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RESEARCH PAPER

The associations of two SNPs in miRNA-146a and one SNP in ZBTB38-RASA2 with the disease susceptibility and the clinical features of the Chinese patients of sCJD and FFI

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ABSTRACT

Prion diseases are a group of fatal neurodegenerative disorders that affect humans and animals. Besides of the pathological agent, prion, there are some elements that can influence or determine susceptibility to prion infection and the clinical phenotype of the diseases, e.g., the polymorphism in *PRNP* gene. Another polymorphism in ZBTB38–RASA2 has been observed to be associated with the susceptibility of sporadic Creutzfeldt-Jacob disease (sCJD) in UK. MicroRNAs are endogenous small noncoding RNAs that control gene expression by targeting mRNAs and triggering either translation repression or RNA degradation. In this study, two polymorphic loci in miR-146a (rs2910164 and rs57095329) and one locus in ZBTB38-RASA2 (rs295301) of 561 Chinese patients of sCJD and 31 cases of fatal familial insomnia (FFI) were screened by PCR and sequencing. Our data did not figure out any association of those three SNPs with the susceptibility of sCJD. However, a significant association of the SNP of rs57095329 in miR-146a showed the association with the susceptibility of FFI. Additionally, the SNP of rs57095329 showed statistical significances with the appearances of mutism and the positive of cerebrospinal fluid (CSF) protein 14-3-3 in sCJD patients, while the SNP of ZBTB38-RASA2 was significantly related with the appearance of myoclonus in sCJD patients. It indicates that the SNPs of ZBTB38-RASA2 and miR-146a are not associated with the susceptibility of the Chinese sCJD patients, but may influence the appearances of clinical manifestations somehow.

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Introduction

Prion diseases, also called transmissible spongiform encephalopathies (TSEs), are progressive fatal neurodegenerative disorders that affect humans and animals. The human prion diseases comprise Kuru, Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI). The majority (85%) of human prion diseases are sporadic. Approximately 10%-15% of human prion diseases are inherited, associated with the mutations in the prion protein gene (*PRNP*), and less than 1% is acquired [1].

PRNP is located on chromosome 20p12 in humans. More than 50 mutations have been found in the open reading frame (ORF) of *PRNP*, which are directly linked with the genetic prion diseases [2]. In addition to these mutations, some polymorphisms have also been observed inside or outside the ORF of *PRNP* [3], particularly the polymorphisms at codon 129 and 219. Although the well-known polymorphism at codon 129 is nonpathogenic, it does influence, even determine, the sensitivity and susceptibility to prion infectious agents, as well as the clinical phenotype of the diseases [2].

Recently, some other candidate genes have been investigated for their potential association with human prion disease, using genome-wide association studies (GWAS) and other new methods [4]. ZBTB38–RASA2 (rs295301) loci are in the vicinity of RASA gene. The product of this gene is member of the GAP1 family of GTPase-activating proteins, which stimulates the GTPase activity of normal RAS p21 but not its oncogenic counterpart [5]. Although the highest score gene locus of ZBTB38-RASA2 was not significant at genome-wide levels, a study performed in UK has proposed that the SNPs at the ZBTB38–RASA2 (rs295301) locus are associated with CJD. However, the subsequent study in Germany did not figure out the significant correlation [6].

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MicroRNAs (miRNAs) are 20–24 base-pair (bp) long non-coding RNAs that participate in post-transcription of gene expression in various cells by affecting both the stability and translation of mRNAs. MiR-146a locates on chromosome 5 in humans. MiR-146a has the activity in immuno-inflammatory regulation [7], showing direct down-regulation for the production of pro-inflammatory cytokines by acting as a negative-feedback effector of the inflammatory signaling pathway initiated by NF-κB [8]. Recently, alteration in expression of miR-146a has been observed in some neurodegenerative disorders, including multiple sclerosis (MS), pro-inflammatory neurodegeneration and prion disease [9,10]. Our study also identified that the amount of miR-146a in the blood of sCJD patients is higher than the health control (data unpublished). The SNPs of miR-146a have been also screened in some kinds of diseases, such as AD, diabetes and hepatocellular cancer [11,12]. However, the possible linkage of the SNPs of miR-146a with the susceptibility of CJD remains unknown.

In this study, two polymorphic loci in miR-146a and one locus in ZBTB38–RASA2 of 561 Chinese patients of sporadic CJD (sCJD) and 31 cases of FFI were screened. The possible associations of those three loci with the disease risk, main clinical manifestations and some examination results were further evaluated.

Materials and methods

Subjects

Totally 561 cases of probable diagnosed sCJD, 31casesof definite diagnosed FFI and 231 health controls were enrolled into study. All subjects were Chinese. The diagnoses of sCJD and FFI were performed by China CJD Surveillance Center, according to the diagnostic criteria for human prion diseases issued by World Health Organization (WHO)[13,14] and by National Health and Family Planning Commission of the People's Republic of China (http://www.nhfpc.gov.cn/ewebeditor/uploadfile/2017/07/20170727150307976.pdf). The detailed processes for the diagnosis of human prion diseases in China CJD Surveillance System were described previously [14,15,16]. Briefly, the clinical data (including the results of EEG and MRI) and specimens of the suspected patients were collected by the clinician from hospitals, while their epidemiological data were collected by the staff of provincial CDCs. The collected data and sample were referred to the national reference laboratory for human prion disease in China CDC for laboratory tests and final diagnosis. The health controls were obtained from blood donors in Beijing area.

Ethics statement

Usage of human blood samples in this study was approved by the Ethical Committee of National Institute for Viral Disease Prevention and Control, China CDC. Samples were obtained with the adequate understanding and consent. All the data were analyzed anonymously, and clinical investigations have been conducted according to the principles Declaration of Helsinki.

PCR and genotyping

For *PRNP* analysis, genomic DNA was extracted from peripheral blood leukocytes by using Qiagen's DNA purification kit according to the manufacturer's instructions. The specific primers were synthesized based on the sequences issued by NCBI, including the primers for miR-146a rs2910164: forward 5′-TGGTCTCCTCCAGATGTTTAT-3′ and reverse 5′- GCTACTTGGAACC CTGCTTA-3′; the primers for miR-146ars57095329:forward 5′-TGAAACTCAGCCTGCGCG-3′ and reverse 5′-ATCCCTCCTCGGCACAGC -3′; the primers for ZBTB38-RASA2 rs295301:forward 5′-CAGTTGCATTC TGTTGGC-3′ and reverse 5′- CTATCTCATAACTGA GCAAATC-3′. The PCR reactions were performed in a total volume of 20 μl, containing 100 ng of genomic DNA, with the experimental conditions of pre-denaturation at 94℃ for 1 min, 30 cycles of denaturation at 94℃ for 10 s, annealing at 60℃ for 20 s and extension at 72℃ for 20 s. The amplified PCR products of rs2910164, rs57095329 and rs295301 were sequenced and analyzed.

Statistical analysis

Pearson's chi-square test was used to compare genotype and allele frequencies between patients and controls. Odds ratio (OR) together with 95% confidence interval (CI) was also estimated, and a p-value less than 0.05 (two-tailed) was considered as statistically significant. The association was also tested under log-additive model. The Hardy-Weinberg equilibrium was performed using a Fisher's exact test. All statistical analyses were performed using the SPSS 11.5 computer software program.

Results

The associations of the three SNPs with the susceptibility to sCJD or FFI

Totally the blood samples from 561 patients of probable sCJD, 31 patients of FFI, and 231 healthy blood donors were collected and specific PCRs for miR-146a rs2910164, miR-146a rs57095329 and ZBTB38-RASA2 rs295301 were conducted for each sample separately.

Table 1. Distributions of the three SNPs and risks of sCJD patients.

	sCJD n (%)	Controls n (%)	OR	95% CI	P value
Rs2910164 genotype					
CC	194 (34.6%)	69 (29.9%)	\		
CG	26 (47.4%)	115 (49.8%)	0.8227	0.5790–1.169	0.2881
GG	101 (18.0%)	47 (20.3%)	0.7643	0.4913–1.189	0.2542
Rs2910164 allele					
C	654 (58.3%)	253 (54.8%)	\		
G	468 (41.7%)	209(45.2%)	0.8662	0.6963 –1.078	0.1993
Rs57095329 genotype					
AA	363 (64.9%)	142 (62.0%)	\		
AG	181 (32.4%)	81 (35.4)	0.8741	0.6309– 1.211	0.4507
GG	15 (2.6%)	6 (2.6%)	0.978	0.3720– 2.571	1
Rs57095329 allele					
A	904 (81.1%)	365 (79.7%)	\		
G	210 (11.3%)	93 (20.3%)	0.9117	0.6941–1.198	0.5267
Rs295301 genotype	558	229			
GG	204 (36.6%)	86 (37.2%)	\		
GA	258(46.2%)	113 (48.9%)	0.9625	0.6883– 1.346	0.8644
AA	96 (17.2%)	30 (13.0%)	1.349	0.8336–2.183	0.2362
Rs295301 allele					
G	666(59.7%)	285 (62.2%)	\		
A	450 (40.3%)	173 (37.8%)	1.113	0.8900–1.392	0.3642

The genotypes and allele frequencies of those three polymorphisms in the groups of sCJD, FFI and health control were summarized in Table 1 and 2.

As shown in Table 1, the genotypes and allele frequencies of the tested three loci were similar between the groups of sCJD and health control, without statistical difference. It indicates little influence of those three polymorphisms on the risk of sCJD among Chinese. Two significant associations were identified in the comparison between the groups of FFI and health control (Table 2). One was in the allele of miR-146a rs2910164. FFI cases showed higher rate of CC homozygosis (41.9%) than health controls (29.9%). Statistical analysis showed significance between CC and GG genotypes (P = 0.04869). Analysis of the frequencies of C and G in this allele also revealed statistical difference between the groups of FFI and health control (P = 0.057). The other association was in the allele of ZBTB38-RASA2 rs295301. Higher rate of GA genotype (74.2%) was detected in the FFI patients compared with that of health controls (48.9%), showing statistical difference (P = 0.0252). However, no difference in the frequency of G and A in this allele was addressed between FFI and control. Additionally, analysis of the genotype and allele frequency of miR-146a rs57095329 did not find out difference between FFI and control.

The association of the three SNPs with the main clinical manifestations and the examinations results of sCJD

Besides of dementia, other four clinical manifestations are included in the diagnostic criteria for sCJD, including pyramidal or extrapyramidal dysfunction, myoclonus,

Table 2 Distributions of the three SNPs and risks of FFI patients.

	FFI n (%)	Controls n (%)	OR	95%CI	P value
Rs2910164 genotype					
CC	13(41.9%)	69 (29.9%)	\		
CG	16 (51.6%)	115 (49.8%)	0.7385	0.3350–1.628	0.5387
GG	2 (6.5%)	47 (20.3%)	0.2259	0.04869–1.048	**0.0485**
Rs2910164 allele					
C	42 (67.7%)	253 (54.8%)	\		
G	20 (32.3%)	209(45.2%)	0.5764	0.3282–1.012	0.057
Rs57095329 genotype					
AA	24 (77.4%)	142 (62.0%)	\		
AG	7 (22.6%)	81 (35.4)	0.5113	0.2110–1.239	0.1604
GG	0 (0%)	6 (2.6%)	\	\	
Rs57095329 allele					
A	55 (88.7%)	365 (79.7%)	\		
G	7 (11.3%)	93 (20.3%)	0.4995	0.2202–1.133	0.1208
Rs295301 genotype					
GG	6 (19.4%)	86 (37.2%)	\		
GA	23 (74.2%)	113 (48.9%)	2.917	1.138–7.479	**0.0252**
AA	2 (6.5%)	30 (13.0%)	0.9556	0.1828–4.994	1
Rs295301 allele					
G	35 (56.5%)	285 (62.2%)	\		
A	27 (43.5%)	173 (37.8%)	1.271	0.7432–2.173	0.4055

visual or cerebellar disturbance and akinetic mutism. Since dementia is recorded in almost all enrolled sCJD cases, the four main clinical manifestations were analyzed for their possible associations with those three alleles. As summarized in Table 3, miR-146a rs2910164 did not reveal any significant association with the appearances of the four symptoms and signs. However, the patients with G allele in rs57095329 had a higher positive rate of kinetic mutism than those with A allele (P = 0.016). The patients with A allele in ZBTB38-RASA2 rs295301 also showed a higher frequency in appearance of myoclonus than the patients with G allele (P = 0.013).

Further, the potential associations of the genotypes and frequencies of those three alleles with the results of main clinical examinations and laboratory tests in the group of sCJD were analyzed, including positive in CSF protein 14-3-3, periodic sharp wave complexes (PSWC) in EEG and high signal in caudate/putamen in MRI. As shown in Table 4, the genotypes and allele frequencies of miR-146a rs2910164 and ZBTB38-RASA2 rs295301 did not reveal statistical association with the results of CSF 14-3-3, EEG and MRI. The genotype and allele frequency of miR-146a rs57095329 also showed no correlation with the results of EEG and MRI. However, it revealed statistical significance with the result of CSF 14-3-3 test, that the sCJD patients with allele A in rs57095329 had a higher positive rate of CSF 14-3-3 than that of allele G (P = 0.011).

Discussion

As the most common types of human prion diseases, sCJD constitutes up to 85% of all prion diseases, characterized by highly sporadic occurrence without linkage among the cases. The highest incidence of Chinese sCJD cases is at the age group of 60–69 year-old with the onset median of 62 year-old. The clinical duration is relatively short with the median of 5.3 months. FFI is a common genetic prion disease worldwide with a mutation of D178N and a M129M homozygous within *PRNP* gene. In China, FFI is the most frequently identified genetic prion disease. About 2/3 Chinese FFI cases show positive family history. The highest incidence of FFI cases is at the age group of 50–59 year-old with the onset median of 51 year-old. The survival time of FFI is relative long with the median of 10 months [14,15]. Although the key mechanism in the pathogenesis of prion diseases is the conversion from the cellular prion protein (PrP^{C}) to pathogenic PrP^{Sc},[17] it is obvious that there are some other factors that affect the developments of prion diseases. The polymorphism of codon 129 in PRNP gene is a well-documented example, which not only affects the susceptibility of the diseases, such as sCJD and variant CJD (vCJD), but also determines the clinical phenotype of some genetic prion diseases, such as D178N/M129M FFI and D178N/M129V gCJD. Along with the development of gene sequencing, many research groups have conducted the studies in different populations in order to address the possible relationships of various SNP polymorphisms with prion diseases in the past decade [3].

In this study, we have analyzed three SNPs based on 561 sCJD cases, 31 FFI cases and 231 healthy persons. Two of three SNPs are associated with miRNA-146a. MiRNA-146a is known as an immune inflammation regulatory factor that play as a key regulator in astrocyte-mediated inflammatory response [9]. It has been found that miRNA-146a is up-regulated in the brain of GSS and sCJD patients, as well as in prion-infected mice [18]. From the data of this study, we do not figure out any association of those two SNPs with the susceptibility of sCJD. However, one SNP (rs57095329) shows statistical significances with the appearance of mutism and the CSF 14-3-3 positive in sCJD patients. The rs57095329 (A/G) polymorphic locus locates at the binding site of the transcription factor ETS-1 within the promoter region of miR-l46a. It has been reported that the G allele can attenuate the binding of ETS-1 in the promoter region, thereby reducing the transcription efficiency of miR-l46a and ultimately affecting the expression of mature miR-l46a [19]. The exact association of the expression of miR-146a in the brain tissues with CSF 14-3-3 still remains unknown. It is described that miR-l46a may modulate the innate immune response and the microglial activation state in prion disease [20]. Actually, we have already reported activations of microglia and complement system in the brains of sCJD patients and many scrapie-infected rodent models [21]. Unfortunately, most of the sCJD cases in this study are probable diagnosed sCJD without post-mortem brain tissues. The correlation between the brain miR-l46a level and activation of innate immune activity deserves further study. This site has been also reported to be involved in the genetic susceptibility to AD, and this risk AA genotype may increase the expression of miR146a and influence certain proinflammatory cytokines, thus playing a role in the pathogenesis of AD [22].

Recently, an international collaborative based on GWAS technique has conducted. In this large patient cohorts study, the SNP at the ZBTB38-RASA2 locus (rs295301) shows to be associated with sCJD in UK

Table 3. Distributions of the three SNPs and laboratory results of sCJD patients.

		Laboratory tests				Clinical tests							
		Protein 14-3-3 in CSF				EEG				MRI			
SNP		n (%)	Positive (%)	Negative (%)	p Value	n (%)	Positive (%)	Negative (%)	p Value	n (%)	Positive (%)	Negative (%)	p Value
Rs2910164	CC	172 (34.8)	131 (33.8)	41 (38.7)	\	127 (35.3)	77 (35.0)	50 (35.7)	\	131 (35.9)	64 (37.0)	67 (34.9)	\
	GC	236 (47.8)	184 (47.4)	52 (49.1)	0.668	167 (46.4)	103 (46.8)	64 (45.7)	0.855	168 (46.0)	78 (45.1)	90 (46.9)	0.677
	GG	86 (17.4)	73 (18.8)	13 (12.2)	0.105	66 (18.3)	40 (18.2)	26 (18.6)	0.997	66 (18.1)	31 (17.9)	35 (18.2)	0.803
	C	580 (58.7)	446 (57.5)	134 (63.2)	\	421 (58.5)	257 (58.4)	164 (58.6)	\	430 (58.9)	206 (59.5)	224 (58.3)	\
	G	408 (41.3)	330 (42.5)	78 (36.8)	0.133	299 (41.5)	183 (41.6)	116 (41.4)	0.966	300 (41.1)	140 (40.5)	160 (41.7)	0.741
Rs57095329	AA	324 (64.7)	237 (61.7)	87 (74.4)	\	229 (64.0)	144 (65.2)	85 (62.0)	\	233 (64.2)	106 (60.9)	127 (67.2)	\
	AG	164 (32.7)	135 (35.2)	29 (24.8)	**0.025**	117 (32.7)	67 (30.3)	50 (36.5)	0.312	117 (32.2)	59 (33.9)	58 (30.7)	0.384
	GG	13 (2.6)	12 (3.1)	1 (0.8)	0.222	12 (3.3)	10 (4.5)	2 (1.5)	0.259	13 (3.6)	9 (5.2)	4 (2.1)	0.166
	A	812 (81.0)	609 (79.3)	203(86.8)	\	575 (80.3)	355 (80.3)	220 (80.3)	\	583 (80.3)	271 (77.9)	312 (82.5)	\
	G	190 (19.0)	159 (20.7)	31 (13.2)	**0.011**	141 (19.7)	87 (19.7)	54 (19.7)	0.994	143 (19.7)	77 (22.1)	66 (17.5)	0.114
Rs295301	GG	187 (37.5)	145 (37.7)	42 (36.8)	\	137 (38.4)	85 (38.6)	52 (38.0)	\	136 (37.2)	65 (37.6)	71 (37.0)	\
	GA	229 (45.9)	173 (44.9)	56 (49.1)	0.634	163 (45.7)	101 (45.9)	62 (45.3)	0.989	163 (44.7)	76 (43.9)	87 (45.3)	0.841
	AA	83 (16.6)	67 (17.4)	16 (14.1)	0.558	57 (15.9)	34 (15.5)	23 (16.7)	0.756	66 (18.1)	32 (18.5)	34 (17.3)	0.927
	G	603 (60.4)	463 (60.1)	140 (61.4)	\	437 (61.2)	271 (61.6)	166 (60.6)	\	435 (59.6)	206 (59.5)	229 (59.6)	\
	A	395 (39.6)	307 (39.9)	88 (38.6)	0.729	277 (38.8)	169 (38.4)	108 (39.4)	0.788	295 (40.4)	140 (40.5)	155 (40.4)	0.979

Table 4. Distributions of the three SNPs and clinical manifestations of sCJD patients.

			Myoclonus			Visual or Cere-bellar Disturbance			Pyramidal or Extrapyramidal Disfunction			Akinetic Mutism		
SNP		n (%)	Positive (%)	Negative (%)	p	Positive (%)	Negative (%)	p	Positive (%)	Negative (%)	p	Positive (%)	Negative (%)	p
Rs2910164	CC	179 (34.4)	129 (72.1)	50 (27.9)	/	115 (64.2)	64 (35.8)	/	137 (76.5)	42 (23.5)	/	62 (34.6)	117 (65.4)	/
	GC	251 (48.3)	165 (65.7)	86 (34.3)	0.165	152 (60.6)	99 (39.4)	0.438	174 (69.3)	77 (30.7)	0.099	78 (31.1)	173 (68.9)	0.438
	GG	90 (17.3)	64 (71.1)	26 (28.9)	0.87	49 (54.4)	41 (45.6)	0.121	66 (73.3)	24 (26.7)	0.565	36 (40.0)	54 (60.0)	0.389
	C	609 (58.6)	423 (69.5)	186 (30.5)	/	382 (62.7)	227 (37.2)	/	448 (73.6)	161 (26.4)	/	202 (33.2)	407 (66.8)	/
	G	431 (41.4)	293 (68.0)	138 (32.0)	0.612	250 (58.0)	181 (42.0)	0.125	306 (71.0)	125 (29.0)	0.361	150 (34.8)	281 (65.2)	0.583
Rs57095329	AA	335 (64.5)	231 (69.0)	104 (31.0)	/	211 (63.0)	124 (37.0)	/	237 (70.7)	98 (29.3)	/	104 (31.0)	231 (69.0)	/
	AG	169 (32.6)	118 (69.8)	51 (30.2)	0.842	95 (56.2)	74 (43.8)	0.142	130 (76.9)	39 (23.1)	0.142	70 (41.4)	99 (58.6)	**0.021**
	GG	15 (2.9)	9 (60.0)	6 (40.0)	0.655	9 (60.0)	6 (40.0)	0.815	11 (73.3)	4 (36.7)	0.829	7 (46.7)	8 (53.3)	0.323
	A	839 (80.8)	580 (69.1)	259 (30.9)	/	517 (61.6)	322 (38.3)	/	604 (72.0)	235 (28.0)	/	278 (33.1)	561 (66.9)	/
	G	199 (19.2)	136 (68.3)	63 (31.7)	0.829	113 (56.8)	86 (43.2)	0.209	152 (76.4)	47 (23.6)	0.211	84 (42.2)	115 (57.8)	**0.016**
Rs295301	GG	194 (37.5)	132 (68.0)	62 (32.0)	/	127 (65.5)	67 (34.5)	/	144 (74.2)	50 (25.8)	/	69 (35.6)	125 (64.4)	/
	GA	234 (45.3)	171 (73.1)	63 (26.9)	0.255	144 (61.5)	90 (38.5)	0.402	182 (77.8)	52 (22.2)	0.391	90 (38.5)	144 (61.5)	0.538
	AA	89 (17.2)	73 (82.0)	16 (18.0)	**0.015**	62 (69.7)	27 (30.3)	0.487	72 (80.9)	17 (19.1)	0.221	40 (44.9)	49 (55.1)	0.133
	G	622 (60.2)	435 (70.0)	187 (30.0)	/	398 (64.0)	224 (36.0)	/	470 (75.6)	152 (24.4)	/	228 (36.7)	394 (63.3)	/
	A	412 (39.8)	317(77.0)	95 (23.0)	**0.013**	268 (65.0)	144 (35.0)	0.727	326 (79.1)	86 (20.8)	0.183	170 (41.3)	242 (58.7)	0.136

($p = 3.13 \times 10^{-8}$) [4]. However, such SNP is confirmed being not associated with German sCJD patients and in Kuru based test. Here, the results of our study also do not propose any correlation of polymorphic ZBTB38-RASA2 locus with the susceptibility with the development of sCJD in Chinese population. However, the SNP of ZBTB38-RASA2 seems to be significantly related with the appearance of myoclonus in sCJD patients. Myoclonus is a frequently observable sign in sCJD. Meanwhile, myoclonus can be also noticed in many other neurological diseases. Whether such correlation is symptom-associated or disease-associated is an interesting topic.

Based on the limited numbers of FFI cases in this study, we have observed a significant association of a SNP (rs57095329) in miR-146a with the susceptibility of FFI. The rs2910164 C/G locates in the hairpin region of miR-l46a, leading to a C:U instead of G:U pairing, which may affect the maturation of the precursor miR-l46a and the expression of mature miR-l46a [23]. In addition, the genotype of ZBTB38-RASA2 shows also association with the susceptibility of FFI. Majority of Chinese FFI patients has positive family history [15], meanwhile, the FFI cases also show regional-association (data not published). Thereby, it is probably acceptable that SNPs can be addressed in FFI patients, and even in other genetic prion diseases with positive family history. Additionally, asymptomatic carriers with D178M mutation are repeatedly identified in many FFI families, and some of them are in the parent-generation of probands. Whether such associations can influence the occurrence and development of this special disease needs further exploration.

In this study, we have defined a significant difference in the P value of 0.05. We have to admit that although P value target of 0.05 shows significance between disease and healthy groups, it is sometimes impossible to use as a possible candidate in further usage, such as predication, diagnosis and prognosis. Additionally, all P values showing significance between disease and healthy groups in this study are higher than 0.01. Together with the limited patient numbers of those rare diseases, we have to raise the limitation of the observations in this study. Study with multiple centers and larger-size samples are definitely benefit to get more realistic conclusion.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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