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Zinc transporter-3 [*SLC30A3* (rs11126936)] polymorphism is associated with major depressive disorder in Asian subjects

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ABSTRACT: Major depressive disorder (MDD) compromises the individual's capacity for self-care and productivity. Single nucleotide polymorphisms (SNP) of a number of genes have been associated with MDD. The zinc transporter-3 protein, encoded by the ZnT3 (*SLC30A3*) gene, maintains zinc-glutamate homeostasis at the glutamatergic synapse, a disruption of which increases risk of MDD. We hypothesise that variation in *SLC30A3* (rs11126936) SNP increases risk of MDD. We recruited 300 MDD cases and 300 controls, matched in the ratio of 1:1 by age, gender and ethnicity. PCR-restriction fragment length polymorphism analysis was used in DNA genotyping, validated by sequencing 10% of samples. Deviation from the Hardy-Weinberg equilibrium was tested using the chi-square test. Conditional logistic regression was used to estimate adjusted odds ratios, controlling for age, gender, ethnicity, occupation and family monthly income. Genotypes G/G and G/T showed two times greater odds of developing MDD compared to variant genotype T/T (OR=1.983, 95% CI=1.031-3.815; p=0.040 and OR=2.232, 95% CI=1.100-4.533; p=0.026 respectively). Carriers of genotypes G/G and G/T of the SNP rs11126936 in *SLC30A3* are associated with increased risk of MDD.

Keywords: depression; genetics; mood disorders; biological markers; zinc transporter (ZnT3) gene; SLC30A3 (rs11126936) SNP;

1.0 INTRODUCTION

Major depressive disorder (MDD) is a chronic disorder associated with high rates of non-recovery, recurrence and comorbidity [1]. DSM-5 defines MDD as "discrete episodes of at least 2 weeks' duration with clear-cut changes in affect, cognition, and neuro-vegetative functions, and inter-episode remissions" [2]. MDD adversely affects psychosocial function associated with reduced work productivity and days lost at work [2] and is the leading cause of disability in developed and developing countries, contributing to substantial health and economic burdens [3,4]. Depression when severe enough, compromises one's capability of self-care and independent living [5] and results in suicidal attempts in 4% of 19,723 inpatients reported in a systematic review [6].

The prevalence of MDD varies between 11.1% to 23.0% across different regions and populations [7-10]. According to the World Health Organization, 3.8% of the Malaysian population is affected by depressive disorders [4]. Local studies have reported the prevalence of depressive illness to be between 10.3% to 32.7% [11-15].

Genetic influence on the development of MDD is indicated by the heritability of between 40 to 75% [16-21]. Single nucleotide polymorphisms of a number of genes such as tryptophan hydroxylase-1 (TPH1), tryptophan hydroxylase-2 (TPH2) gene and brain-derived neurotrophic factor (BDNF) gene have been associated with MDD [22-26]. A point mutation in the *SLC30A3* gene, particularly *rs11126936* SNP, has been shown to be associated with schizophrenia in genome-wide association studies [27] and low blood zinc levels [28]. The *SLC30A3* gene encodes for the zinc transporter-3 (ZnT3) protein [29], which "is the sole mechanism for concentrating zinc ions within synaptic vesicles in a subset of the brain's glutamatergic neurons" [30]. Zinc efflux and influx, controlled by zinc transporter genes, play a crucial role in zinc-glutamate homeostasis at the synaptic junction, which modulates the balance of excito-inhibitory impulses of the glutamatergic neurons [31,32]. Lower levels of ZnT3 have been found on autopsy in suicidal cases diagnosed with MDD [33]. Although there is extensive literature on blood zinc level and MDD, none have described the relationship between genetic variation of ZnT3 and risk of MDD. This study aims to elucidate the effect of ZnT3 *(SLC30A3* rs11126936*)* SNP on risk of MDD.

2.0 MATERIALS AND METHODS

2.1 Ethics approval and consent to participate

This study was approved by the Medical Research and Ethics Committee of the Ministry of Health Malaysia (NMRR No.: NMRR-14-688-19696). All recruited subjects gave written informed consent to participate.

2.2 Study design and subject recruitment

Three hundred case-control pairs matched on a 1:1 ratio by age (±5 years), gender and ethnicity, were recruited from psychiatry clinics in four public hospitals from 2014 to 2017. Cases included those who were 18 to 65 years of age diagnosed with single-episode non-psychotic Major Depressive Disorder (using the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5)) who presented with a history of MDD less than 2 years prior to recruitment.

Patients who had (i) significant suicidal risk as assessed by the psychiatrist at the point of inclusion, or diagnosed with (ii) dementia (iii) schizophrenia or other

psychotic disorder (iv) bipolar I or II disorder, and (v) anxiety disorders including panic disorder, generalized anxiety disorder, obsessive-compulsive disorder and post-traumatic stress disorder were excluded from the study. Controls without a history of psychiatric disorders were recruited from patients attending otorhinolaryngology and ophthalmology clinics from the same hospitals where the cases were recruited. The sample size was estimated based on a power of 80%, and a level of significance of 0.05 to detect an odds ratio of 2 or greater [34].

2.3 Blood Collection

Using evacuated EDTA tubes (Vacutainer Tubes, Becton-Dickinson, USA), 5 ml of venous blood were collected from each subject and stored at 4°C prior to genomic DNA extraction within 24 h.

2.4 Genotyping of *SLC30A3* rs11126936 SNP

Genomic DNA was extracted from the buffy coat of the collected whole blood by using QIAamp DNA Mini and Blood Mini kit in accordance with the manufacturer's protocol (Qiagen, USA). Purity was determined using a NanoPhotometer®Classic (Implen, USA) and the acceptable range for the ratio A260/A280 was 1.8-2.0.

Genotyping protocol was adapted from Fujihara *et al.* 2018 [28]. Oligonucleotide primers (both sense-F 5'-TCCCAGAACCTCCACTCCTGGATCCTG-3' and antisense-R 3'-CCCCAGCTCTGGAATCTAGCCATCAGTTCT-5') were used to amplify the targeted SNP. Genotyping of *SLC30A3* rs11126936 was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). A region of 146 bp carrying the polymorphic restriction sites of *MspI* was amplified in a 20 µl PCR cocktail containing 100 ng of genomic DNA. PCR composition, PCR condition, and RFLP digestion are listed in Table 1. Amplified fragments were subjected to RFLP analysis. Using the restriction endonuclease *MspI*, which cuts at 5'… C^CGG…3', the variant (T/T) genotype [35] produced a single undigested product with 146 bp, G/T genotype produced partially digested product with 146 bp, 109 bp and 37bp, while G/G genotype was fully digested into 109 bp and 37 bp fragments.

Ten percent of randomly selected PCR products were sequenced using ABI PRISM 3730xl Genetic Analyzer (Thermo Scientific, USA) to confirm accuracy of the genotyping method. The cycle sequencing reaction was performed following manufacturer's specifications (BigDye® Terminator v3.1 Cycle Sequencing Kit, Thermo Scientific, USA). The DNA sequence was then viewed on a sequence analysis software (Sequence Scanner Software 2.0, Thermo Scientific, USA).

Table 1. PCR composition, PCR condition, RFLP digestion of *SLC30A3* rs11126936 polymorphisms

Polymorphisms	*SLC30A3* (rs11126936)
PCR composition	20 µL of total PCR reaction consisted of 1X DreamTaq green PCR mastermix with DreamTaq DNA polymerase, 1X DreamTaq Green buffer, dATP (0.4 mM), dCTP (0.4 mM), dGTP (0.4 mM) and dTTP (0.4 mM), and 4 mM $MgCl_2$ (Thermo Fisher Scientific, USA), 10 µM of each primer, 100 ng of genomic DNA and nuclease free water.
PCR condition	94°C for 3 minutes, followed by 30 cycles of denaturation at 96°C for 1 minute, annealing at the melting temperature 63°C for 1 minute and extension at 72°C for 1 minute, with a final 10 min extension at 72°C and stored at 4°C.
RFLP digestion	15 µL of total reaction mixture consisting of ~8 µL of PCR product, 1X CutSmart® Buffer, 20 U of *Msp*I (New England Biolabs, USA) was prepared in nuclease-free water. The mixture was incubated at 37°C for 2 hours followed by 15 minutes at 65°C.

2.5 Data analysis

McNemar test was used to determine differences in sociodemographic variables between cases and controls using IBM Statistical Package for the Social Sciences (IBM SPSS) version 22. Conditional logistic regression (using STATA 10) was used to estimate odds ratios and 95% confidence intervals (CIs), controlling for confounding variables of age, gender, ethnicity, occupation, education and family monthly income. Statistical significance was set at alpha=0.05. Court Lab Calculator [36] was used to perform the chi-square test for deviations from Hardy-Weinberg equilibrium (HWE) for *SLC30A3* (rs11126936) gene polymorphism in the controls.

3.0 RESULTS

3.1 Socio-demographic characteristics of cases and controls

Significant differences were found between cases and controls ($p<0.05$) in educational level, occupation, monthly income and family history of psychiatric illnesses. A higher proportion of controls (70.3%) obtained tertiary education compared to cases (51.0%) while MDD cases had lower monthly family incomes, where half of them earned less than RM 2,000 (USD 497.00) per month. In contrast to controls (4.7%), a much higher proportion (26.7%) of MDD cases had a family history of psychiatric illnesses while a greater proportion of controls worked in government or semi-government sectors (40.3%) (Table 2).

3.2 Laboratory findings

The representative images of the original gels are shown in Figure 1A. Ten percent of total samples from each genotype sent for sequencing were 100 % identical with the results of PCR-RFLP. Figure 1B shows the partial sequence chromatograms of *SLC30A3* (rs11126936) polymorphism.

The genotypic distribution of *SLC30A3* rs11126936 polymorphism for controls was T/T: 19% (57/300); G/T: 23% (69/300); G/G: 58% (174/300) while for cases, it was T/T: 12.7% (38/300); G/T: 31.7% (95/300); G/G: 55.7% (167/300). *SLC30A3* rs11126936 polymorphism deviated from Hardy-Weinberg Equilibrium ($p <0.001$). Conditional logistic regression showed that subjects

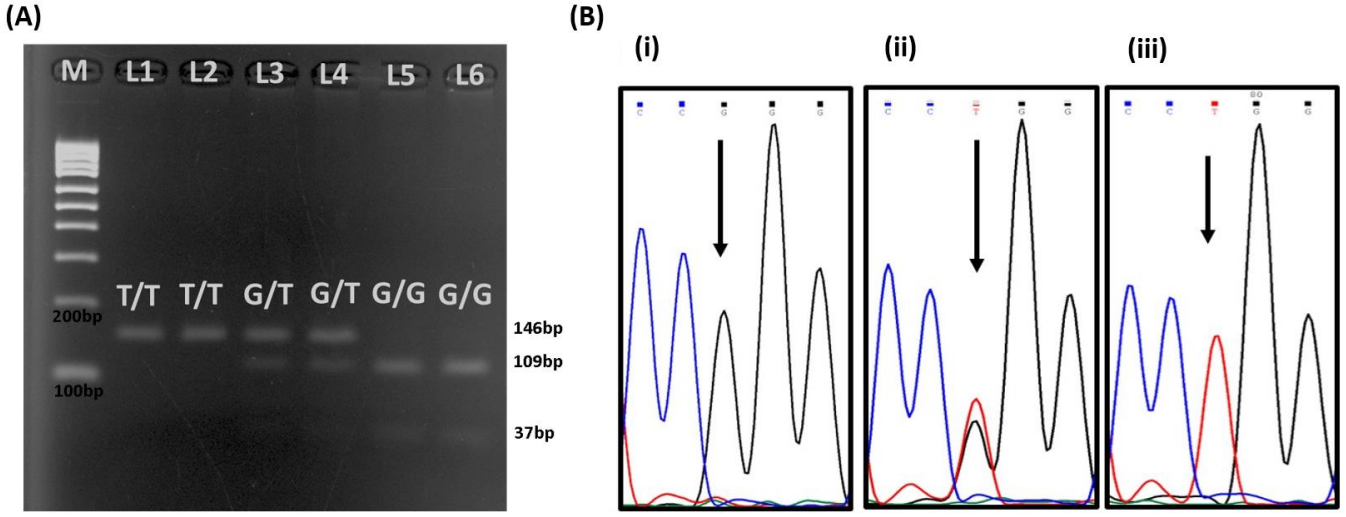

Figure 1. (A) Gel electrophoresis of PCR-RFLP products for *SLC30A3* rs11126936 polymorphisms. M on the figure represents DNA ladder marker; L1 and L2 represent T/T genotype (146bp), L3 and L4 represent G/T genotype (146 bp, 109 bp and 37 bp); L5 and L6 represent G/G genotype (109 bp and 37 bp). **(B)** Partial sequence chromatograms of *SLC30A3* rs11126936 polymorphism from study subjects. Arrow indicates the location of the nucleotide changes. Partial sequence chromatogram (i) represents G/G genotype; (ii) represents G/T genotype; (iii) represents T/T genotype.

with genotypes G/T (OR=2.23, 95% CI=1.10 - 4.53; p=0.026) and G/G (OR=1.98, 95% CI=1.03 - 3.82; p=0.040) had two times higher odds of developing major depressive disorder compared to subjects with variant genotype T/T after adjusting for age, gender, ethnicity, occupation, education and family monthly income. Those without a family history experienced a fifth of the odds of MDD (OR=0.18, 95% CI=0.09 - 0.36; p=<0.001) compared with those with a family history (Table 3).

Table 2. Sociodemographic characteristics of the study population.

Variables	Cases, n=300%		Controls, n=300 (%)		χ^2	*P* value
Age (years)						
18-25	52	(17.3)	55	(18.3)	4.675	0.320
26-35	76	(25.3)	89	(29.7)		
36-45	83	(27.7)	62	(20.7)		
46-55	48	(16.0)	55	(18.3)		
56-65	41	(13.7)	39	(13.0)		
Gender						
Male	97	(32.3)	97	(32.3)	0.000	1.000
Female	203	(67.7)	203	(67.7)		
Ethnicity						
Malay	149	(49.7)	149	(49.7)	0.000	1.000
Chinese	90	(30.0)	90	(30.0)		
Indian and others	61	(20.3)	61	(20.3)		
Family income						
< RM 1000	87	(29.0)	28	(9.3)	46.863	<0.001[a]
RM1001-RM2000	65	(21.7)	57	(19.0)		
RM2001-RM3000	64	(21.3)	74	(24.7)		
RM3001-RM4000	25	(8.3)	51	(17.0)		
>RM4000	59	(19.7)	90	(0.0)		
Education level						
Primary/Secondary	147	(49.0)	89	(29.7)	27.062	<0.001[a]
Certificate	25	(8.3)	27	(9.0)		
Diploma	45	(15.0)	82	(27.3)		
Degree/Postgraduate	83	(27.7)	102	(34.0)		
Family History						
No	220	(73.3)	286	(95.3)	54.949	<0.001[a]
Yes	80	(26.7)	14	(4.7)		
Occupation						
Private	85	(28.3)	66	(22.0)	78.640	<0.001[a]
Government/ Semi-government	47	(15.6)	121	(40.3)		
Student	41	(13.7)	64	(21.3)		
Retired	29	(9.7)	23	(7.7)		
Others	98	(32.7)	26	(8.7)		

[a] $p < 0.05$.

4.0 DISCUSSION

SLC30A3 rs11126936 (ZnT3 protein) is found abundantly in the brain, mostly in areas regulating emotions where glutamatergic neurons are distributed; namely the hippocampus, amygdala and frontal cortex [31,37-39]. It was found that genotypes G/G and G/T was associated with twice the odds of MDD compared to the variant T/T. Fujihara *et al.* (2018) [28] postulated that increased expression of the *SLC30A* family of genes might increase blood zinc levels. This is plausible as

Table 3. Association of single nucleotide polymorphism of ZnT3 gene (rs11126936), with MDD using conditional logistic regression (239 cases and 263 controls).

Variables		Crude OR	95% CI	p	OR[a]	95% CI	p
SLC30A3	T/T[b]	1			1		
(rs11126936)	T/G	2.1816	(1.2152 - 3.9165)	0.009*	2.2324	(1.0995 - 4.5326)	0.026[c]
	G/G	1.4075	(0.8345 - 2.3739)	0.200	1.9834	(1.0311 - 3.8150)	0.040[c]
Family history	Yes	1			1		
	No	0.1430	(0.0754 - 0.2714)	<0.001*	0.1771	(0.0867 - 0.3620)	<0.001[c]

[a] odds ratio, adjusted by age, gender, ethnicity, family monthly income, occupation and education by conditional logistic regression.

[b] Variant genotype (T/T) as reference for *SLC30A3* genotypes.

[c] $p<0.05$.

SLC30A3 rs11126936 causes efflux of zinc ions out of the neurons, thus increasing zinc in the extracellular space. Fujihara *et al.* also found that the G/G genotype was associated with reduced blood zinc concentration [28].

A considerably higher proportion (26.7%) of MDD cases had a positive family history of psychiatric illnesses compared to 4.7% in controls (χ^2=54.949, p<0.01). Sullivan *et al.* concluded in their meta-analysis that MDD is a familial disorder with heritability between 40% to 75% [16,17,19-21]. Although *SLC30A3* (rs11126936) SNP was not consistent with HWE, this is unlikely to be due to genotyping assay error as 10% of samples sequenced were in 100% concordance with RFLP results and the distribution for the genotypes of the SNP mentioned above in controls does not deviate towards an excess of heterozygotes [40]. Secondly, we do not think this is due to selection bias, as care was taken to recruit controls from the same hospitals as the cases so that the characteristics of controls would reflect those of the population from which cases arose. Thirdly, the deviation from HWE is likely due to "non-random mating" effect - leading to genetic segregation in which marriages within the same ethnicity or cultural background are still widely being practised in Malaysia [41]. Thus, the deviation from HWE does not preclude further analysis as the SNP may not be in equilibrium due to unidentified association with some other traits [40].

One of the potential limitations of the study could be selection bias introduced by the case-control study design. We have attempted to minimize this bias by recruiting controls from the same hospitals as the cases; differences in terms of sociodemographic characteristics were minimized by recruiting cases and controls matched by age, gender and ethnicity and also, by controlling for age, gender, ethnicity, family income, occupation and education by regression at the stage of data analysis.

5.0 CONCLUSIONS

Carriers of genotypes G/G and G/T of the *SLC30A3* gene conferred twice the odds of MDD compared to the variant T/T. A significantly higher proportion (26.7%) of MDD cases had a positive family history of psychiatric illnesses compared to 4.7% in controls. This study is an important first step to uncovering the effect of *SLC30A3* (rs11126936) polymorphism on the risk of MDD. To the best of our knowledge, this is the first report of *SLC30A3* (rs11126936) SNP increasing risk of MDD.

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Conflicts of Interest: The authors declare no conflict of interest.

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