1.566	0.83	t3711165
rs10420252	<i>COX6B1</i>	163/1263	237/1473	0.042	0.802	298/1828	0.025	0.792	0.34	8.231	0.009	t3830649

^aControl-1 includes 859 normal Han Chinese in this study^bP-Value, Fisher's exact test^cControl-2 include 1067 normal controls (859 normal Han Chinese in this study, 103 Han Chinese from Beijing and 105 Han Chinese from South China in the 1000 Genomes Project phase 3 (<http://www.1000genomes.org>) [53, 54]^dThe IGAP P-values for these SNPs were retrieved from IGAP datasets [7]^eThe PHRED-like scaled CADD-score: a score >10 indicates that the variant belongs to the top 10% most deleterious substitutions in human genome [66]^fThe expression quantitative trait loci (eQTLs) data were retrieved from the BRAINEAC database (<http://www.braineac.org/>) [70]**Table 3.** Variants identified in the exon and flanking regions of the COX-related genes in 107 genetically unrelated patients with a high probability of hereditarily transmitted AD

Gene	rsID ^a	Position	Variant	Function	AD ^b	Control ^c	P-Value ^d	ExAC ^e	ExAC P-Value ^d	IGAP ^f	PHRED ^g
<i>COX15</i>	rs11190255	chr10: 101474499	C/T	intron	48/152	234/502	0.04	2295/5507	0.10	0.323	14.75
<i>COX4I1</i>	rs2599091	chr16: 85838653	C/T	p.L62L	211/1	736/0	0.22	7866/0	0.03	—	4.913
<i>COX10</i>	rs2108683	chr17: 14005386	C/T	intron	151/49	600/136	0.07	6448/1390	0.02	—	3.942
<i>COX10</i>	.	chr17: 14110383	T/C	p.L395L	2/206	0/160	0.51	0/7866	0.0007	—	15.77
<i>COX10</i>	.	chr17: 13977637	C/T	spliceSite	2/210	0/160	0.51	4/7858	0.01	—	1.724
<i>COX10</i>	.	chr17: 14110542	T/C	3-UTR	1/209	2/318	1	0/4670	0.04	—	4.395
<i>COX6B1</i>	rs7991	chr19: 36142187	T/C	p.T14T	74/138	209/527	0.07	2208/5640	0.04	0.046	19.31
<i>COX6B2</i>	rs201970981	chr19: 55865767	A/G	intron	2/210	0/366	0.13	6/7840	0.02	—	3.491
<i>SURF1</i>	rs62637580	chr9: 136219594	G/A	p.F181F	65/147	171/565	0.03	1907/5313	0.18	—	12.33

^aSNPs showed significantly different frequencies between AD cases and controls were marked in bold^bAD: including 107 Chinese AD patients with familial history and/or with an early onset age ≤ 55 years old^cControl: including 160 in-house controls [52], the data of Han Chinese in Beijing (CHB, *N* = 103) and Southern Han Chinese (CHS, *N* = 105) from the 1000 Genomes Project phase 3 [53, 54]^dP-Value, Fisher's exact test^eThe allele frequency in East Asian population in ExAC dataset (<http://exac.broadinstitute.org>) [65]^fThe IGAP P-values for these variants were retrieved from IGAP datasets [7]^gThe PHRED-like scaled CADD-score: a score >10 indicates that the variant belongs to the top 10% most deleterious substitutions in human genome [66]

potential risk effect of this variant in different populations (Table 3). Pathogenicity assessments of these variants by using the CADD database [66] indicated that rs7991 (*COX6B1*), rs11190255 (*COX15*), NC_000017.10:g.14110383C>T (*COX10*) and rs62637580 (*SURF1*) belong to the top 10% most deleterious variants in the human genome (Table 3). To sum up, through sequencing the coding and flanking regions of the COX-related genes in AD patients with a high probability of hereditarily transmitted AD, we identified several potentially pathogenic variants in the COX-related genes associated with AD risk.

mRNA expression levels of the COX-related genes were significantly decreased in brains of AD patients. Considering the fact that the significantly decreased activity of COX is a characteristic of AD [20, 30, 37–43], we compared mRNA expression profiles of 269 AD brain tissues and 271 control brain tissues from four brain regions (entorhinal cortex, hippocampus, temporal cortex, and frontal cortex), which were integrated and normalized by the AlzData database (www.alzdata.org) ([73] and

references therein), to investigate whether the mRNA expression levels of the COX-related genes were altered in AD brains. Among the 17 analyzed genes, the mRNA levels of 13 genes were significantly decreased in AD patients (Table 4). In particular, we observed a remarkable downregulation of mRNA levels of the *COX5B*, *COX7C*, *COX7A2*, *NDUFA4*, *COX6C*, *COX8A*, *COX5A*, *COX4I1*, *COX6A1*, and *COX6B1* genes in the entorhinal cortex and hippocampus tissues of AD patients (Table 4 and Fig. 2), which were the most affected brain regions in AD [2]. Furthermore, the mRNA levels of *COX5B*, *NDUFA4*, *COX6C*, *COX8A*, *COX5A*, *COX4I1*, and *COX6B1* were also significantly downregulated in the temporal cortex of AD patients, and *COX5B* and *COX8A* were significantly decreased in all four brain tissues of AD patients (Table 4). It is of note that all of the above significantly regulated genes in entorhinal cortex and hippocampus tissues of AD patients were subunits of COX [33], while the mRNA expression level of assembly factors were not consistently altered in AD brains. Further analysis of the Stage II data of the Alzdata database ([73] and references therein) (containing 415 AD brain tissues

Table 4. Comparison of mRNA levels of COX-related genes between 269 AD brain and 271 control brain tissues

Gene	Entorhinal cortex ^a			Hippocampus ^b			Temporal cortex ^c			Frontal cortex ^d		
	log2 fold change	P-Value	FDR	log2 fold change	P-Value	FDR	log2 fold change	P-Value	FDR	log2 fold change	P-Value	FDR
<i>LRPPRC</i>	−0.11	0.227	0.410	−0.23	0.002	0.029	−0.38	0.001	0.006	−0.12	0.026	0.091
<i>COX5B</i>	−0.41	1.45E-04	0.006	−0.26	0.001	0.015	−0.53	1.16E-05	0.0005	−0.14	0.009	0.046
<i>COX7C</i>	−0.21	0.018	0.084	−0.25	1.43E-04	0.007	—	—	—	0	0.971	0.982
<i>COX7A2</i>	−0.44	6.47E-07	0.001	−0.21	0.009	0.073	−0.06	0.618	0.791	−0.14	0.012	0.053
<i>NDUFA4</i>	−0.49	1.31E-04	0.005	−0.37	2.85E-05	0.003	−0.63	6.85E-05	0.001	−0.04	0.580	0.721
<i>COX6C</i>	−0.32	0.01	0.059	−0.31	0.001	0.017	−0.43	0.009	0.045	−0.14	0.075	0.185
<i>SURF1</i>	0.02	0.877	0.931	0.05	0.575	0.772	—	—	—	0.13	0.024	0.086
<i>COX15</i>	0.04	0.566	0.723	−0.07	0.244	0.494	−0.03	0.777	0.887	−0.08	0.087	0.203
<i>COX6A1</i>	−0.42	2.39E-05	0.002	−0.2	1.53E-04	0.007	—	—	—	−0.08	0.090	0.208
<i>COX8A</i>	−0.37	1.63E-04	0.006	−0.3	0.002	0.027	−0.34	0.020	0.082	−0.17	0.010	0.048
<i>COX5A</i>	−0.31	0.011	0.064	−0.17	0.032	0.156	−0.31	0.009	0.047	−0.06	0.412	0.577
<i>COX4I1</i>	−0.26	0.004	0.033	−0.22	2.07E-04	0.008	−0.36	0.003	0.019	−0.05	0.445	0.607
<i>SCO1</i>	−0.18	0.177	0.353	−0.42	0.006	0.057	−0.47	0.031	0.112	—	—	—
<i>COX10</i>	0.08	0.515	0.682	−0.21	0.056	0.215	−0.19	0.162	0.354	−0.09	0.154	0.602
<i>COX6B1</i>	−0.5	7.30E-06	0.001	−0.26	0.001	0.021	−0.64	0.001	0.009	−0.08	0.319	0.487
<i>COX6B2</i>	0.19	0.166	0.338	0.4	0.001	0.024	0.37	0.069	0.197	—	—	—
<i>SCO2</i>	−0.84	1.50E-05	0.002	0.11	0.410	0.653	—	—	—	0.03	0.753	0.846

The expression data were retrieved from the NCBI Gene Expression Omnibus (GEO, <https://www.ncbi.nlm.nih.gov/geo>) and were normalized and analyzed in the AlzData database (www.alzdata.org) [73]. Genes showed significantly different expression level between AD cases and controls were marked in bold

^aFour expression datasets of entorhinal cortex from 39 AD patients and 39 controls were retrieved from the GEO: GSE26927 [75], GSE48350 [80], GSE26972 [84], and GSE5281 [19]

^bFive expression datasets of hippocampus from 74 AD patients and 65 controls were retrieved from the GEO: GSE28146 [74], GSE48350 [80], GSE5281 [19], GSE29378 [78], and GSE36980 [76]

^cFour expression datasets of temporal cortex from 52 AD patients and 39 controls were retrieved from the GEO: GSE37263 [83], GSE29652 [82], GSE36980 [76], and GSE5281 [19]

^dSeven expression datasets of frontal cortex from 104 AD patients and 128 controls were retrieved from the GEO: GSE48350 [80], GSE66333 [81], GSE53890 [77], GSE5281 [19], GSE36980 [76], GSE12685 [86], and GSE15222 [85]

and 291 control tissues from temporal cortex and frontal cortex) gave similar results and revealed one more gene, *COX10*, to be significantly decreased in the frontal cortex of AD patients (Supplementary Table S7).

Taken together, though a systematic analysis of mRNA expression profiles of AD brain tissues, we found that most of the subunits of the COX were downregulated at the mRNA levels in AD brain tissues and this was consistent with the reduced COX activity in AD brains as reported in previous studies [20, 37, 39–41].

The AD risk SNPs in the COX-related genes were associated with altered gene expression level

Based on the finding that the mRNA levels of the COX-related genes were significantly downregulated in AD brains, we investigated whether the AD risk SNPs in these genes would account for the altered gene expression. Through data-mining of the brain eQTL data from the BRAINEAC database (<http://www.braineac.org/>) [70], which provides the brain eQTL data across 10 brain tissues of 134 neurological normal individuals, we found that the carriers of allele A of SNP rs10420252 have significantly decreased *COX6B1* mRNA expression level in the brain (Table 2 and Fig. 3a). Investigation of the BrainCloud database (<http://braincloud.jhmi.edu/>; [71]) identified that the AD risk genotype CC of rs10778233 was associated with a reduced *COX10* mRNA expression level in prefrontal cortex (Fig. 3b). An analysis of the eQTL data in the CTE project [72] (GTEx, <http://www.gtexportal.org/home/>) revealed that the AD risk genotype CC of rs571549 was prominently associated with an increased *SURF1* mRNA expression level in hippocampus and cortex tissues (Fig. 3c), which was consistent with the upregulated *SURF1*

mRNA level in the frontal cortex tissues of AD patients (Table 4 and Fig. 3d). Collectively, these results suggested that variants in the COX-related genes may be associated with AD by affecting gene expression.

The mRNA expression levels of the COX-related genes were correlated with the level of Aβ plaques in AD mouse models. In order to explore whether the mRNA expression levels of the COX-related genes were associated with AD pathology in species other than in man, we retrieved and analyzed the gene expression data and pathology data of AD mouse models from Mouseac (www.mouseac.org) [87]. By analyzing the gene expression levels in hippocampus and cortex tissues of wild type and HO₂TASTPM mice (with homogenous APP^{K670N/M671L} and PSEN1^{M146V} mutations) at different life stages, we found that most of the COX-related genes were downregulated in hippocampus at the late life stage of the AD mouse models, though only some of them (*Cox6a1*, *Cox7a2*, *Cox7c*, *Surf1*, and *Lrpprc*) reached a significant level (Fig. 4). An analysis of the mRNA expression data in the cortex tissues gave similar results (Supplementary Figure S2). Strong negative correlations between the mRNA expression levels of *Lrpprc*, *Cox7a2*, *Ndufa4*, *Cox5a*, and *Cox10* and the level of Aβ plaques were observed in hippocampus and cortex tissues of mouse models with the APP^{K670N/M671L} mutation, while the *Cox6b2* gene exhibited a significantly positive correlation (Fig. 4 and Supplementary Figure S2). Overall, these observations further supported the association between COX-related genes and AD, especially at the pathological level.

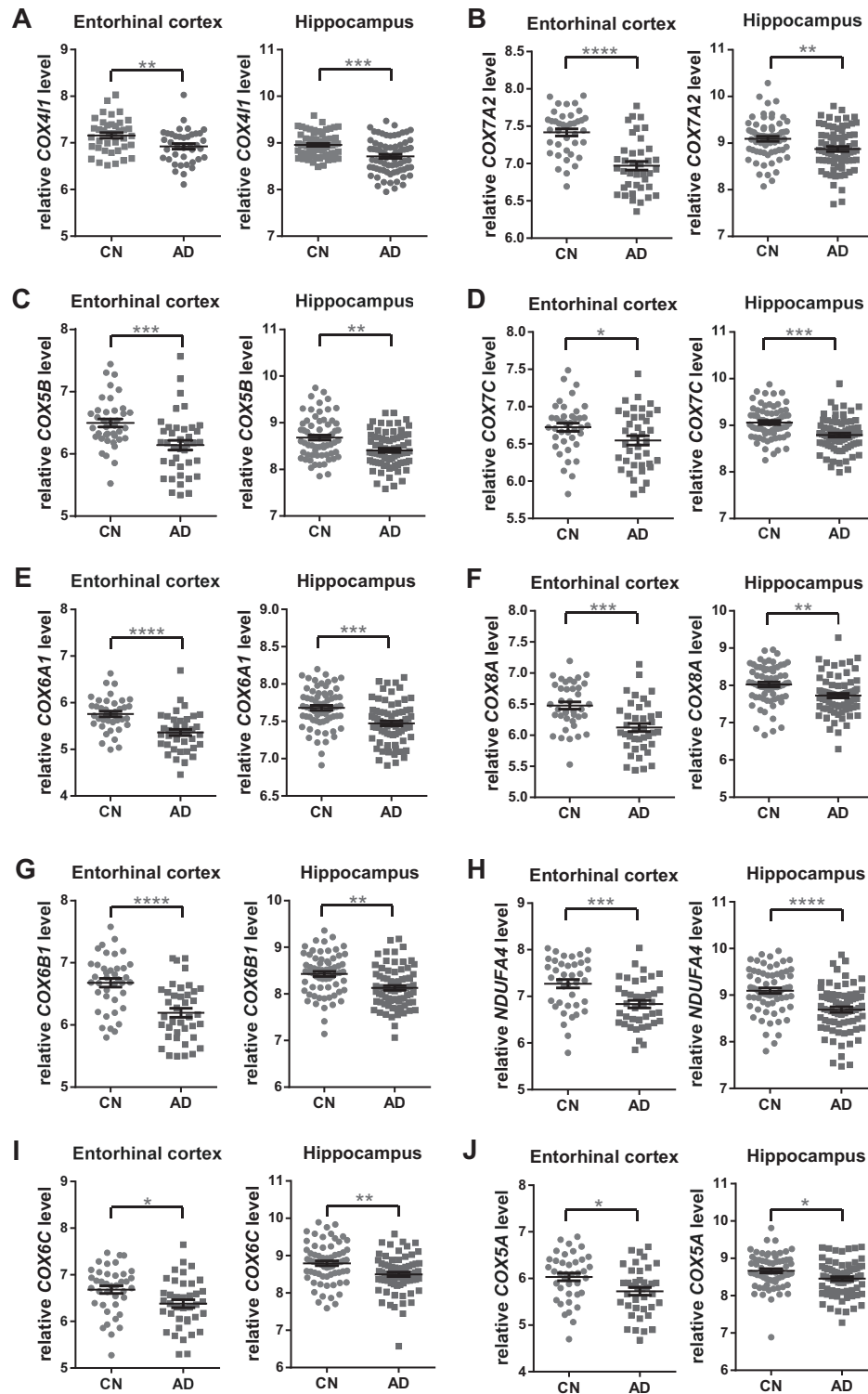


Fig. 2 The mRNA levels of the COX-related genes were significantly decreased in entorhinal cortex and hippocampus tissues of AD patients. **a–j** The mRNA expression data of the COX-related genes in entorhinal cortex and hippocampus tissues were retrieved from the NCBI Gene Expression Omnibus (GEO, <https://www.ncbi.nlm.nih.gov/geo>) and were normalized and analyzed in the AlzData database (www.alzdata.org) [73]. The expression data for the entorhinal cortex came from 39 AD patients and 39 controls in the 4 GEO datasets: GSE26927 [75], GSE48350 [80], GSE26972 [84], and GSE5281 [19]. The expression data for the hippocampus came from 74 AD patients and 65 controls in the 5 GEO datasets: GSE28146 [74], GSE48350 [80], GSE5281 [19], GSE29378 [78], and GSE36980 [76]. The difference between AD patients and controls was quantified by using a two-tailed Student's *t*-test. Error bars represent the standard error of the mean. **P*-value < 0.05; ***P*-value < 0.01; ****P*-value < 0.001; *****P*-value < 0.0001

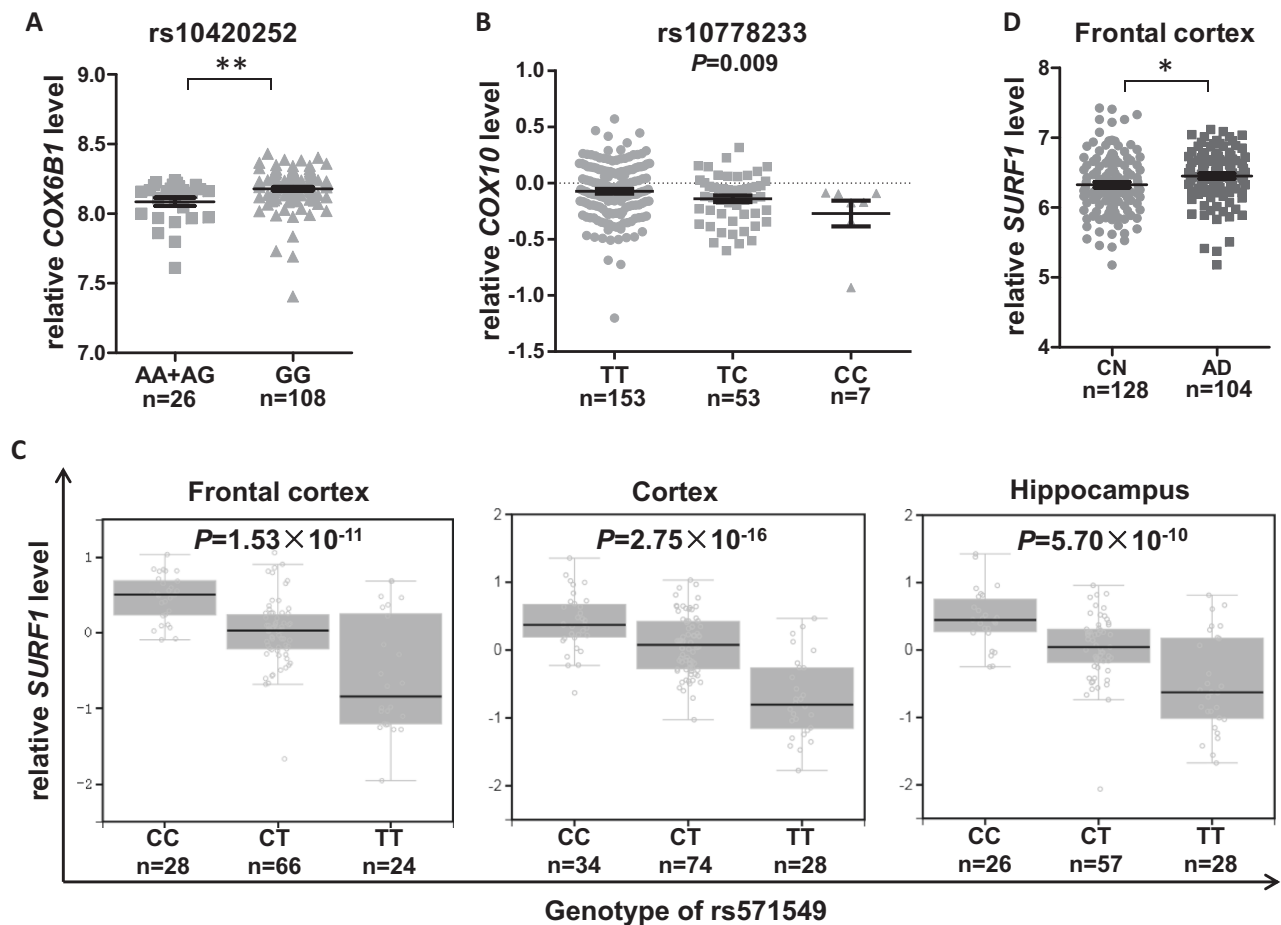


Fig. 3 The AD-associated SNPs in the COX-related genes were eQTLs and affected mRNA expression in brain tissues. **a** The carriers of the A allele of SNP rs10420252 showed a significantly decreased *COX6B1* mRNA expression level. Data were retrieved from the brain eQTL database BRAINEAC [70], and was analyzed by using a two-tailed Student's *t*-test. **b** Genotype CC of SNP rs10778233 was associated with a decreased *COX10* mRNA expression level in cortex. Data were retrieved from the BrainCloud database (<http://braincloud.jhmi.edu/>) [71] and was analyzed by using a linear regression test. **c** Genotype CC of SNP rs571549 was prominently associated with an increased *SURF1* mRNA expression level in frontal cortex, cortex, and hippocampus. Data were retrieved from the CTE project (GTEx, <http://www.gtexportal.org/home/>) [72] and was analyzed by using a linear regression test. **d** The mRNA expression level of *SURF1* was increased in frontal cortex tissues from AD patients relative to controls. The data were retrieved from the 7 datasets of GEO: GSE48350 [80], GSE66333 [81], GSE53890 [77], GSE5281 [19], GSE36980 [76], GSE12685 [86], and GSE15222 [85], and were normalized in the AlzData database (www.alzdata.org) [73]. Error bars represent the standard error of the mean. **P*-value <0.05; ***P*-value <0.01; two-tailed Student's *t*-test

CFG analysis prioritized *COX6B1*, *COX10*, *NDUFA4*, and *SURF1* genes as being associated with AD

In order to identify the highly AD-relevant genes, the COX-related genes were subjected to a CFG analysis [73, 88, 89]. By using multiple lines of evidence (including the genetic association in Han Chinese and in IGAP populations [7], eQTLs [70–72], gene expression in AD patients [73] and AD mice, and pathology correlation in AD mice [87]), each gene was scored and ranked (Table 5). *COX6B1* and *COX10* showed the highest CFG score of 5, followed by *NDUFA4* and *SURF1* with a CFG score of 4, indicating that these genes were possibly the most significant of the 17 COX-related genes considered to be associated with AD (Table 5).

DISCUSSION

Despite the fact that the downregulation of the COX-related genes and defects of the COX activity have been recognized as characteristics of AD [20, 30, 37–43], there has been little evidence produced to show the genetic association between the COX-related genes and AD [46], especially in populations of Asian origin. We present here an association study exploring the

involvement of the COX-related genes in contributing genetic risk to AD. By screening for the common variants (Tag SNPs) and variations in the exon regions of 17 COX-related genes in Han Chinese populations, followed by an integrative analysis with data of eQTL, expression and pathology, we have been able to show an active role of the COX-related genes in AD. To our knowledge, this is the first extensive study evaluating the association of the COX-related genes with AD.

Among the 11 subunit genes of COX, the *COX6A1*, *COX6B1*, and *NDUFA4* genes showed associations with AD genetic risk (Table 2). Further analysis of the exon regions in patients with a high probability of hereditarily transmitted AD also identified variants in *COX6B1*, *COX6B2*, and *COX4I1* to be associated with increased AD risk (Table 3). Most of these genes, except for *COX6B2*, were prominently downregulated in the entorhinal cortex and hippocampus tissues of AD patients (Table 4 and Fig. 2). *NDUFA4*, a newly recognized subunit of COX [36], exhibited a similar mRNA expression pattern with the other COX subunits (Fig. 2 and Table 4), and was negatively correlated with Aβ plaque burden in AD mouse models (Fig. 4). The data further supported the previous reports for decreased mRNA levels of nuclear-encoded

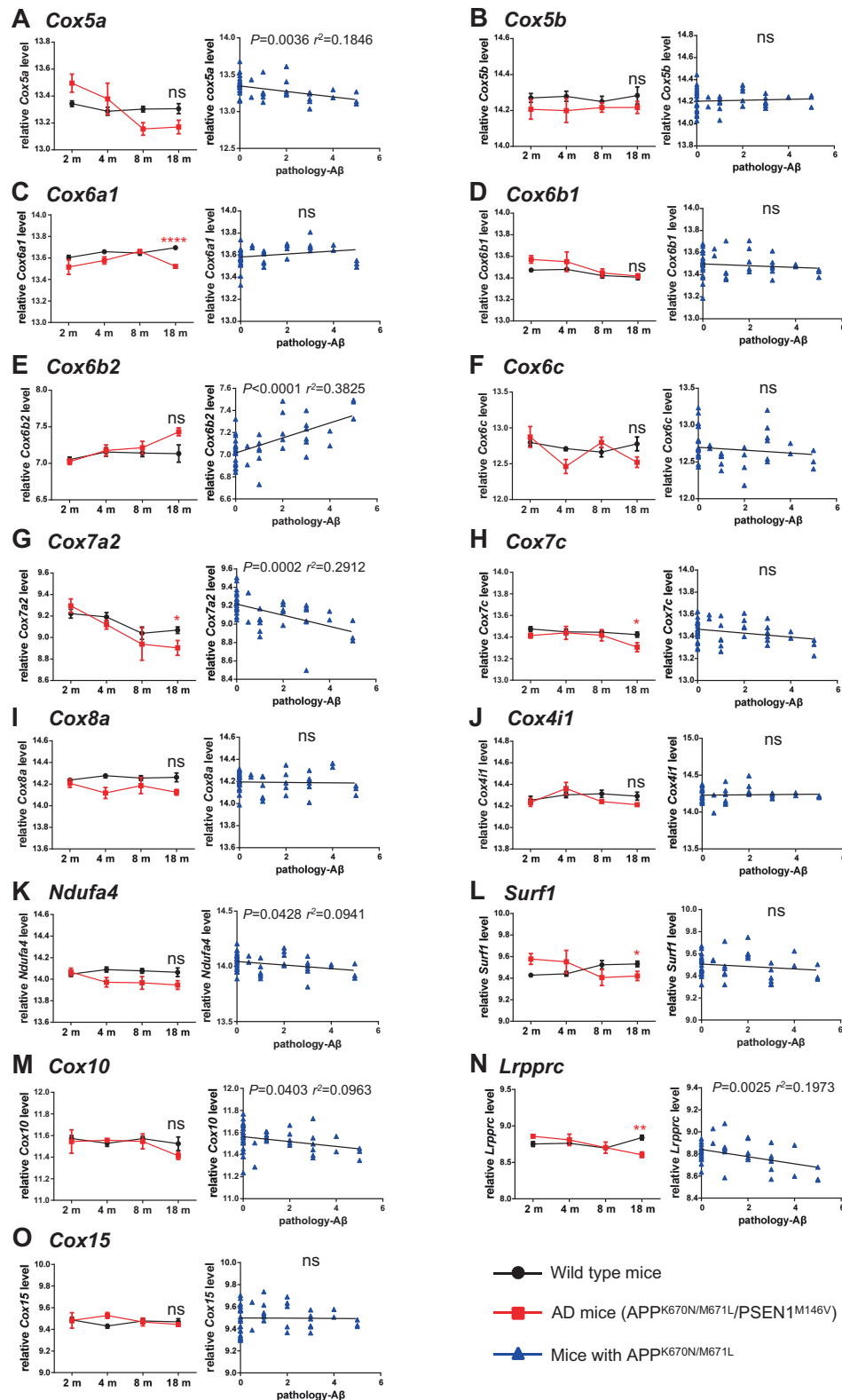


Fig. 4 The mRNA expression levels of the COX-related genes were correlated with the level of A β plaque in hippocampus tissues of AD mouse models. The gene expression data and the pathology data were retrieved from Mouseac (www.mouseac.org) [87]. Data shown were the age-related mRNA expression (left) and the correlation between mRNA level and A β plaque (right) of each of 15 genes (a–o). *Sco1* and *Sco2* had no data available in Mouseac [87]. The age-related mRNA expression level was measured in hippocampus tissues of wild type ($n=7$) and HO_{TASTPM} mice (with homogenous APP^{K670N/M671L} and PSEN1^{M146V} mutations, $n=3$) at different life stages (2, 4, 8, and 18 months). Error bars represent the standard error of the mean. * P -value <0.05 ; ** P -value <0.01 ; **** P -value <0.0001 ; ns, not significant; two-tailed Student's t -test. The correlation between mRNA level and A β plaques in mice with APP^{K670N/M671L} mutation ($n=44$) was measured using the Pearson correlation test. A P -value <0.05 was considered as statistically significant, ns: not significant

Table 5. Convergent functional genomics (CFG) for the COX-related genes in AD

Gene	Association ^a	IGAP ^b	eQTL ^c	DEG in AD patients ^d	DEG in AD mice ^e	Pathology (Aβ) ^f	CFG ^g
<i>LRPPRC</i>	0	0	0	1	1	1	3
<i>COX5B</i>	0	0	0	1	1	0	2
<i>COX7C</i>	0	0	0	1	1	0	2
<i>COX7A2</i>	0	0	0	1	1	1	3
<i>NDUFA4^h</i>	1	0	0	1	1	1	4
<i>COX6C</i>	0	0	0	1	1	0	2
<i>SURF1^h</i>	1	0	1	1	1	0	4
<i>COX15</i>	0	0	0	0	0	0	0
<i>COX8A</i>	0	0	0	1	0	0	1
<i>COX6A1</i>	1	0	0	1	1	0	3
<i>COX5A</i>	0	0	0	1	1	1	3
<i>COX4I1</i>	0	0	0	1	0	0	1
<i>SCO1</i>	0	0	0	1	—	—	1
<i>COX10^h</i>	1	0	1	1	1	1	5
<i>COX6B1^h</i>	1	1	1	1	1	0	5
<i>COX6B2</i>	0	0	0	1	1	1	3
<i>SCO2</i>	0	0	0	1	—	—	1

^aGenetic association in Han Chinese. If a gene had at least one locus being associated with AD in the combined Han Chinese population, 1 point was assigned; otherwise zero

^bIf the identified AD-associated variant in Han Chinese was also associated with AD in the IGAP population [7], 1 point was assigned to the relative gene; otherwise zero

^cIf a gene was associated with eQTLs [70–72] showing an AD risk in Han Chinese population, 1 point was assigned; otherwise zero

^dIf a gene was a differentially expressed gene (DEG) between AD patients and controls [73], 1 point was assigned; otherwise zero

^eIf a gene was a DEG between AD mouse models and controls [87], 1 point was assigned; otherwise zero

^fIf the mRNA expression level of a gene was correlated with AD pathology in AD mice [87], 1 point was assigned; otherwise zero

^gCFG score was the sum of the above scores of the multiple lines of evidence

^hGenes with a CFG score ≥ 4 were marked in bold.

subunits of COX and impaired COX activity in AD brains [20, 37, 39–41]. It is of note that three of the subunit genes, *COX6A1*, *COX6B1*, and *COX6B2*, identified as being risk genes in the genetic analysis were members of COX subunit 6. The mRNA expression levels of *COX6A1* and *COX6B1* were decreased in AD patients and AD mouse models (Table 4, Figs. 2 and 4). However, eQTL analysis showed that the risk genotype of rs10420252 was associated with an increased *COX6B1* mRNA level in AD brain (Fig. 3). Interestingly, *COX6B2* exhibited a remarkably increased mRNA expression level in the hippocampus tissues of AD patients and mouse models, and there was a positive correlation between its mRNA expression level and the pathological level of Aβ plaque burden in hippocampus and cortex tissues of AD mouse models (Figs. 2 and 4, and Supplementary Figure S2). The increased mRNA expression level of *COX6B2* in AD was in contrast to those of *COX6A1* and other COX subunits, suggesting that there might be underlying mechanisms to regulate the balance of expression of COX subunits in AD.

Assembly factors are key regulators for the COX structure and function [33, 34, 44]. Many diseases, including Leigh syndrome, myopathy, encephalopathy, to name a few, are caused by mutations of the COX assembly factors [44]. In this study, we screened variants in *LRPPRC*, *SURF1*, *SCO1*, *SCO2*, *COX15*, and *COX10*, which were reported to be mutational hotspots for the COX-related diseases [44]. Variants in *SURF1*, *COX10*, and *COX15* were associated with AD in Han Chinese population (Table 2 and Table 3). However, the mRNA levels of the three genes were not consistently altered in AD patients. An increased mRNA expression level of the *SURF1* gene was observed in the frontal cortex tissues of AD patients (Table 4 and Fig. 3). Consistently, the risk genotype of rs571549 in the *SURF1* gene was found to be associated with a significantly increased mRNA level of *SURF1* in frontal cortex,

cortex, and hippocampus tissues (Fig. 3). The mRNA expression of *COX10* was decreased in the frontal cortex of AD patients (Supplementary Table S7). The mRNA level of this gene was also decreased in the hippocampus and cortex tissues from the AD mouse models, and was negatively correlated with Aβ plaque burden in both hippocampus and cortex tissues (Fig. 4 and Supplementary Figure S2). Accordingly, carriers with the risk genotype of rs11078233 had a decreased mRNA level of *COX10* in brain tissues (Fig. 3). All of the data indicated that variants in the COX-related genes may be associated with AD through affecting the mRNA expression.

By using an integrative analysis of mRNA expression data of AD patients [73] and references therein) and mouse models [87], we obtained a more comprehensive dataset (684 AD brain tissues and 562 control tissues; and the brain expression and pathology data of 44 AD mice) to explore the mRNA expression alterations of the COX-related genes in AD. Almost all of the COX-related genes, except for *COX15*, *SURF1*, and *COX2*, which have been discussed above, were significantly downregulated in AD patients (Table 4 and Supplementary Table S7). In particular, *Cox7a2* and *Lrpprc* were significantly downregulated in mouse models, and we observed a negative correlation of the mRNA expression levels and Aβ plaque burden levels in hippocampus and cortex tissues (Fig. 4 and Supplementary Figure S2). Although, we found no association of genetic variants in these two genes with AD risk in this study, we could not exclude a possibility of rare functional variants or SNPs in the regulatory regions of these genes that would confer genetic risk to AD. Further investigation is needed to clarify this important issue.

Among all the identified variants in this study, only rs7991 in the *COX6B1* gene showed an association with AD in the IGAP dataset [7], but the association could not survive after multiple

testing correction. The inconsistency of the associations of other SNPs and genes with AD between our sample and the IGAP dataset [7] could be explained by different genetic structures between populations of Asian and European origins that may have different AD susceptible genes/variants [8]. Note that none of the identified common variants showed an association with AD after Bonferroni correction if we followed a stringent *P*-value <0.001 (0.05/41), partially because of the limited sample analyzed here. However, the association of these variants with AD could not be simply neglected, as we did identify some variants that could alter gene expression and showed different frequencies between cases and controls. These results provided genetic evidence to further support the critical role of COX in AD as reported by previous studies [20, 30, 37–43].

Whether defects of mitochondria contribute to the pathology of AD is still under debate [20, 91]. Converging lines of evidence have demonstrated that mitochondrial dysfunction occurs before the development of AD pathology [16, 21, 22, 24, 30, 31, 92]. In the early stages of AD, downregulation of many of the OXPHOS related genes has been observed [19, 30–32]. Furthermore, oxidative stress as a result of OXPHOS defects has contributed to neuronal death in AD brains [12, 93]. On the other hand, there are multiple lines of direct evidence showing that mitochondria are the direct target of A β , thus the mitochondrial defects observed in AD should be the consequence of A β toxicity [23, 25–27]. A focused functional characterization of the risk COX-related genes in the context of the above studies is essential to define the roles of mitochondrial genes and mitochondrial dysfunction in AD pathology.

Finally, by using a CFG approach which integrating multiple lines of evidence, we have highlighted *COX6B1*, *COX10*, *NDUFA4*, and *SURF1* as highly AD-relevant genes. Taken together, through an extensive association analysis of the COX-related genes at the genetic, expressional, and pathological levels, we have provided comprehensive evidence to support the important role of the COX-related genes in the development of AD. Further studies with independent populations for validation and functional assays are necessary to confirm the present conclusion and to characterize the mechanisms of the COX-related genes in AD pathogenesis.

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ADDITIONAL INFORMATION

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