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Adiponutrin and Adiponectin Gene Variants in Indonesian Patients with Non-Alcoholic Fatty Liver Disease: a Preliminary Study

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Abstract

Background: Variants of adiponutrin *(PNPLA3)* and adiponectin *(ADIPOQ)* genes were considered to be associated with non-alcoholic fatty liver disease (NAFLD). Although the prevalence of NAFLD is increasing, there are limited numbers of studies about the association in Indonesian population.

Objective: To confirm that specific variants of *PNPLA3* and *ADIPOQ* in Indonesian patients are associated with NAFLD.

Methods: Data and DNA of 152 participants were obtained from a previous study in Dr. Kariadi Hospital, Semarang, Indonesia. PCR-RFLP analysis was performed for detection of *PNPLA3* rs738409 and *ADIPOQ* rs2241767 variants. The diagnosis and severity of NAFLD were assessed according to NAFLD activity score (NAS) based on histopathology assessment of liver biopsy.

Results: Allele G of *PNPLA3* rs738409 was associated with NAFLD in both bivariate (p=0.009, OR 2.52, CI 95% 1.25–5.07) and multivariate (p=0.008, OR 2.62, CI 95% 1.29%–5.32%) analysis, while *ADIPOQ* rs2241767 had no significant association. In NAFLD participants, both genotypes showed allele G was higher in the group of possible non-alcoholic steatohepatitis (NASH) – NASH (NAS >2) than in the simple steatosis group (NAS ≤2) i.e. 40.0% vs. 3.75% for the rs2241767 variant and 23.75% vs. 1.25% for the rs738409 variant, without significant association.

Conclusion: Variant *PNPLA3* rs738409 was associated with NAFLD incidence in studied population. Among NAFLD participants, the frequency of both variants were found higher in the possible NASH – NASH group, yet needs to be confirmed with more participants and a multicenter study.

Keywords: *Non-alcoholic fatty liver disease; adiponectin; adiponutrin; Indonesia*
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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common type of chronic liver injury in many countries.[1] The prevalence of NAFLD increased variably from 9–40% in Asia and Western countries.[1,2] The spectrum of NAFLD ranges from slowly progressive simple hepatic steatosis to non-alcoholic steatohepatitis (NASH) and hepatic cirrhosis.

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NASH increases liver related mortality and is considered as a progressive liver disease. Approximately 25-35% of NASH develops fibrosis and is more likely progressing to hepatic cirrhosis, hepatoma and liver failure.[3–5] The NAFLD activity score (NAS) is commonly used to diagnose and grade NAFLD based on the histopathological assessment and is considered as the gold standard for NAFLD diagnosis. It could distinguish NASH from simple steatosis and predict the prognosis of the disease.[6–8]

The pathogenesis of NAFLD is explained as 'multiple parallel hits hypothesis', in which insulin resistance, nutritional factors, gut microbiota, genetic, and epigenetic factors take part.[9] There are candidates genes involved in the NAFLD pathogenesis, which includes gene encoding adiponutrin and adiponectin.[10–12] Adiponutrin, encoded by *PNPLA3*, has triacylglycerol lipase and acylglycerol O-acyltransferase activities that involve TAG hydrolysis and the acyl-CoA independent transacylation of acylglycerols,[10] it maintains the triglyceride balance in the liver Variant *PNPLA3* rs738409 causes triglyceride accumulation in the liver, which accelerates the progression of NAFLD.[13]

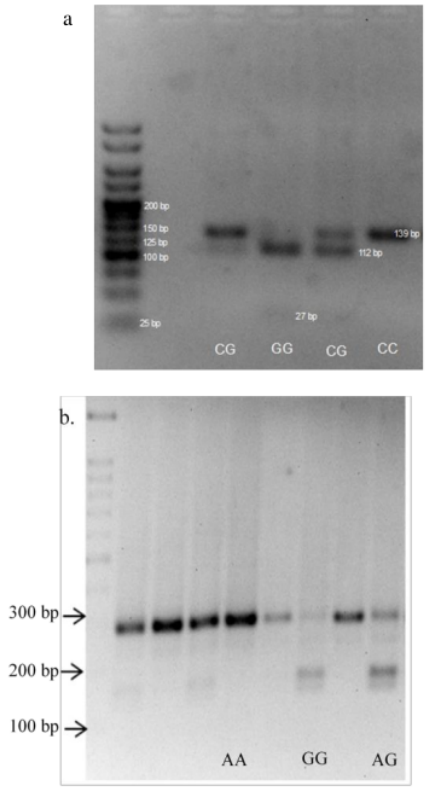

Figure 1. Electrophoresis of Restriction Fragments. PCR product of *PNPLA3* rs738409 was cut into 112 bp and 27 bp fragments (a). While PCR product of *ADIPOQ* rs2241767 was cut into 163 bp and 129 bp fragments (b).

Adiponectin, encoded by *ADIPOQ*, has anti-atherogenic, anti-diabetic and anti-inflammatory effects which is involved in pathogenesis of NAFLD.[6,12,14] A systemic low adiponectin levels was associated with the development of NAFLD,[15–17] and was considered as a diagnostic test severity of NAFLD.[5,15] Variant *ADIPOQ* rs2241767 was reported as a risk factor of susceptibility and progression to NAFLD for it is involved in adiponectin dysregulation.[3,18,19] However, the findings from previous studies on *ADIPOQ* rs2241767 variant were inconsistent and inconclusive.

A previous study in Indonesian population reported that low level of adiponectin plasma was an independent risk factor for NAFLD incidence and severity,[15] yet there was lack of data of the association of rs2241767 and rs738409 with NAFLD in this population. Since the prevalence of NAFLD in Indonesia is considered high, further research of these variants is needed. This study aimed to confirm whether *PNPLA3 rs738409* and *ADIPOQ* rs2241767 variants are associated with NAFLD in Indonesian patients.

MATERIALS AND METHODS

A total of 152 participants were included in this study, 80 patients with NAFLD and 72 were controls. The inclusion criteria of case and control group were listed in the previous study.[15] Participants were classified as NAFLD according to NAS as per NASH Clinical Research Network (NASH CRN) system based on histopathology of liver. Score of NAS ≤2 indicates 'simple steatosis' or 'no NASH', score 3–4 indicates 'possible NASH', and score ≥5 is indicating 'NASH'.[20]

Participants were Indonesian patients, predominantly Javanese. Liver biopsy procedures and other data collections were done at the Dr. Kariadi Hospital Semarang. DNA was obtained from a previous study that used salting out method,[15] while the genotyping was analysed at the Center for Biomedical Research of Universitas Diponegoro. This study was approved by the Human Research Ethics Committee of the Faculty of Medicine Universitas Diponegoro–Dr. Kariadi Hospital Semarang (No.128/EC/FK- RSDK/2014).

The *ADIPOQ* rs2241767 variant (A > G) and *PNPLA3* rs738409 variant (C > G) were genotyped by polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) method according to Li et al and Islek et al.[3,21] The PCR reaction for determination of rs2241767 was pre-degenerated at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, elongation at 72°C for 30 s, final elongation at 72°C for 10 min, and the PCR reaction for determination of rs738409 was pre-degeneration at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 60 s, annealing at 58°C for 30 s, elongation at 72°C for 60 s, final elongation at 72°C for 10 min. The primers were purchased from IDT (Singapore), while restriction enzymes were from NEB (Ipswich, MA, USA). PCR reagent for DNA optimation was MyTaq Mix (Bioline, Singapore). The PCR products were run on 1.5% agarose gels electrophoresis containing 0.1% FloroSafe DNA Stain (1st Base, Singapore) and was analyzed using Gel Doc (Bio-Rad Laboratories, USA). PCR method of rs2241767 showed a 292 bp product of AA genotype, two fragments of 163 and 129 bp product of GG genotype, and three fragments of 292 bp in heterozygous (A/G) genotype participants. PCR method of rs738409 showed a 139 bp product of CC genotype, two fragments of 112 and 27 bp products of GG genotype, and three fragments of 139 bp in heterozygous (C/G) genotype participants. The details of primers, enzymes, PCR product and fragment lengths were shown in Table 1.

To analyze the equilibrium and errors in genotyping, Hardy-Weinberg Equilibrium Test was done for both rs738409 and rs2441767. Bivariate and multivariate analyses of the correlation between genotype and NAFLD were done using SPSS software version 20.0 (Polar Engineering and Consulting, USA). The age of participants was categorized into 3 groups; age 20–40, age 41–60, and age more than 60 years old. To analyse the correlation between genotype and NAFLD severity; simple steatosis, possible NASH, and control participants were sorted as non-NASH. Simple steatosis and possible NASH participants were sorted as NAFLD non-NASH. Differences with a p value of <0.05 were considered statistically significant.

RESULTS

The age of NAFLD participants ranged from 23 to 74 years old with a mean age of 45.52 (1.16) years, while control participants ranged from 20 to 72 years old with a mean age of 44.54 (1.18) years. The result of Hardy-Weinberg Equilibrium test for both rs738409 and rs2441767 showed no significant different (p=0.85 and

Table 1. Primers, Enzymes, and PCR Product and Fragment Lengths

Variant	Primer	Enzyme	PCR Product (bp)	Fragment Length (bp)
rs2241767	F: GGTGAGAAGGGTGAGAAAGGAG R: TACTGGGAATAGGGATGAGGG	Bsu36I	292	163 + 129
rs738409	F: CCCTGCTCACTTGGAGAAAG R: AGGAGGGATAAGGCCACTGT	NlaIII	139	112 + 27

Table 2. Genotype Distribution of rs2241767 and rs738409 Based on Gender

	rs2241767					rs738409				
	Genotypes (%)			Alleles (%)		Genotypes (%)			Alleles (%)	
	AA	AG	GG	A	G	CC	CG	GG	C	G
Total (n=152)										
NAFLD	60 (75.0)	19 (23.8)	1 (1.3)	139 (86.9)	21 (13.1)	45 (56.2)	32 (40.0)	3 (3.8)	122 (76.2)	38 (23.8)
Control	48 (66.7)	24 (33.3)	0 (0.0)	120 (83.3)	24 (16.7)	55 (76.4)	15 (20.8)	2 (2.8)	125 (86.8)	19 (13.2)
Men (n=84)										
NAFLD	37 (75.5)	12 (24.5)	0 (0.0)	86 (87.8)	12 (12.2)	30 (61.2)	17 (34.7)	2 (4.1)	77 (78.6)	21 (21.4)
Control	21 (60.0)	14 (40.0)	0 (0.0)	56 (80.0)	14 (20.0)	28 (80.0)	7 (20.0)	0 (0.0)	63 (90.0)	7 (10.0)
Women (n=68)										
NAFLD	23 (74.2)	7 (22.6)	1 (3.2)	53 (85.5)	9 (14.5)	15 (48.4)	15 (48.4)	1 (3.2)	45 (72.6)	17 (27.4)
Control	27 (73.0)	10 (27.0)	0 (0.0)	64 (86.5)	10 (13.5)	27 (72.9)	8 (21.6)	2 (5.4)	62 (83.8)	12 (16.2)

Table 3. Bivariate Analysis of rs2241767 and rs738409

	rs2241767				rs738409			
	AG+GG	AA	P	OR (CI95%)	CG+GG	CC	P	OR (CI95%)
Group								
Case	20	60	0.26	0.67	35	45	0.009	2.52
Control	24	48		(0.33-1.35)	17	55		(1.25-5.07)
Gender								
Men	26	58	0.55	1.25	26	58	0.39	0.72
Women	18	50		(0.61-2.53)	26	42		(0.37-1.42)
Age								
20-40	17	34		Reference	15	36		Reference
41-60	26	66	0.53	0.79 (0.38-1.65)	34	58	0.36	1.41 (0.67-2.94)
>60	1	8	0.21	0.25 (0.03-2.16)	3	6	0.81	1.20 (0.26-5.44)
Severity 1								
Simple Steatosis	1	10		Reference	3	8		Reference
Possible NASH	9	31	0.34	2.90 (0.37-25.82)	20	20	0.19	2.67 (0,62-11.54)
NASH	10	19	0.14	5.26 (0.59-47.20)	12	17	0.41	1.88 (0.41-8.60)
Severity 2								
NASH	10	19	0.30	1.38	12	17	0.24	1.46
Non NASH	34	89		(0.59-3.26)	40	83		(0.64-3.36)
Severity 3								
NASH	10	19	0.18	0.46	12	17	0.47	1.16
NAFLD non NASH	10	41		(0.16-1.3)	23	28		(0.46-2.93)

p=0.71, respectively). Electrophoresis of restriction fragments of both variants is shown in Figure 1. The frequency of both genotypes is summarized in Table 2.

Allele G in rs2241767 was found higher in men and in the group of 20-40 years old (30.95% and 33.33%, respectively), while allele G in rs738409 was found higher in women and in the group of 41-60 years old (38.24% and 36.96%, respectively), despite no statistically significant association. A significant association was identified between variant rs738409 and NAFLD (p=0.009, OR 2.52, CI 95% 1.25-5.07) (Table 3). In NAFLD participants, both genotypes showed allele G was found higher in the group of possible NASH – NASH (NAS >2) than in the simple steatosis group (NAS ≤2) (40.0% vs. 3.75% for rs2241767 variant and 23.75% vs. 1.25% for rs738409 variant), without significant association (p>0.05). In multivariate analysis, there was significant association between variant rs738409 and NAFLD (p=0.008, OR 2.62, CI 95% 1.29% - 5.32%) (Table 4).

DISCUSSION

Variants in certain genes were reported to play a role in NAFLD occurrence and severity.[5,6] A previous study documented that metabolic syndrome, insulin resistance, and low adiponectin levels were independent risk factors for the incidence of NAFLD.[15] By observing the same participants, this study analyzed the data including the severity degree of NAFLD confirmed by liver biopsy, and *PNPLA3* and *ADIPOQ* variants as susceptibility factors for NAFLD and NASH.[5,6] To the best of our knowledge this is the first study in Indonesia with predominantly Javanese population.

Previous studies showed a significant association between *ADIPOQ* rs2241767 and NAFLD.[3,19] This current study noted that the allele G of variant *ADIPOQ* rs2241767 was found in 13.1% NAFLD participants in Semarang, Indonesia. Variant rs2241767 is an intron variant[22] which can influence gene expression in various ways, including mechanisms through splicing, transcription, polyadenylation, mRNA export, translation, and specific features that boost expression. Nevertheless, which predominate intron mechanism enhances or represses the expression of a gene is not yet known.[23] The mechanism of allele G of *ADIPOQ* rs2441767 in adiponectin dysregulation is also not clearly understood, although there are studies reporting positive effect of this variant and NAFLD.[3,19]

The *PNPLA3* rs738409 variant increases the risk of development and progression of NAFLD.[24,25] Consistent with previous findings, variant *PNPLA3* rs738409 in this study showed significant association with NAFLD incidence (p=0.009, OR 2.52, CI 95% 1.25-5.07).

This study found a higher frequency of allele G in *ADIPOQ* rs2241767 in men and in the group of 20-40 years old, while allele G in *PNPLA3* rs738409 was found higher in women and in the group of 41-60 years old, despite no significant association (p>0.05). It confirmed that sex is one of the NAFLD risk factors. Higher prevalence NAFLD rates were seen in men younger than menopause age in women. After menopause, the risk becomes comparable between both sexes, perhaps due to the effects of estrogen.[26,27] However, whether the age factor could affect the impact of variant *PNPLA3* on NAFLD prevalence is still unclear, as NAFLD could start since childhood.[28,29]

In this study, allele G of both variants was found higher in NASH participants, despite no statistically significant association (p>0.05). Previous results noted an association between variant *PNPLA3* rs738409 and NASH susceptibility,[30,31] The *PNPLA3* rs738409 allele was associated with adipocytes and increased leptin transcription in participants with NAFLD. Therefore, the adipose tissue endocrine modulation activity may be involved in mechanism of NASH susceptibility induced by *PNPLA3*.[24] On the other side, the association between variant *ADIPOQ* rs2241767 and NASH has not been reported. The factors behind the association between *ADIPOQ* rs2241767 and NASH remain unclear. It is not known yet whether homozygous or heterozygous alleles are involved in development of NAFLD, although this study documented higher heterozygous allele frequency in NAFLD participants.

By multivariate analysis, only variant rs738409 was found significantly associated with NAFLD (p=0.008, OR 2.62, CI 95% 1.29% - 5.32%). However, both genotypes showed allele G was found higher in group of possible NASH – NASH than in simple steatosis group, despite no significant association (p>0.05). Interestingly, there was a study documented *PNPLA3* rs738409 variant represented a genetic determinant of serum adiponectin levels.[24] Since systemic adiponectin levels considered involved in development of NAFLD[15–17] and variant *ADIPOQ* rs2241767 associated with risk of NAFLD,[3,4,6] further study is needed to confirm the findings. The potential limitation of this study is the relatively small sample size, therefore larger sample sizes and diverse ethnic groups are needed in future studies to confirm the present data.

CONCLUSION

In line with 'multiple parallel hits pathogenesis' of NAFLD, there are many variations of candidate genes which are considered related to NAFLD and its severity. Two suspected spots of genes considered involved in NAFLD development were observed in this study. As far as we know, this is the first study in Indonesia and confirmed the association between *PNPLA3 rs738409* and NAFLD in Indonesian participants. However, other spots and other genes are needed to be observed, as well as their association with clinical findings. As Indonesia has lots of ethnicities, a multicenter study is needed to collect larger sample sizes and more variation of participants.

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PAGE 1

PAGE 2

PAGE 3

PAGE 4

PAGE 5

PAGE 6