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Original Article

Genetic variation I148M in patatin-like phospholipase 3 gene and risk of non-alcoholic fatty liver disease among Filipinos

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Abstract: Genome-wide association studies have shown that a non-synonymous single nucleotide polymorphism characterized by a C-to-G change encoding an isoleucine-to-methionine substitution at amino acid position 148 in the human patatin-like phospholipase 3 (PNPLA3) gene was found to be associated with non-alcoholic fatty liver disease (NAFLD) and advanced liver damage. A hospital-based study was conducted to determine the distribution of PNPLA3 genotypes among patients clinically diagnosed and histologically confirmed with NAFLD and among normal controls. We also compared the allelic frequencies of PNPLA3 with different ethnic populations. More importantly, we evaluated the association between PNPLA3 genetic variation and risk of developing NAFLD among Filipinos. Real-time PCR was performed using the Taqman SNP genotyping assay for rs738409. Nucleotide sequencing was performed to confirm the PNPLA3 genotypes. Allelic frequencies among normal controls were 0.83 and 0.17 for the PNPLA3 C and PNPLA3 G alleles, respectively. Calculated frequencies in Hardy Weinberg Equilibrium were 72% for PNPLA3 C/C, 22% for PNPLA3 C/G, and 6% for PNPLA3 G/G genotype. There is a significant difference in the distribution of PNPLA3 genotypes between normal controls and NAFLD patients ($p = 0.0172$). However, there was no significant association found between PNPLA3 genotypes and risk of developing NAFLD after controlling for possible confounding effects ($p = 0.0574$). Allelic frequencies of PNPLA3 among Filipinos were statistically different from Hispanics, Japanese, and Han Chinese. In conclusion, genetic variation in PNPLA3 rs738409 C>G seems to be associated with NAFLD among Filipinos. Further studies are needed to replicate our observations in an independent larger population.

Keywords: Genetic variation, I148M, non-alcoholic fatty liver disease, patatin-like phospholipase 3 gene, rs738409 C>G, Filipino

Introduction

Non-alcoholic fatty liver disease (NAFLD) is an emerging health concern, with increasing prevalence worldwide. Several factors have been shown to associate with NAFLD such as obesity, type-2 diabetes, hyperlipidemia, and genetic variations [1-4]. The prevalence of NAFLD in the Philippines was approximately 12%. Female sex, obesity, and diabetes were the characteristic features found among Filipino NAFLD patients [5].

Recently, a non-synonymous genetic variation (rs738409) in the human patatin-like phospholipase domain-containing 3 gene (PNPLA3) that substitutes methionine for isoleucine (I148M) at amino acid position 148 in exon 3, was found to be associated with NAFLD among Hispanics, African Americans, and European Americans [6-8]. Studies have shown that the G allele (risk allele) of rs738409 in PNPLA3 gene was associated with increased propensity of steatosis and severe fibrosis. Moreover, the PNPLA3 rs738409 C>G genetic variation seemed to predispose liver fat deposition despite normal body mass index and absence of dyslipidemia [9-12].

Human PNPLA3 located at chromosome 22q13.31, also known as adiponutrin, encodes a 481-residue protein that is expressed in the

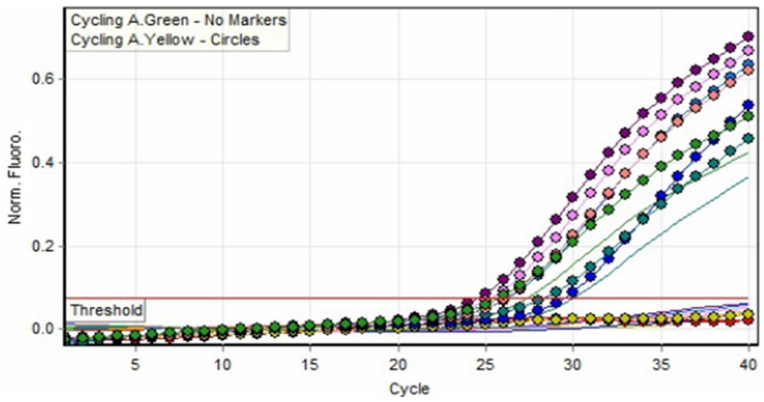

Figure 1. Genotyping of PNPLA3 rs738409 C>G by real-time PCR. Representative allelic discrimination for cycling A. Green FAM-No Markers (G allele, homozygous variant) and cycling A. Yellow VIC-Circles (C allele, homozygous wild-type).

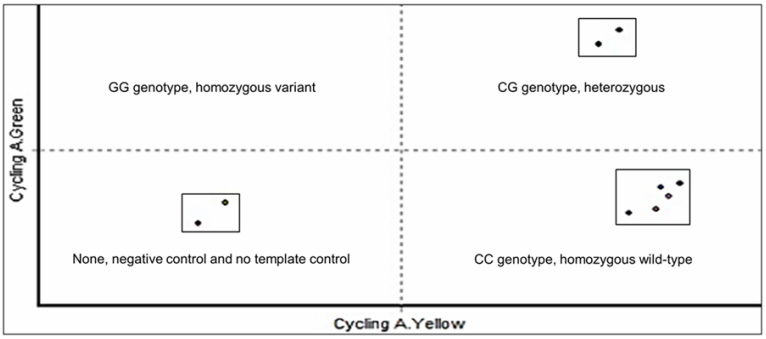

Figure 2. Genotyping of PNPLA3 rs738409 C>G by real-time PCR. Scatter graph for cycling A. Green FAM (G allele, variant) and cycling A. Yellow VIC (C allele, wild-type). Fluorescence was measured at 470 nm (FAM) and 530 nm (VIC). C/C genotype, homozygous wild-type; C/G genotype, heterozygous; G/G genotype, homozygous variant; none, negative control and no template control.

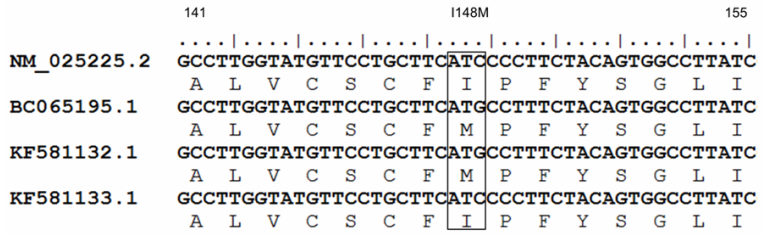

Figure 3. Sequence alignment of PNPLA3 rs738409 C>G samples. At the top, PNPLA3 reference sequences are given (GenBank accession numbers NM_25225.2, BC065195.1). On the left, the GenBank accession numbers are shown (this study). The key amino acid associated with isoleucine-to-methionine substitution (C-to-G change) at position 148 is boxed.

liver, as well as in adipose tissue, and has been shown to hydrolyze triglycerides [6, 13, 14]. The PNPLA3 I148M substitution results in a protein loss of enzyme activity.

The risk to develop hepatic steatosis varies among ethnic populations with Hispanics having a frequency of 45% as compared with European Americans, and African-Americans having a frequency of 33% and 24%, respectively [15-18]. In this study, we investigated the distribution of PNPLA3 genotypes among Filipinos and compared the frequencies with various ethnic populations. We also evaluated whether the PNPLA3 rs738409 C>G genetic variation could play a role in the development of NAFLD. Understanding the prevalence of PNPLA3 genetic variation among various ethnic populations could provide useful information for the improvement of care of patients at risk for developing hepatic steatosis and advanced liver damage.

Materials and methods

Samples and patients

Thirty-two samples collected from unrelated patients clinically diagnosed with NAFLD and 36 normal controls from January 2012 to December 2013 were analyzed. Study participants were enrolled from St. Luke's Medical Center-Quezon City. The diagnosis of NAFLD was based on histopathology. The steatosis grade and stage of fibrosis were graded by the pathologists according to metavir scoring. The age and sex of all the study participants were noted. Body mass index was calculated according to the measured height and weight at the time of recruitment. Peripheral blood samples were obtained from the study participants following an overnight fast (12 to 14 hours). Fasting blood sugar, high density lipoprotein cholesterol, low density lipoprotein cho-

Table 1. Distribution of PNPLA3 genotypes in normal and NAFLD patients

Genotype	NAFLD patients N = 32	Normal controls N = 36	Total N = 68	*p*-value
	N (%)	N (%)	N (%)	
C/G and G/G	18 (56)	10 (28)	28 (41)	0.0172
Wild-type C/C	14 (44)	26 (72)	40 (59)	
p	0.64	0.83		
q	0.36	0.17		

Abbreviations: PNPLA3, patatin-like phospholipase 3 gene; NAFLD, non-alcoholic fatty liver disease; C/G, heterozygous; G/G, homozygous variant; C/C, homozygous wild-type; p, frequency of allele C; q, frequency of allele G.

lesterol, triglyceride, alanine transaminase, and aspartate transaminase were measured using an automated analyzer. The control group was found to be non-reactive for hepatitis A, B, C, with normal biochemical tests, and with normal ultrasound. There were 21 (58%) males and 15 (42%) females with ages ranging from 21 to 54 years old. The study was approved by the St. Luke's Institutional Ethics Review Committee (Ethics Review Board Reference No. 12-002). All study participants gave written informed consent prior to enrollment upon referral to the St. Luke's Liver Disease and Transplant Center. All those who were clinically diagnosed with NAFLD were further requested to undergo liver biopsy to confirm the diagnosis. The benefits, risks and potential complications were discussed extensively by a medical team prior to the procedure. Patients who underwent liver biopsy were monitored closely during the peri-operative time by the attending consultant.

Genomic DNA extraction and genotyping of PNPLA3 by real-time PCR

The nucleic acid was extracted from peripheral blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) or the Taqman sample-to-SNP kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. The nucleic acid concentration and purity were measured using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). All samples were stored at -20°C until use. Real-time PCR was performed on the Rotor-Gene Q instrument (hold at 95°C for 20 sec, followed by 40 cycles of 95°C for 15 sec, and 60°C for 60 sec) with an SNP genotyping assay for rs738409. Negative controls and no template controls were included for every run to ensure the quality of genotyping results. Allelic discrimination and scatter plot analysis were performed using fluorogenic probes and 5' nuclease assay. Human PNPLA3 rs738409 is bi-allelic with three possible genotypes as follows: C/C, homozygous wild-type; C/G, heterozygous; and G/G, homozygous variant. The allelic and genotype frequencies of rs738409 were in Hardy-Weinberg Equilibrium (HWE). Biosafety measures were observed throughout the conduct of the study.

Direct nucleotide sequencing of PNPLA3 gene

The TaqMan assay was validated by direct nucleotide sequencing of randomly selected samples. PCR was performed by using the sense (5'-GCC CTG CTC ACT TGG AGA AA-3') and antisense (5'-TGA AAG GCA GTG AGG CAT GG-3') primers as previously described [18]. PCR was carried out using a gradient thermocycler (G-Storm, Essex, England) at 95°C for 5 min, 35 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, and a final elongation at 72°C for 10 min. The amplicons of PNPLA3 rs738409 were purified using the QIAquick PCR Purification kit (Qiagen, Hilden, Germany) and sequenced using Big Dye Kit (First BASE Laboratories, Selangor, Malaysia). Sequence data were edited and aligned with the reference sequences from NCBI using BioEdit version 7.1.3.0 software.

Nucleotide sequence accession numbers

The GenBank accession numbers of PNPLA3 rs738409 used in the analysis were NM_025225.2, and BC065195.1. The nucleotide sequences reported in this study have been deposited to NCBI and can be retrieved under GenBank accession numbers KF581132.1 to KF581133.1.

Statistical analysis

Data were analyzed using EpiInfo7, OpenEpi version 3.01, and EpiTools [19, 20]. The X^2 test was used to determine significant differences in the allelic frequencies as well as to determine differences in the demographic, clinical, and laboratory characteristics between NAFLD patients and normal controls. To compare means across groups, independent *t* test was used. HWE was tested using X^2 analysis [21]. In

Table 2. Allelic and genotype frequencies of PNPLA3 rs738409 C>G reported from various ethnic populations

Population	Allele			Genotype frequency (%)			Reference
	N	frequency	*p*-value	C/C	C/G	G/G	
Filipino	36	0.17		72	22	6	Present study
Italian	179	0.18	NS	66	31	3	[11]
Chinese	202	0.31	NS	47	45	8	[22]
Han Chinese	553	0.34	0.0355	42	47	11	[23]
Japanese	578	0.44	0.0015	30	52	18	[24]
White	328	0.27	NS	55	36	9	[25]
White	326	0.24	NS	58	36	6	[26]
Hispanic	30	0.50	0.0042	30	40	30	[26]
Black	38	0.13	NS	74	26	0	[26]
German	162	0.19	NS	64	34	2	[27]

Abbreviations: PNPLA3, patatin-like phospholipase 3 gene; C/C, homozygous wild-type; C/G, heterozygous; G/G, homozygous variant; NS, not significant.

this study, the C/G and G/G genotypes containing the risk allele (G) were grouped together as the risk genotype and compared with the C/C genotype. To determine the association between PNPLA3 genetic variation and risk of developing NAFLD among Filipinos, univariate analysis using X^2 and *t* test were used. In order to control for confounding effects of other variables that may be associated with the risk of developing NAFLD among Filipinos, multiple logistic regression was also done. A *p*-value value < 0.05 was considered statistically significant throughout the analysis.

Results

The PNPLA3 gene was successfully amplified by real-time PCR in all NAFLD patients and normal controls (**Figures 1** and **2**). DNA sequencing of randomly selected samples showed identical genotypes (**Figure 3**). **Table 1** shows the distribution of PNPLA3 genotypes among the study participants. Most individuals were found to be homozygous for the C/C genotype (72%). Twenty-two percent were genotyped as C/G, and 6% were G/G. There is a significant difference in the distribution of PNPLA3 genotypes between normal controls and NAFLD patients ($p = 0.0172$). The PNPLA3 genotype frequencies were consistent with HWE in the two groups. The allelic frequencies among normal controls were 0.83 and 0.17 for the PNPLA3 C and PNPLA3 G alleles, respectively. Among NAFLD patients, the allelic frequencies were 0.64 for the PNPLA3 C allele and 0.36 for the PNPLA3 G allele.

Table 2 shows the PNPLA3 allelic and genotype frequencies among various populations and the X^2 *p*-values resulting from the comparison of allelic frequencies between Filipinos and other ethnic populations. The result among Filipino normal controls was used for the purpose of comparison with other populations. Results showed that the allelic frequencies of PNPLA3 were statistically different from Hispanics ($p = 0.0042$), Japanese ($p = 0.0015$), Han Chinese ($p = 0.0355$) but not with Chinese, White, Italian, German, and Black populations.

The association of age, sex, body mass index, blood chemistry, PNPLA3 genotype of normal controls and NAFLD patients was examined. There is a significant difference in age distribution ($p \leq 0.001$) and PNPLA3 genotypes ($p = 0.0172$) but not in sex between the two groups (**Table 3**). The BMI, FBS, HDL-C, triglyceride, ALT and AST levels were also found to be significantly different between normal controls and NAFLD patients.

Logistic regression was done to control for possible confounding effect. However, due to the limited sample size of the dataset, only the effects of age, BMI, FBS, HDL-C, and triglyceride were included in the multiple logistic regression analysis of the association of the PNPLA3 genotype with the risk to have NAFLD. The analysis showed that BMI ($p = 0.0184$) and triglyceride ($p = 0.0362$) as the factors significantly affecting the risk to have NAFLD. The PNPLA3 genotype turned out to be not significant ($p = 0.0574$), although somehow near the borderline (**Table 4**).

The clinical and biochemical characteristics as well as histological findings of the 32 NAFLD patients were also investigated separately. No associations were found between PNPLA3 genotype and BMI, FBS, HDL-C, LDL-C, triglyceride, ALT and AST levels. Due to limited sample size,

Table 3. Clinical and laboratory characteristics of normal controls and NAFLD patients

Parameters	Normal control N = 36	NAFLD patient N = 32	*p*-value
	N (%)	N (%)	
Age			< 0.001
21-40	24 (67)	7 (22)	
41-70	12 (33)	25 (78)	
Sex			NS
Male	21 (58)	16 (50)	
Female	15 (42)	16 (50)	
PNPLA3 rs738409			0.0172
C/G and GG	10 (28)	18 (56)	
C/C	26 (72)	14 (44)	
	Mean (SD)	Mean (SD)	
BMI kg/m^2	22.1 (1.8)	28.50 (6.0)	0.0001
FBS (mg/dl)	89.6 (8.5)	109.7 (30.7)	0.0012
HDL-C (mg/dl)	58.3 (15.3)	44.0 (12.9)	0.0001
LDL-C (mg/dl)	123.0 (28.0)	125.8 (41.6)	NS
Triglyceride (mg/dl)	79.7 (48.0)	164.2 (69.0)	< 0.0001
ALT (U/L)	41.0 (13.4)	84.8 (44.3)	< 0.0001
AST (U/L)	20.2 (6.1)	44.5 (25.8)	< 0.0001

Abbreviations: BMI, body mass index; FBS, fasting blood sugar; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; ALT, alanine transaminase; AST, aspartate transaminase; PNPLA3, patatin-like phospholipase 3 gene; SD, standard deviation; NS, not significant.

statistical analyses for the degree of steatosis and fibrosis were not done. In the group of NAFLD patients, the results showed that there were no significant differences between genotypes, considering the factors that significantly affect the risk of having NAFLD.

Discussion

Based on current literature, the present study is the first to have documented the distribution of PNPLA3 alleles and genotypes among Filipinos, and the first to evaluate the association between PNPLA3 rs738409 C>G genetic variation in Filipino NAFLD patients. Previous studies have shown differences in PNPLA3 genotypes among various ethnicity and geographical location of the population. Particularly, the PNPLA3 allele has been reported at frequencies of 24% among White populations, and 13% among Black populations. The frequency of PNPLA3 rs738409 G allele was 0.31 among Chinese normal controls, 0.24 among Malay normal controls, and 0.18 among Indian normal controls. Other studies reported that the PNPLA3 allelic frequency among Italian, German, and Hispanic populations were 18%, 19% and 50%, respectively [11, 26-29]. Our data show that allelic frequency of PNPLA3 among Filipino normal controls was 17% and that majority of the study participants were homozygous (72%) for the C/C genotype. Differences in the allelic and genotype frequencies may reflect ethnic variety among various races [15, 29].

Traditionally, genotyping for single nucleotide polymorphism (SNP) has been analyzed by PCR-RFLP and visualized on agarose or polyacrylamide gels. However, this technique is time-consuming and its sensitivity relies on the quality of the amplicons, as well as the staining intensity of the digested PCR products. In this study, we have demonstrated that allelic discrimination for SNP in PNPLA3 rs738409 C>G using 5' nuclease assay with fluorogenic probes provides a rapid and sensitive method for detecting SNP. The isoleucine-to-methionine substitution at codon 148 of PNPLA3 was confirmed by direct nucleotide sequencing. Other high-throughput techniques used for SNP genotyping include iPLEX Sequenom MassARRAY platform and allele-specific PCR [9, 30].

Several associations of PNPLA3 rs738409 C>G genetic variations with NAFLD have been reported [8, 31-35]. This study cannot confirm previous findings that genetic variation in PNPLA3 is associated with NAFLD among Filipinos. Univariate analysis showed strong association of the genotypes with NAFLD. However, after controlling for the effects of possible confounding factors, the significant association could not be demonstrated. This does not mean that there is no genetic association between PNPLA3 and NAFLD. One possible explanation is that the genetic effect in this study population might be too small and cannot be demonstrated due to the limited sample size.

Our study has some other limitations. First, the study investigated the association between PNPLA3 genetic variation and NAFLD using hospital-based participants. Thus, further research will be necessary to replicate our observations in an independent larger population

Table 4. Association of PNPLA3 genotype with the risk of NAFLD

Term	OR	95%	C.I	Coefficient	S.E	Z-Statistic	*p*-value
CG or GG/CC	31.2045	0.8977	1084.6905	3.4406	1.8105	1.9004	0.0574
Age group	4.1483	0.2946	58.4056	1.4227	1.3494	1.0543	0.2917
BMI	2.5475	1.1711	5.5414	0.9351	0.3965	2.3583	0.0184
FBS	1.0569	0.9234	1.2098	0.0554	0.0689	0.8032	0.4219
HDL-C	0.8912	0.7453	1.0656	-0.1152	0.0912	-1.2628	0.2067
Triglyceride	1.0286	1.0018	1.0562	0.0282	0.0135	2.0943	0.0362
Constant	*	*	*	-28.6085	11.1006	-2.5772	0.0100

Abbreviations: BMI, body mass index; FBS, fasting blood sugar; HDL-C, high density lipoprotein cholesterol; OR, odds ratio; CI, confidence interval; SE, standard error.

considering all other possible confounding factors that might affect the result. Secondly, liver biopsies were not performed among normal controls due to ethical reasons. Lastly, the possible interactions of the PNPLA3 rs738409 C>G genetic variation and environmental factors such as tea drinking were not addressed in this study. Recent studies have shown that the risk of developing NAFLD in non-tea drinkers was 3-fold as compared with tea drinkers among individuals carrying the heterozygous C/G and homozygous G/G variant genotype [23].

In conclusion, there is a significant difference in the distribution of PNPLA3 genotypes between normal controls and NAFLD patients. Allelic frequencies of PNPLA3 among Filipinos were statistically different from Hispanics, Japanese, and Han Chinese. Genetic variation in PNPLA3 rs738409 C>G seems to be associated with NAFLD among Filipinos. Further studies are needed to replicate our observations in an independent larger population.

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Disclosure of conflict of interest

None.

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