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Nanopore sequencing-based genotyping suggested an association between CYP2D6 function and susceptibility to anxiety and depression

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Abstract

Objective CYP2D6 activity has been inconsistently associated with anxious and depressive personality traits. The inconsistency may stem from limitations of targeted genotyping, employed in most previous studies, leading to undetected errors in metabolic classification. Using a nanopore sequencing-based method, we comprehensively genotyped *CYP2D6* alleles in a small cohort of 96 Malaysians and re-examined the relationship between CYP2D6 activity and susceptibility to anxiety and depression.

Results In keeping with prior studies, *CYP2D6*10* was found to be the most common defective allele. Nearly half of the (48.5%) participants were classified as intermediate and poor metabolizers. Linear regression analysis suggested that impaired CYP2D6 activity could be a predictor of anxiety and depression, consistent with the putative role of CYP2D6 in the synthesis of serotonin and dopamine, the mood-boosting neurotransmitters. We hope this brief report will prompt larger-scale studies to further elucidate the contribution of *CYP2D6* to the genetic underpinnings of mental well-being.

Keywords CYP2D6, Anxiety, Depression, Nanopore sequencing

Introduction

The CYP2D6 enzyme, which metabolizes a variety of drugs and endogenous substances, has been implicated in the development of personality traits linked to anxiety or depression. However, the supporting evidence has been conflicting (study findings summarized in Supplementary Table 1), with some studies detecting an insignificant influence of CYP2D6 on predispositions to depression or anxiety [1–4], and others inconsistently ascribing heightened risks to normal [5, 6] or impaired metabolic function [7–9]. We surmised that the discrepancy could be caused by non-exhaustive genotyping leading to undetected errors in the classification of CYP2D6 function.

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Thus, in this study, we used a nanopore sequencing-based method to comprehensively genotype *CYP2D6* alleles in a group of 96 Malaysians; then, we re-examined the role of CYP2D6 in mental well-being.

Methods

Clinical cohort

The clinical cohort described in this study was part of a larger project examining factors that could influence non-communicable disease risks and general health among the staff ($n = 399$) of a university-affiliated hospital, Hospital Canselor Tuanku Muhriz. Depression, Anxiety and Stress Scales (DASS) scores were obtained through interviews guided by a questionnaire, medical records were reviewed, and whole-blood samples were obtained for genetic analyses. DASS is a general health screening tool used to gauge stress levels and the risk of depression or anxiety. It is not intended to diagnose clinical depression or anxiety disorders.

DNA extraction

DNA was extracted from the blood samples using the Genomic DNA Purification Kit (Promega Corp, USA) according to the manufacturer's instructions. Briefly, white blood cells were lysed to release DNA, which was then precipitated in isopropanol, pelleted by centrifugation, washed with ethanol, and finally dissolved in the DNA Rehydration Solution. DNA quality was assessed by $A_{280/260}$ ratios and agarose gel electrophoresis.

Genotyping of *CYP2D6* deletion and duplication alleles

A subset of the participants ($n = 96$) was selected for comprehensive *CYP2D6* genotyping, based on their DASS scores. Two separate duplex PCRs were performed initially to detect *CYP2D6* deletion and duplication alleles. Both PCRs amplified the entire *CYP2D6* gene, producing a 6.6-kb amplicon [10], and the residual repeat segments that result from whole-gene duplication or deletion, yielding a 3.5-kb amplicon [11]. The duplex PCRs were performed in 10-µL reactions consisting of 0.4 µM *CYP2D6*-specific primers, 0.3 µM primers for detection of the deletion or duplication allele, 1× Kapa Long Range buffer solution, 0.3 mM deoxynucleotide triphosphates (dNTPs), 1.75 mM $MgCl_2$, 1 M betaine, 1.25 U of Kapa LongRange Taq Polymerase (Sigma-Aldrich), and 50 ng of DNA. The thermal profile comprised initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 25 s, 68 °C for 10 s, 68 °C for 7 min, and final extension at 68 °C for 7 min. Controls known to be positive for the deletion and duplication alleles were included in all PCR runs. For each sample, 5 µL of the resultant long amplicons were visualised by 1% agarose gel electrophoresis.

After the initial round of screening for duplication and deletion alleles, the samples were re-amplified in singleplex PCRs with tailed primers to yield only the 6.6-kb amplicons:

5'-TTTCTGTTGGTGCTGATATTGC-[forward primer]-3'.

5'-ACTTGCCTGTCGCTCTATCTTC-[reverse primer]-3'.

Preparation of amplicon libraries for nanopore sequencing

The tailed 6.6-kb PCR amplicons were purified using the Agencourt AMPure XP Beads (Beckman Coulter™), which removed small fragments and unwanted contaminants. The volume of the magnetic beads required for each sample was calculated according to the following formula: Volume of Agencourt AMPure XP Beads = 1.8 × Reaction Volume. Thus, 5 µL of PCR products were mixed thoroughly with 9 µL of the magnetic beads in a clean microfuge tube, and set aside for 5 min; this captured DNA fragments >100 bp. Then, the beads were separated from the mixture, and the resultant clear solution was discarded. The bead-bound amplicons were washed thrice with 70% ethanol, eluted in 40 µL of PCR-grade water, and stored at −20 °C until use.

The concentrations of the purified products were measured using Qubit. Sample-specific barcodes were subsequently added to the purified 6.6-kb amplicons using the PCR Barcoding Expansion 1–96 Kit (EXP-PBC096; Oxford Nanopore Technologies, UK). The barcodes were added in a second-round PCR, which contained approximately 0.5 nM of the purified 6.6-kb PCR products, 1× LongAMP PCR buffer, 2 mM $MgCl_2$, 0.3 mM dNTPs, and 5 U of LongAmp Taq DNA Polymerase (New England Biolabs, Ipswich, USA) in a 50 µL reaction. The PCR was initiated by heating at 95 °C for 3 min, followed by 15 cycles of 95 °C for 15 s, 62 °C for 15 s, 65 °C for 7 min, and a final extension of 65 °C for 7 min.

Then, the amplicon library was prepared using the Ligation Sequencing Kit (SQK-LSK109; Oxford Nanopore Technologies, UK), according to the supplier-provided protocol for 1D PCR amplicon sequencing. All the barcoded amplicons were pooled; 200 fM of the DNA pool was end-repaired, and ONT adapters were ligated. After purification, approximately 350 ng of the pooled amplicon library was loaded into a Flongle flow cell to be sequenced for 48 h.

Bioinformatics analysis

DNA sequences were extracted from fast5 files using Guppy v2.2.2 with flip-flop basecalling (ONT, Oxford, UK). Only DNA reads with quality scores exceeding 7 were used for downstream analysis. Demultiplexing was performed with Porechop (https://github.com/rrwick/ Porechop) and NanoFilt [12]. The resultant fastq

data were aligned to the reference sequence hg19 using minimap2 [13], yielding alignments in the BAM format. DNA variants were then called with nanopolish [14] to produce variant lists in the VCF format. Where more than one heterozygous variant was present in the same sample, the haplotype was ascertained using WhatsHap [15] with 25× maximum depth of coverage in lieu of the default cutoff 15×. Quality control metrics were generated by NanoOK and MinIONQC.

Then, the CYP2D6 genotypes were converted into activity values using Stargazer [16]. The assignment of the activity value for an allele followed the guidelines published by PharmGKB [17] and the Clinical Pharmacogenetics Implementation Consortium (CPIC) (Please refer to the CYP2D6 Allele Functionality Table available from https://www.pharmgkb.org/page/cyp2d6RefMaterials). Then, the sum of the activity values for a diploty pe was calculated to yield the activity score, which was used to determine the metabolic status according to the latest consensus scheme [18]: Activity score >2.25, ultrarapid metabolizer; 1.25–2.25, normal metabolizer; >0 and <1.25, intermediate metabolizer; 0, poor metabolizer.

Statistical analysis

RStudio was used to perform linear regression and examine the relationship between DASS scores and CYP2D6 activity. Separate regression analyses were performed to test the impact of CYP2D6 activity on depression, anxiety, and stress scores, with gender, number of comorbidities, and body mass indices (BMIs) also included as independent variables. Poor and intermediate metabolizers were grouped together in the analysis. A p-value of <0.05 was considered statistically significant. The R code used to perform the analysis can be found in the Supplementary Materials.

Results and discussion

Of the 96 samples sequenced, 93 were successfully genotyped and assigned to a metabolic category. In two of the samples, *CYP2D6* variants with uncertain functional effects were detected, and metabolic classification was not feasible. One sample did not meet the quality control criteria, and no genotypes were identified. A final group of 93 participants were available for analyses of *CYP2D6* allele frequencies and susceptibility to depression, anxiety, and stress; they comprised 54 men and 39 women, with an average age of 47. All the participants, except one, were Malay. Based on BMIs, over 38% of the participants were overweight, and some had multiple self-reported comorbidities, namely hypertension, diabetes, and hypercholesterolemia (Table 1).

*CYP2D6*10* (allele frequency, 0.442) was the most common defective allele, followed by equally prevalent **5* and **41* (0.079), **4* (0.032), and low-frequency **43* and **71* (0.005), both detected in only one participant as a heterozygote (Table 2). This is in keeping with prior studies reporting that **10* is a common reduced-function allele among Asians [19, 20]. **4* and **5* are well known loss-of-function alleles. **4* shifts a splice site in intron 3 by a single base and derails the reading frame for exons 4–9; **5* results from whole-gene deletion, caused by loss of gene segments to neighbouring pseudogenes during large-scale crossover events.

Table 1 Participants' demographic characteristics and mental well-being assessed based on the Depression, Anxiety, and Stress Scales (DASS)

	Intermediate / Poor Metabolizer	Normal Metabolizer
Gender, n (%)	23 (50%)	31 (66.0%)
Male	23 (50%)	16 (34.0%)
Female		
Mean age (years)	47	48
Ethnicity, n (%) Malay	45 (97.8%)	47 (100%)
Others	1 (2.2%)	-
Body mass index, n (%)	2 (4.3%)	2 (4.3%)
Underweight	14 (30.4%)	12 (25.5%)
Normal	17 (37%)	18 (38.3%)
Overweight	13 (28.3%)	15 (31.9%)
Obese		
Comorbidities[1], n (%)	12 (26.7%)	10 (22.2%)
Hypertension	3 (6.8%)	4 (8.9%)
Diabetes	11 (24.4%)	8 (17.8%)
Hypercholesterolemia		
DASS scores, mean ± SD	7.4 ± 2.5	3.2 ± 2.5
Depression	5.7 ± 3.0	2.9 ± 2.5
Anxiety	5.7 ± 2.9	5.7 ± 3.5
Stress		

[1]Data on self-reported hypertension and hypercholesterolemia were available for 45 intermediate and poor metabolizers, while the prevalence of diabetes was calculated based on data from a total of 44 intermediate and poor metabolizers. Data on all three comorbidities were available for 45 normal metabolizers

Table 2 Frequencies of *CYP2D6* alleles among Malay Malaysians

CYP2D6 Allele	Count[1] (Allele Frequency)	Activity Value	Remarks
Normal-function allele			
**1*	48 (0.253)	1.0	Wild-type allele
**2*	17 (0.089)		Contains an expression-decreasing variant, 2850 C > T, whose effect is offset by two distant enhancer variants
**39*	3 (0.016)		Defined by two variants also present in **2*, 1661G > C and 4180G > C
Decreased-function allele			
**10*	84 (0.442)	0.25	Prevalent in Asian populations
**41*	15 (0.079)	0.5	Decreases the level of full-length transcripts
Loss-of-function allele			
**4*	6 (0.032)	0	CYP2D6 protein truncated by erroneous splicing and frameshifting; a common defective allele in Caucasians
**5*	15 (0.079)		Whole-gene deletion
Uncertain functional significance			
**43*	1 (0.005)	Unknown	A decreased-function allele that has not yet received an official CPIC activity value
**71*	1 (0.005)		May be a poor-metabolizer allele, through its adverse effect on the insertion of the CYP2D6 protein into the membranes of endoplasmic reticulum
TOTAL	190 (1)		

[1]One sample could not be genotyped

Abbreviation: CPIC, Clinical Pharmacogenetics Implementation Consortium

The **41* allele has been detected in different ethnic subgroups of Malaysian aborigines [21], with allele frequencies ranging from 0.071 to 0.455. Our study is the first to have documented the frequency of **41* in Malays. Likewise, **43* has not been reported in Malaysians. Both alleles diminish CYP2D6 function and can, therefore, alter clinical responses to CYP2D6 substrates. However, **43* has not been officially assigned an activity value by CPIC, despite having been shown to decrease enzyme function in vitro [22]. Another *CYP2D6* allele worth noting was **71*, first reported in Han Chinese [23]. Causing an amino acid switch at the N-terminal, **71* may eliminate CYP2D6 function by hindering the attachment of the protein to the endoplasmic reticulum; however, this has not been empirically confirmed.

Based on the CYP2D6 activity scores, the majority of the participants were classified as normal (49.5%) or intermediate (46.3%) metabolizers (Fig. 1). Only two participants (2.1%) were classified as poor metabolizers, consistent with the findings of prior surveys that complete CYP2D6 deficiency is relatively uncommon among Asians [24, 25]. The metabolic status for the remaining two participants was undeterminable as they were carriers of the **43* and **71* alleles (Fig. 1). Given the novelty of the **41* allele, we then assumed a hypothetical scenario where a targeted approach was adopted to genotype only the more commonly known alleles such as **10*. We found that if **41* had not been genotyped, ~12% (11/95) of the study participants who were intermediate or poor metabolizers would have been misclassified as normal metabolizers. This underlines the importance of sequencing-based approaches in genotyping the large assemblage of *CYP2D6* alleles and accurately ascertaining metabolic status.

Subsequent linear regression analysis revealed that anxiety and depression scores were negatively correlated with CYP2D6 activity, where poor and intermediate metabolizers were more susceptible to mood-related disturbances. Notably, the largest intergroup difference was identified in the depression scores (7.4 ± 2.5 and 3.2 ± 2.5 for poor and intermediate vs. normal metabolizers). No significant association was found between stress scores and CYP2D6 metabolic status (Fig. 2). Of the variables tested for association with depression scores, CYP2D6 activity and age were found to be potential predictors, accounting for 35.9% of the variance (adjusted R-squared, Fig. 2); similarly, CYP2D6 activity and age explained 19.4% of the variance in the anxiety scores. The findings of the regression analysis coincided with the prior hypothesis that impaired CYP2D6 activity reduces serotonergic and dopaminergic neurotransmission, which regulates mood and induces positive feelings such as happiness and motivation [3, 7, 8]. Interestingly, the association remained significant when some of the participants were intentionally misclassified to simulate the results

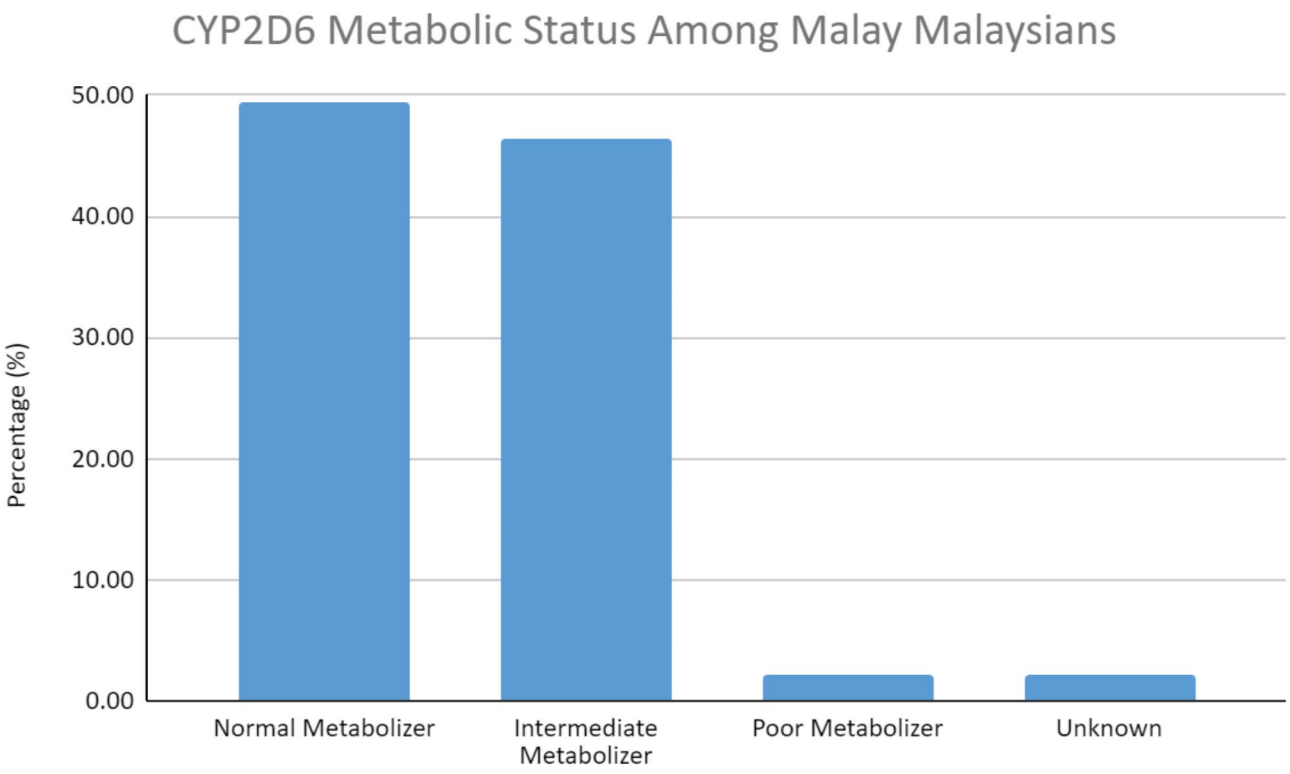

Fig. 1 Metabolic status in a predominantly Malay group of Malaysians, assigned according to the following phenotypic scheme [18]: Activity score >2.25, ultrarapid metabolizer; 1.25–2.25, normal metabolizer; >0 and <1.25, intermediate metabolizer; 0, poor metabolizer. Participants with unknown metabolic status harbored *CYP2D6* alleles whose functional significance had not been confirmed. The *CYP2D6* alleles were genotyped by nanopore sequencing (Oxford Nanopore Technologies, UK)

that would have been obtained from a targeted genotyping method (data not shown).

It is noteworthy that some of the previous studies did not detect a significant association between CYP2D6 function and personality [1, 2] or risks of anxiety and depression [3]. We have shown that the methodological limitations of targeted genotyping were unlikely to have caused the inconsistency, so other confounding factors should be considered. Firstly, the definition of the phenotype under investigation could affect the magnitude of interindividual differences and, consequently, the power of a study to uncover significant associations. Unlike previous studies which performed broad assessments of personality traits using a variety of specialized instruments (Supplementary Table 1), we homed in on susceptibility to depression, anxiety, and stress. Nuanced differences in personality traits can be difficult to pinpoint; the results of the regression analysis seem to support this hypothesis, as the genotype-phenotype association was found to weaken with decreasing severity of the phenotype and eventually become negligible for the stress scores (Fig. 2).

Secondly, CYP2D6 activity is highly variable. While the accuracy of genotyping may suffer from the omission of uncommon alleles, even direct measurement of enzyme activity using probe drugs has yielded conflicting findings [5, 7, 8]. Efforts to standardise the conversion of *CYP2D6* genotypes into phenotypes have led to some major changes. Most notably, the activity value for **10* has been reduced from 0.5 to 0.25, taking into account recent findings that the extent of CYP2D6 deficiency caused by **10* is greater than that of a typical decreased-function allele [18]. The update underscores the long-standing challenge of rendering the continuum of CYP2D6 activity into precise metabolic categories.

Thirdly, *CYP2D6* may be associated with as-yet-unknown causative genes which are activated only within specific (epi)genetic context or backgrounds. A recent systematic review has disputed the accuracy of the "serotonin theory" that has long been the basis for contemporary antidepressant treatments, on grounds of inadequate evidence [26]. This begs the question of whether the function served by CYP2D6 in synthesizing serotonin and dopamine is meaningful. Moreover, personality is a complex trait governed by a nexus of neurophysiological pathways and processes [27]. The connection between *CYP2D6* and other key genes also participating in the development of anxious and depressive traits may be "lost" in certain ethnic groups, making its effects undetectable.

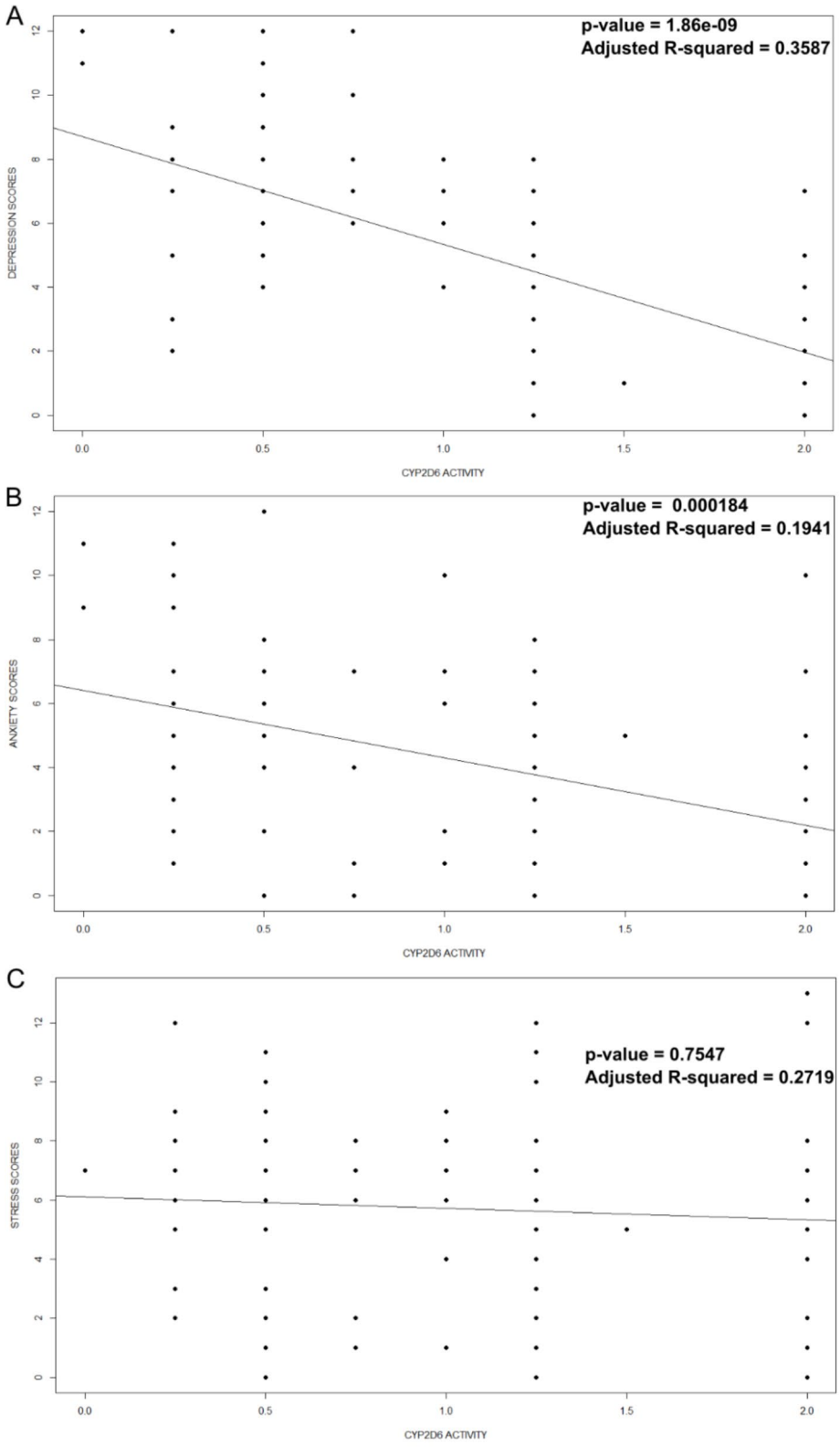

Fig. 2 Linear regression was performed to determine the association between CYP2D6 activity and (**A**) depression, (**B**) anxiety, and (**C**) stress scores

Conclusions

Our study adds to the mixture of evidence supporting or rejecting the hypothesis that CYP2D6 activity impacts susceptibility to anxiety and depression. We hope this brief report will prompt larger-scale follow-up studies to elucidate the contribution of CYP2D6 to the genetic underpinnings of mental well-being. We suggest that whole-genome or -exome sequencing, combined with focused assessments of anxious and depressive traits, may constitute a fruitful avenue of analysis.

Study limitations

A major limitation of our study is the small sample size. Detailed subgroup analyses could not be conducted to explore the influence of demographic characteristics such as age, a known risk factor for anxiety and depression [28]. Additionally, the comorbidities were self-reported by the participants and were not confirmed through on-site examination or diagnosis. However, high levels of agreement have been reported previously between self-reported comorbidities and data obtained from medical records and hospital registers [29].

Abbreviations

CYP2D6	Cytochrome P450 2D6
DASS	Depression, Anxiety and Stress Scales
DNA,	Deoxyribonucleic acid
PCR	Polymerase chain reaction
kb	Kilobase
dNTPs	Deoxynucleotide triphosphates
bp	Base pair
CPIC	Clinical Pharmacogenetics Implementation Consortium
BMI	Body mass index

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13104-025-07156-9.

Supplementary Material 1: **Supplementary Table 1**: Prior studies that examined the relationship between CYP2D6 metabolic status and personality traits or susceptibility to stress, anxiety, and depression.

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Author contributions

EWC conceived and designed the experiments, contributed research materials, analyzed the data, and wrote the final draft of the manuscript submitted for review and publication. MRAM assembled the original cohort from which a subset of participants was selected for ***CYP2D6*** genotyping. PYN and KY collected the blood samples and extracted DNA. HK performed the long PCRs, helped analyze the data, and wrote the initial manuscript draft. SM, PSK, and MK sequenced the PCR products and processed the resultant data for downstream analysis.

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Data availability

The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (GSA-Human: HRA007810) and are publicly accessible at https://ngdc.cncb.ac.cn/gsa-human.

Declarations

Ethics approval and consent to participate

The study was approved (UKM PPI.800-1/1/5/JEP-2019-391) by the Research Ethics Committee UKM (Human). Informed consent was obtained from all participants. The study was performed in accordance with the Declaration of Helsinki.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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