Polymorphisms in *CAMKK2* may Influence Domain-Specific Neurocognitive Function in HIV+ Indonesians Receiving ART

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Background: Despite effective antiretroviral therapy (ART), milder forms of HIV-associated neurocognitive disorders remain prevalent and are characterized by neuroinflammation, synaptic dysfunction, and neuronal loss.

Methods: We explore associations between neurocognitive impairment in HIV+ Indonesians and 17 polymorphisms in adjacent genes involved in inflammation and neuronal growth/repair pathways, *P2X4R* and *CAMKK2*. HIV+ Indonesians (n = 59) who had received ART for 12 months were assessed to derive Z-scores for the attention, fluency, memory, executive, and motor speed domains relative to local control subjects. These were used to determine total cognitive scores.

Results: No alleles of *P2X4R* displayed significant associations with neurocognition in bivariate or multivariable analyses. In *CAMKK2*, rs2686344 influenced total cognitive scores in bivariate analyses (P = 0.04). Multivariable linear regression modeling independently associated rs2686344 with higher executive function Z-scores (P = 0.05) after adjusting for CD4 T-cell counts (adjusted R^2 = 0.103, model P = 0.034), whereas rs1653588 associated with lower and rs1718120 (P = 0.05) with higher fluency Z-scores (P = 0.05) after adjusting for education and $\log_{10}$ HIV RNA copies/mL (adjusted R^2 = 0.268, model P = 0.001).

Conclusions: Polymorphisms in *CAMKK2* may influence neurocognitive outcomes in specific domains in HIV+ Indonesians receiving ART for 12 months.

Key Words: HIV, neurocognitive impairment, P2X4R and CAMKK2, single nucleotide polymorphisms, Indonesia

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INTRODUCTION

Despite effective antiretroviral therapy (ART), milder forms of HIV-associated neurocognitive disorders (HAND) remain prevalent[1,2] and can affect ability to work, quality of life, and adherence to ART. Approximately 50% of our cohort of HIV+ Indonesians beginning ART with <200 CD4 T cells/μl experienced HAND.[1,3] Neurocognitive function improved over 6 months at rates influenced by age, education, and/or CD4 T-cell counts, but memory function remained poor.[1,3] Neuroinflammation, synaptic dysfunction, and neuronal degeneration are hallmarks of HAND.[4] Accordingly, markers of microglial activation and synaptic dysfunction are associated with neurocognitive impairment in HIV+ individuals.[5,6] Purinergic P2X receptor 4 (P2X4R) and calcium/calmodulin-dependent kinase 2 (CaMKK2) are involved in neuroinflammatory and neuronal growth and repair pathways[7,8] and so may play a role in HAND.

P2X4R is an ATP-gated cation channel protein found abundantly in the central nervous system and highly expressed by microglia.[7] The stimulation of P2X4R drives an influx of calcium ions and activation of p38 mitogen-activated protein kinase (MAPK).[9] In microglia, p38 MAPK signaling regulates the expression of cytokines including interleukin 1-beta and tumor necrosis factor-alpha.[10] This can promote synaptic and neuronal loss in cocultured microglia and neurons treated with lipopolysaccharide.[10] In addition, cultured satellite glial cells from rat dorsal root ganglia treated with HIV envelope glycoprotein 120 exhibited decreased viability correlated with increased phosphorylation of p38 MAPK and production of interleukin 1-beta. These increases were attenuated by inhibition of P2X4R.[11]

CaMKK2 activates the AMP-activated protein kinase[8] which activates p38 MAPK pathways[12] and so has potential to elicit a cytokine response. However, CaMKK2 has a clearer neurological role, involved in synapse and dendrite development, axonal growth and repair, neuronal survival,

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J.G. conducted statistical analyses and wrote the manuscript. R.E. designed and conducted the neurocognitive assessments. D.D. assisted with neurocognitive assessments. S.H. performed the genotyping. P.K. provided statistical advice. P.P. coordinated the project.

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and learning and memory formation.[8] In cultured hippocampal neurons, CaMKK2 phosphorylates calcium/calmodulin-dependent kinase I (CaMKI), which phosphorylates and complexes with βPak-interacting exchange factor. In turn, this activates Rac1, a member of the Rho family GTPases which stimulates spine and synapse formation.[13] The inhibition of CaMKK2 or CaMKI decreased spine formation, and silencing of CaMKK2 impaired maturation of spines, thus inhibiting synapse formation.[13]

P2X4R and *CAMKK2* are adjacent genes in a region of high linkage disequilibrium.[14] We have associated single nucleotide polymorphisms (SNPs) in *P2X4R* and *CAMKK2* with altered concentrations of tumor necrosis factor-alpha in vitro[15] and with HIV-associated sensory neuropathy (HIV-SN) affecting peripheral nerves.[14,16,17] SNPs were selected based on (in order) exonic location, published links with inflammatory/neurological diseases, location in proximal untranslated regions, and presence in more than 1 human population. A *CAMKK2* intronic SNP, rs1063843, was associated with decreased expression of CaMKK2 in the dorsolateral prefrontal cortex, deficits in working memory and executive function, and increased risk of schizophrenia.[18] Here, we use multivariable regression models to address the hypothesis that associations between polymorphisms in *P2X4R* and/or *CAMKK2* influence cognitive function in HIV+ Indonesians treated with ART for 12 months. Because HIV does not affect neurocognitive domains equally, this may illuminate which pathways are affected by *P2X4R* and *CAMKK2*.

MATERIALS AND METHODS

Eighty-two HIV+ Indonesians were recruited at Cipto Mangunkusumo National General Hospital in Jakarta, Indonesia.[1,3] Participants were ART-naive, had <200 CD4 T cells/μl, a Karnofsky performance score of 70–100, and were living in Jakarta. The exclusion criteria included history of recurrent seizures, severe depression, stroke, head injury, neurological deficits which may interfere with neurocognitive evaluation, pregnancy, breastfeeding, and current use of illicit drugs. An additional 82 age-matched, gender-matched, and education-matched local healthy controls were recruited with the same criteria plus no declared HIV risk behavior. Patients were assessed for pulmonary tuberculosis, plasma HIV RNA was quantitated using COBAS AmpliPrep/COBAS TaqMan HIV-1 Tests (version 2.0), and CD4 T-cell counts were determined using standard flow cytometric techniques. All participants provided written and informed consent, and the study was approved by the Human Research Ethics Committees of the Faculty of Medicine Universitas Indonesia, Cipto Mangunkusumo National General Hospital, and Curtin University.

Neurocognitive assessments over 5 domains[19] were completed at baseline and after 12 months on ART for HIV+ participants and once for healthy controls to establish demographically adjusted normative values.[1,3] In brief, attention was assessed using the Forward Digit Span, fluency by the Animal Naming Test, memory by the Rey Auditory Verbal Learning Test (immediate recall, delayed recall, and learning over trials), executive function by the Trail Making Test A and B, and motor speed by the Grooved Pegboard test. The test results were subtracted from the mean normative value and then divided by the SD of the normative value to calculate Z-scores for each domain. The average of the Z-scores for each domain was calculated to derive the total cognitive function Z-scores. Of the 82 participants, 61 completed baseline and follow-up neurocognitive assessment after 12 months of ART. DNA samples were available for 59 of the 61 participants for genotype analyses.

The 59 DNA samples were diluted to 50 ng/μL and mixed with TaqMan OpenArray Genotyping Master Mix at a 1:1 ratio. Samples were genotyped for 17 SNPs across *P2X4R* and *CAMKK2* with custom TaqMan OpenArray Real-Time PCR Plates on the QuantStudio 12K Flex Real-Time PCR System (Life Technologies, NY). Genotypes were determined manually using TaqMan Genotyper Software. Four SNPs from *P2X4R* (rs2303998, rs10849860, rs11608486, and rs7961979) and 3 SNPs from *CAMKK2* (rs11065502, rs3714454, and rs3817190) were carried by less than <10% of participants or failed to genotype in more than 10% of samples and so were excluded from analyses. *P2X4R* (rs25643) and *CAMKK2* (rs1560568) were excluded because they were coinherited with tested SNPs (rs7298368 and rs7975295, respectively).

Z-scores of individuals carrying 1 or 2 copies of the minor alleles were compared with Z-scores of individuals with homozygous carriage of the major allele (dominant models; see Table 1, Supplemental Digital Content, http://links.lww.com/QAI/B737) with the Wilcoxon rank-sum tests in the R statistical programming environment (v1.3.959).[20] No corrections were made for multiple comparisons. Linear regression modeling was completed using the "olsrr" v0.5.3 package for R[21,22] and included variables previously associated with neurocognitive scores [age, CD4 T cells/μl, and years of education[1]], $\log_{10}$ HIV RNA copies/mL, plus SNPs from *P2X4R* or *CAMKK2*. Optimal models were determined for each domain using backward elimination until only variables achieving $P < 0.1$ remained.[22] Only models achieving an adjusted R-squared (R^2) ≥0.1, a model $P < 0.05$, and with at least 1 SNP are discussed.

RESULTS AND DISCUSSION

The demographic, clinical, and neurocognitive outcomes in this subset of 59 participants reflects the parent cohort previously described.[1,3] The median (range) age was 31 (19–48) years, education completed was 12 (6–16) years, CD4 T-cell counts were 288 (44–763) cells/μL, and $\log_{10}$ plasma HIV RNA was 1.30 (1.30–6.32) copies/mL after 12 months on ART (see Table 2, Supplemental Digital Content, http://links.lww.com/QAI/B737). Pulmonary tuberculosis was identified in 27 of 59 participants (46%) at baseline but did not influence Z-scores after 12 months of ART [*P* values = 0.20 to 0.92; Supplemental Digital Content (see Table 2, http://links.lww.com/QAI/B737)].

P2X4R SNPs were not Retained in Linear Regression Models

No alleles of *P2X4R* achieved $P < 0.05$ in bivariate analyses for any domain or total cognitive function after 12 months of ART (Table 1). Optimal linear regression models were based on age, CD4 T-cell counts, $\log_{10}$ HIV RNA copies/mL, education, and did not retain either SNP (see Table 3, Supplemental Digital Content, http://links.lww.com/QAI/B737). This suggests a limited role for *P2X4R* in HAND. However, we only tested 2 SNPs, and it is pertinent that animal and *in vitro* studies suggest a role for P2X4R in neuropathic pain, memory loss and anxiety, and synaptogenesis.[23–25] We were able to validate the effect of rs7298368 in Australian patients with HIV.[26] They were men (n = 72), aged 55 (42–74) years and of European descent, and had been on ART for more than 2 years.[26] We identified significant effects on mean T-scores for mental flexibility [51.1 (29–78) vs 47.9 (24–68), $P = 0.01$] and verbal fluency [54.1 (33–69) vs 47.7 (36–64), $P = 0.03$] (unpublished data). Genetic studies of other *P2X4R* SNP in larger cohorts should consider ethnicity because we have shown that this has clear effects on linkage disequilibrium influencing the haplotypes that predominate in the populations.[14,16,17]

Fluency and Executive Function Domain Z-scores were Influenced by SNP in *CAMKK2*

In bivariate analyses, 5 *CAMKK2* SNPs achieved $P = 0.06$–0.18 in at least 1 of the 5 domains or total cognitive function. rs2686344 associated significantly with total cognitive function ($P = 0.04$; Table 1). Of the 5, rs11065504 and rs7975295 associated with a reduced risk of HIV-SN in Africans[17] and Indonesians[16] treated without stavudine (respectively).

Models for 2 domains retained *CAMKK2* SNP and achieved an adjusted $R^2 > 0.1$ and model $P < 0.05$ (Table 2). Those failing these criteria are presented but are not discussed further. The optimal model for fluency (adjusted $R^2 = 0.268$, $P = 0.001$) included education, $\log_{10}$ HIV RNA copies/mL, rs1653588, and rs1718120. The minor allele of rs1653588 associated with poor fluency ($P = 0.04$; Table 2) and the minor allele of rs1718120 associated with higher fluency Z-scores ($P = 0.05$; Table 2). rs1653588 is a noncoding variant located in the 3-prime untranslated region of *CAMKK2*, so carriage of this allele may affect neurocognitive function through altered expression of *CAMKK2* or neighboring genes. The Genotype-Tissue Expression (GTEx) Portal (version 8) is an online reference resource that reports associations between gene expression levels and genotype in nondiseased human tissue. The GTEx Portal associates rs1653588*A with altered *P2X4R* expression and splice variants (https://gtexportal.org/; accessed November 2020).

The optimal model for executive function retained rs2686344 and rs1718120 after adjusting for CD4 T-cell counts (Table 2). Carriage of the minor alleles of both SNPs was linked with higher Z-scores. rs2686344 is an intronic variant associated with an altered *CAMKK2* expression in the GTEx Portal (https://gtexportal.org/) but yields conflicting results. Here, it approached significant associations with fluency and total cognitive Z-scores and not executive function in bivariate analyses (Table 1), so interactions with clinical factors may be important. rs2686344 associated with lower rates of peripheral neuropathy in Africans treated with stavudine but showed no effect in Africans and Indonesians treated without stavudine.[14,16,17] Stavudine probably inhibits mitochondrial function in peripheral nerves, so the ART regimen

TABLE 1. rs2686344 in *CAMKK2* Associated With Total Cognitive Function in HIV+ Indonesians After Receiving ART for 12 months

		Cognitive Domain					
	MAF	**Attention**	**Fluency**	**Memory**	**Executive**	**Motor Speed**	**Total**
Z-scores median (range)	—	−0.11 (−1.94 to 2.23)	−0.10 (−2.48 to 3.48)	−2.72 (−6.19 to 1.25)	0.93 (−2.88 to 1.57)	0.65 (−2.82 to 1.75)	−0.25 (−2.05 to 1.17)

		P* values for the Mann–Whitney *U* tests					
	MAF	**Attention**	**Fluency**	**Memory**	**Executive**	**Motor Speed**	**Total**
P2X4R SNP ID minor/major allele							
rs2686387 G/C	0.43	**0.20**†	0.47	0.90	0.43	0.27	0.99
rs7298368 T/C	0.45	0.43	0.56	0.79	0.87	**0.09**	0.88
CAMKK2 SNP ID minor/major allele							
rs1653587 T/A	0.09	0.38	0.48	0.79	0.62	0.52	0.54
rs1653588 A/T	0.08	0.77	**0.11**	0.85	0.87	0.79	**0.15**
rs11065504 C/G	0.32	0.27	0.80	0.28	0.71	**0.18**	0.83
rs7975295 C/T	0.32	0.92	0.83	**0.17**	**0.16**	0.97	0.39
rs2686344 T/C	0.24	0.93	**0.06**	0.23	0.21	1.00	*0.04*‡
rs1718120 G/T	0.26	**0.10**	**0.12**	**0.17**	**0.12**	**0.12**	0.45

*Dominant model; heterozygous or homozygous minor allele versus homozygous major allele.
†Variables achieving $P < 0.20$ are bolded.
‡Variables achieving $P < 0.05$ are italicized.
MAF, minor allele frequency.

TABLE 2. SNP in *CAMKK2* Differentially Influence Neurocognitive Outcomes After 12 months of ART

Variable	β*	95% CI		*P*
		2.5%	97.5%	
Attention: adjusted $R^2 = 0.050$, model $P = 0.054$				
rs1718120	0.47	−0.01	0.94	0.05
Fluency: adjusted $R^2 = 0.268$, model $P = 0.001$†				
Education	0.20	0.08	0.32	0.002
HIV RNA copies/mL	−0.24	0.05	0.29	0.10
rs1653598	−0.93	−1.79	−0.06	0.04
rs1718120	0.65	−0.01	1.32	0.05
Memory: adjusted $R^2 = 0.103$, model $P = 0.018$				
Age	−0.09	−0.16	−0.02	0.02
HIV RNA copies/mL	−0.35	−0.74	0.03	0.07
Executive: adjusted $R^2 = 0.103$, model $P = 0.034$†				
CD4 T cells/μl	−0.001	−0.002	0.000	0.06
rs2686344	0.39	0.01	0.77	0.05
rs1718120	0.36	−0.01	0.74	0.06
Motor speed: no individual variables achieved $P < 0.1$				
Total cognitive: adjusted $R^2 = 0.119$, model $P = 0.011$				
Age	−0.03	−0.06	−0.01	0.02
HIV RNA copies/mL	−0.14	−0.29	0.01	0.08

*β represents the regression coefficient.
†Models achieving study criteria (Adjusted R2 ≥0.1, model $P<0.05$ and includes at least one SNP)
CI, confidence interval.

may be important. We sought validation of associations between rs2686344 and cognitive deficits in the Australian patients with HIV as described above. These individuals were tested in 2009–2011, so some had used stavudine or related drugs at some time.[26] Carriage of the minor allele associated with higher T-scores for speed information processing [51.9 (42–66) vs 47.6 (17–69), $P = 0.038$], with a marginal effect on verbal memory [44 (10–63) vs 49.5 (23–61), $P = 0.11$] (unpublished data).

rs1718120 displayed weak links with Z-scores in all 5 domains in bivariate analyses (Table 1) and was included in the optimal models for fluency and executive function (Table 2). The association with carriage of the minor allele in the optimal models was positive but weak ($0.05 < P < 0.10$). rs1718120 is intronic and has been associated with an altered expression of *CAMKK2* and the upstream gene *ANAPC5* (https://gtexportal.org/). We associated SNP in *ANAPC5* with large fiber neuropathy in HIV+ Indonesians.[27] *ANAPC5* encodes a subunit of the anaphase-promoting complex (APC) which initiates cell progression from metaphase into anaphase. Replication of neurons would disturb signal transmission, so aberrant reentry into the cell cycle elicits apoptosis.[28] Accordingly, Almeida et al[28] demonstrated that dysregulation of APC pathways in cultured primary rat cortical neurons triggers apoptotic neuronal death.

The exploratory nature of this study and the modest number of participants limited our ability to assess rare SNP and correct for multiple comparisons. Nonetheless, our study identifies SNP in *CAMKK2* which may contribute to HIV-associated neurocognitive impairment in specific domains. Overall, these results suggest a role for *CAMKK2* in neurocognitive impairment in HIV+ individuals and warrant further investigation.

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