ORIGINAL ARTICLE

Association analysis of four candidate genetic variants with sporadic amyotrophic lateral sclerosis in a Chinese population

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Abstract Recently, four single nucleotide polymorphisms (SNPs), including rs2814707 in the 9p21, rs12608932 in the *UNC13A* gene, rs13048019 in the *TIMA1* gene, and rs2228576 in the *SCNN1A* gene have been reported to be associated with the risk for developing amyotrophic lateral sclerosis (ALS) in Caucasian population. However, this association is not consistent among different studies and yet to be tested in ALS patients in Mainland China. This study included 397 sporadic ALS (SALS) patients and 287 unrelated Chinese healthy controls from Southwest China. Four SNPs listed above were genotyped by using Sequenom's iPLEX assay. No significant differences in the genotype distributions or minor allele frequencies in all SNPs were found between ALS group and control group, between the spinal-onset group and bulbar-onset group, and between the early-onset group and the late-onset group. Our results suggest that these SNPs are unlikely to be common cause of SALS in Chinese population.

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Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease, involving both upper and lower motor neurons in the brain and spinal cord [1]. Most people with ALS suffered progressive muscle weakness and atrophy throughout the body, and most of them died from respiratory failure within 3–5 years after diagnosis. Until now the definite etiology of ALS remains unclear. Genetic factors are considered to be involved in the pathogenesis of ALS [2]. Mutations in several genes including Cu/Zn superoxide dismutase gene (SOD1), alsin (ALSIN, ALS2), senataxin (SETX, ALS4), FUS/TLS (AlS6), vesicle-associated membrane protein B (VAPB, ALS8), dynactin (DCTN1), angiogenin (ANG), and transactive-responsive DNA binding protein (TARDBP) [3–5] have been identified to cause familial ALS (FALS). However, only about 10 % of ALS cases are FALS. The majority of ALS patients (90 %) have no family history of ALS, known as sporadic ALS (SALS) [6]. Recent studies have demonstrated that a substantial proportion of FALS cases were traced to an expansion of the intronic hexanucleotide repeat sequence in *C9orf72*. The frequency of expansion in worldwide sampling is 7–36 % among individuals with FALS, and about 4–7 % of SALS cases are associated with these expansions, depending on the population studied [7, 8]. Meanwhile, the frequency of SOD1 mutations in SALS is less than 1 % in a population-based study [9]. This *C9orf72* genetic breakthrough further reinforces the concept that ALS pathogenesis involves multiple pathways. Furthermore, environmental factors are also considered to be the potential risk factors for ALS patients, including military service, life

style factors such as smoking, the use of statins, and the exposure to β-N-methylamino-ʟ-alanine (BMAA) [2, 10]. Therefore, identification of genetic susceptibility and assessment of gene-environment interactions for ALS susceptibility loci contributing to SALS can help us to better understand the pathogenic mechanisms of ALS. To date, Genome-wide association studies (GWAS) have provided genetic evidence that several modifier loci and associated genes were involved in the developing of complex diseases, such as Parkinson disease [11] and SALS [12, 13]. In SALS, consistent GWAS results indicate that the chromosome 9 locus appears to be aberrant in 5–8 % of SALS [14].

Recently a locus on chromosome 9p21 has been linked to be the autosomal dominant ALS with fronto-temporal dementia (FTD) [15, 16]. SNP rs2814707 located in chromosome 9p21 has been identified as a potential susceptibility factor for both FALS and SALS, using the GWAS approach in Finnish patient population [17].The SNP rs12608932, located in an intron of the UNC13A gene [18], has been found to be associated with susceptibility to ALS in different populations of European ancestry [19–21]. However, the association of SNP rs2814707 and rs12608932 with susceptibility to SALS was considered negative in East Asians [22]. The SNP rs13048019 within the *TIAM1* gene has also been reported to be a potential susceptibility factor for ALS [17]. In addition, the SNP rs2228576 of *SCNN1A* gene in ALS [23], has found to have no statistically significant difference in genotype frequency, however; another study found that SNP rs2228576 can affect the fasting glucose level, which was thought to be abnormal in ALS patients [24, 25].

Ethnic differences have important implications in genetic analysis. For example, *C9orf72* mutations were identified as a major cause of ALS in Caucasians, but they were not a common cause of ALS in Chinese population [26]. Therefore, we performed a large case–control study including 684 individuals from mainland China to clarify if the above SNPs (rs2814707, rs12608932, rs13048019 and rs2228576) were risk factors contributing to SALS in Chinese population.

Materials and methods

Subjects

The recruitment of 397 patients was carried out in the Department of Neurology, West China Hospital of Sichuan University, from May 2006 to April 2011. All SALS patients fulfilled EI Escorial revised criteria [27]. Patient with no family history of ALS in first-degree and second-degree relatives was defined as SALS. The diagnosis of SALS was based on neurological history, neurological examination, electromyography test, and laboratory tests. The Awaji-criteria, which are based on the El Escorial criteria was applied in recruitment [28]. For comparison, 287 unrelated Chinese healthy controls (HC) matching in gender, age, and area of residence were recruited. Written informed consent was obtained from each subject enrolled in the study. Detailed clinical data were recorded, and peripheral blood samples were collected from all subjects. Genomic DNA was prepared from isolated leukocytes using standard phenol–chloroform procedures [29]. All patients and control subjects gave their written informed consent, and this study was approved by the Ethical Committee of West China Hospital of Sichuan University.

Genetic analysis

All patients and controls were genotyped for SNPs using Sequenom iPLEX Assay (Sequenom iPLEX Assay, San Diego) according to the manufacturer's instructions. Locus-specific polymerase chain reaction (PCR) and detection primers were designed with the MassARRAY Assay Design 3.0 software (Sequenom, San Diego). Approximately 10 ng of genomic DNA was amplified by primers flanking the targeted sequence. The extension products were purified and transferred onto a 384-element Spectro CHIP bioarray for purification. Parameters were obtained through Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS). Data processing and analysis were complete using the Sequenom MassARRAY Workstation software (Sequenom).

Statistical analysis

Fisher's exact test was performed to verify Hardy–Weinberg equilibrium (HWE) of each SNP. The Fisher's exact test was also performed to examine the genotype distributions and minor allele frequency (MAF) for each SNP between SALS patients and HC, between the spinal-onset and the bulbar-onset groups, between the early-onset and the late-onset groups. Differences of age at onset between groups were analyzed by using Student's t test. A p value of less than 0.05 was considered statistically significant. The Bonferroni correction was applied to the p value to account for the nature of multiple comparisons. All statistical analysis was performed using SPSS11.0 (SPSS Inc., Chicago, IL, USA).

Results

Clinical data

Among 397 SALS patients, 236 were males and 161 were females. The mean age of onset was

51.32 ± 11.55 years (ranging from 18 to 84 years), which was comparable to the mean age of HC (51.38 ± 11.07 years, $p = 0.31$). One hundred and one patients (25.44 %) presented difficulty in speaking or swallowing which was the initial symptom in the bulbar form of ALS, while 296 (74.56 %) patients presented with initial spinal symptoms. Two hundred patients had upper limb onset and 96 patients had lower limb onset. The mean age of onset in the bulbar-onset group (54.98 ± 10.28 years) was significantly higher than that in the spinal-cord onset group (50.07 ± 11.71 years, $p < 0.001$). The mean age at onset of all bulbar-onset patients was significantly greater than that of the spinal-cord onset group. A total of 74 patients were diagnosed with early-onset ALS (age of onset younger than 40 years), whereas 323 patients were divided to late-onset group (age of onset older than 40-years-old). No gender differences were found between the bulbar-onset and the spinal-cord onset group ($p = 0.059$), and between the early-onset and the late-onset group ($p = 0.601$). Sixty-eight patients died during the follow-up.

Genetic finding

The allele and genotype distributions for each SNP in the SALS patients and HC did not significantly deviate from that expected for a HWE (all p value > 0.05). There were no significant differences in the genotype distributions and MAF for all SNPs between the SALS patients and controls (all $p > 0.05$, the Bonferroni adjustment; Table 1). No significant differences in the genotype distributions and MAF for all SNPs were found between the spinal-onset and bulbar-onset group, and between the early-onset and late-onset group after conservative Bonferroni' correction (Table 2, Table 3). Moreover, compared with the "AA"/"AC" genotypes of rs12608932, the homozygote "CC" of rs12608932 could not significantly modify the survival time of SALS patients (29.58 ± 20.79 vs 31.83 ± 15.87 months; $p = 0.620$).

Discussion

In the current study, we found that four SNPs, including rs2814707, rs12608932, rs13048091 and rs2228576, do not

Table 1 MAF and Genotype distributions for all four tested SNPs

Genotype	All of subjects				MAF (%)			
	ALS (n = 397)	HC (n = 287)	HWE (ALS, HC)	P value	ALS (n = 794)	HC (n = 574)	P value	OR (95 %CI)
MOBKL2B rs2814707 AA AG GG	0, 41, 356	0, 27, 260	0.39, 0.40	0.692	41 (5.16)	27 (4.70)	0.699	1.548 (0.982–2.441)
UNC13A rs12608932 CC CA AA	166, 173, 58	123, 132, 32	0.56, 0.70	0.411	505 (63.60)	378 (65.85)	0.390	0.906 (0.723–1.135)
TIMA1 rs13048019 TT CT CC	0, 10, 387	0, 10, 277	0.75, 0.76	0.460	10 (1.25)	10 (1.74)	0.463	0.719 (0.297–1.740)
SCNN1A rs2228576 AA GA GG	120, 180, 97	65, 155, 67	0.08, <0.01	0.046[a]	420 (52.90)	285 (49.65)	0.236	1.139 (0.919–1.412)

Represent the p value of genotype compared between patients and HC

HC health control

[a] No significant difference after bonferroni adjustment

Table 2 Genotype analysis among the subgroups

Genotype	Initial symptoms			Age of onset			HC (n = 287)	P value[a]
	Spinal-onset (n = 296)	Bulbar-onset (n = 101)	P value	Early-onset (n = 74)	Late-onset (n = 323)	P value		
rs2814707 AA AG GG	0, 30, 266	0, 11, 90	0.825	0, 13, 61	0, 28, 295	0.023[b]	0, 27, 260	NS
rs12608932 CC CA AA	119, 135, 42	47, 38, 16	0.374	30, 33, 11	136, 140, 47	0.970	123, 132, 32	NS
rs13048019 TT CT CC	0, 6, 290	0, 4, 97	0.284	0, 3, 71	0, 7, 316	0.350	0, 10, 277	NS
rs2228576 AA GA GG	85, 135, 76	35, 45, 21	0.442	98, 146, 79	22, 34, 18	0.992	65, 155, 67	NS

[a] Represent the p value of genotype compared between each of subgroup; NS means no significant differences

[b] No significant difference after bonferroni adjustment

Table 3 Minor allele frequency analysis among the subgroups

MAF	Initial symptoms			Age of onset			HC ($n = 574$)	P value[a]
	Spinal-onset ($n = 592$)	Bulbar-onset ($n = 202$)	P value	Early-onset ($n = 148$)	Late-onset ($n = 646$)	P value		
rs2814707 AA AG GG	30 (5.06)	11 (5.44)	0.183	13 (8.78)	28 (4.33)	0.027[b]	27 (4.70)	NS
rs12608932 CC CA AA	373 (63.01)	132 (65.35)	0.834	93 (62.84)	412 (63.78)	0.830	378 (65.85)	NS
rs13048019 TT CT CC	6 (1.01)	4 (1.98)	0.551	3 (2.03)	7 (1.08)	0.353	10 (1.74)	NS
rs2228576 AA GA GG	305 (51.52)	115 (56.93)	0.287	78 (52.70)	342 (52.94)	0.958	285 (49.65)	NS

NS no significant

[a] Represent the *p* value of MAF compared between each of subgroup

[b] No significant difference after bonferroni adjustment

modulate the susceptibility and phenotype of SALS in Chinese population. The general features of recruited SALS patients in the current study were similar to those in our previous study [30].Thus, the profile of patients represented the SALS patient population in China.

A susceptibility locus SNP rs2814707 on chromosome 9p21 for ALS is first replicated in different GWAS studies and replication studies across multiple populations, demonstrating the importance of this locus in ALS. Subsequently, one study revealed the association of SNP rs2814707 and ALS-FTLD [31]. Another study on Belgian population of ALS, ALS-FTLD, and FTLD found that SNP rs2814707 showed highly significant allelic and genotypic associations in ALS and ALS-FTLD subpopulation, and a maximal odds ratio reached 3.27 in ALS patients with homozygote of the minor allele "A" of rs2814707. A meta-analysis of SNP rs2814707 on chromosome 9p21 indicated that carriers of the minor allele "A" of rs2814707 are at increased risk to develop ALS or FTLD [32]. Although this locus has been reported to increase the risk of ALS in European Caucasians [17, 19, 20, 33], the present study failed to provide replication in our Chinese SALS patients. This finding was consistent with the East Asian cohort study which recruited 684 Han Chinese SALS cases [22]. The SNP rs2814707 is simply a tag SNP for the *C9orf72* repeat expansion, and this *C9orf72* mutation was found to be present in both SALS and FALS patients in Caucasian population, but this mutation was not a common cause of SALS and FALS in Chinese population [26]. Therefore, the effect of SNP rs2814707 on ALS present in Chinese patients can be explained by the effect of ethnic difference. Furthermore, because of the unique homogeneous genetic structure of Chinese population, the extent and structure of linkage disequilibrium (LD) may be different from other European populations.

Large GWAS studies showed that the common variant rs12608932 located within an intron of *UNC13A* on chromosome 19p13.3, was associated with susceptibility to ALS, as well as survival, among populations of north European descent [20], and similar findings were noted in two cohorts of 450 SALS patients from Netherlands and 1,767 individuals of Dutch, Belgian, or Swedish descent [21]. However, the association between rs12608932 and ALS susceptibility was not found in another population-based cohort of 500 Italian ALS patients, but rs12608932 was significantly associated with survival, and considered to be an independent prognostic factor to influence survival ("AA"/"AC" genotypes compared with "CC") [34]. A meta-analysis of the International consisting of 4,243 ALS from 13 European ancestry cohorts from across the United States and Europe provided evidence of association between rs12608932 and ALS, and rs12608932 was also associated with age at onset of ALS [35]. Thus, UNC13A plays a role in ALS pathogenesis and considered to be a modifier of prognosis among SALS patients. However, in the present study, the association between SNP rs12608932 in *UNC13A* and SALS susceptibility was negative in Chinese SALS patients, which was also supported by the East Asian study (22) Furthermore, we found that homozygote "CC" of rs12608932 did not affect survival time of Chinese SALS patients compared with the "AA"/"AC" genotypes of rs12608932. *UNC13A* gene encodes a member of the UNC13 family. UNC13 proteins bind to phorbol esters and diacylglycerol, regulating the release of neurotransmitters at synapses, including glutamate [18]. Therefore, we appeal that the ethnic factor may play an important role in GWAS studies, and more studies on different races and ethnicities are need to be done to clarify the involvement of *UNC13A* gene in ALS pathogenesis. Recently, a GWAS of ALS in the Han Chinese population has demonstrated that SNP rs2814707 and rs12608932 did not have any evidence of association with ALS [36]. Consisting with our results, these findings suggested the heterogeneity of ALS genetic susceptibility in the Chinese and European populations, and these susceptibility loci might be population-specific genetic risk factors for ALS.

We found that rs13048019 in *TIMA1* gene did not contribute to the development and phenotype of Chinese

ALS patients. This finding was inconsistent with a Finnish ALS study [17]. *TIMA1* gene is a T-lymphom invasion and metastasis gene encoding Tiam1 protein which could span the interacting domain within the superoxide dismutase 1 gene (SOD1) by binding to a region of Rac1, this binding can help SOD1 directly regulate the NADPH oxidase-dependent (Nox-dependent) super oxide ($O_2^{\bullet -}$) production [37]. This SNP rs13048019 locus corresponds to the autosomal recessive D90A allele of SOD1, which is known to cause ALS in Scandinavian populations, Thus, rs13048019 may be a tag SNP for *SOD1* gene, and correlate to the susceptibility to FALS by affecting the autosomal dominate D90A allele in *SOD1* gene. However, more studies were needed to verify this hypothesis.

Rs2228576 located in *SCNN1A* was reported to have no association with ALS susceptibility in Italian patients [23]. The finding in the current study was consistent with the Italian cohort study. But Irvin et al. [25] found that hypertension patients with "GG" genotype of *SCNN1A* rs2228576 have higher fasting glucose level with treatment of amlodipine and chlorthalidone compared to patients with "AA" or "AG" genotype, indicating that *SCNN1A* rs2228576 "GG" genotype may be involved in glucose metabolism. The abnormality of fasting glucose level was also found in ALS patients [24].However, the exact mechanism of *SCNN1A* rs2228576 "GG" genotype modulating glucose metabolism in ALS needs further studies.

The consistency and inconsistency among different studies were probably affected by various factors, including ethnic factors and the size of sample set. Ethnic categories should be a significant consideration in genetic analysis. For example, MAF "A" of rs2814707 is varied in different racial and ethnic groups, and it is 23.9 % in European population, 3.5–5.8 % in Asian population, and 5.61 % in Chinese population (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=2814707); MAF "T" of rs13048019 is also different in people of various ethnicities, and it is 15–50 % in European population, and 1.46 % in Chinese population (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=13048019). In general, a real association or linkage is affected by the background noise in the population, consisting of all possible combinations of environmental and genetic factors. More genetically isolated populations will be more homogeneous, and environmental variation can be reduced in culturally and genetically isolated population, having a more similar way of living, eating habits and natural environment. In the present study, all the recruited Chinese SALS patients came from the Southwest of China. This geographical restriction could diminish allelic heterogeneity, increase tagging efficiency, and reduce false positive association rate [38]. The sample size should be large enough to yield reliable estimates and small sample size is a common

factor resulting in a different finding. In heterogeneous populations, large sample sizes would be required to get sufficient statistical power to detect genetic risk factors. The sample size of our cohort has sufficient power to detect the moderate association, although it is smaller than that of previous GWAS studies. In addition, our finding of the lack of replication of these SNP variants leads us to hypothesize that population-specific difference would account for the findings.

Overall, the four SNPs in the present study are not associated with SALS susceptibility in Chinese population.

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