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PNPLA3 GENE POLYMORPHISMS IN HCV/HIV COINFECTED INDIVIDUALS

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Abstract

BACKGROUND AND AIMS: The patatin-like phospholipase domain containing 3 (PNPLA3) gene has been associated with development of alcoholic and non-alcoholic steatohepatitis. Using a newly developed and validated assay for PNPLA3, we explored the prevalence of gene polymorphisms in a cohort of HCV/HIV coinfecting individuals to determine if there was an association with insulin resistance or hepatic fibrosis.

METHODS: A high-resolution melting point (HRM) assay was developed and validated. The assay was used to evaluate samples obtained in the context of a clinical trial performed at ACTG sites across the U.S. in HIV-infected patients. Clinical features and treatment outcomes were assessed in relation to the PNPLA3 genotype.

RESULTS: The HRM methodology demonstrated 100% concordance with results obtained by Sanger sequencing. Among 241 participants tested, 66.0% had the wildtype allele (CC) and the remainder had the aberrant PNPLA3 gene polymorphism in the homozygous (GG) or heterozygous (CG) form. Race and ethnicity were associated with PNPLA3 genotype but fibrosis stage, HOMA (Homeostatic Model Assessment of Insulin Resistance), and HCV treatment outcome was not.

CONCLUSION: The HRM method is an effective, rapid technique for characterizing PNPLA3 genotype. In those with HCV/HIV infection, nearly 40% carry gene polymorphisms associated with development of NASH or ASH. Prospective studies should focus on this group to determine if they represent a subset of HIV-infected persons at increased risk of fibrotic progression.

Keywords

patatin-like; human immunodeficiency; AIDS; fibrosis; steatosis

Introduction

Persons with HIV infection are at risk for development of non-alcoholic fatty liver (NAFL) and for non-alcoholic steatohepatitis (NASH). The reasons for this appear to be multifactorial and include combination antiretroviral therapy (cART) associated oxidative stress, alteration of lipid pathways due to HIV and gp120, presence of coinfections including hepatitis C virus (HCV), and increased exposure to bacterial breakdown products in the portal circulation resulting from impairment of the gut mucosal barrier by HIV infection.¹ It is likely that one or more host factors may represent determinants of liver injury. One of those described in this regard is PNPLA3 (patatin-like phospholipase domain containing 3). This gene, located on chromosome 22 encodes for a protein that is a tri-acyl glycerol lipase. This enzyme mediates hydrolysis in adipocytes. A key gene polymorphism in PNPLA3 (rs738409 C/G) has been described that appears to have close association with development of NASH and ASH (alcoholic steatohepatitis), though the specific mechanism(s) are unknown.^{23–5}

Limited data are available regarding the association of the PNPLA3 gene polymorphism and development/modulation of liver disease in those with HIV infection. Price et al. evaluated the prevalence of PNPLA3 among HIV-infected men in the Multicenter AIDS Cohort Study who had CT-defined fatty liver. Fatty liver was associated with PNPLA3 polymorphism, as well as visceral fat and insulin resistance.⁶

To examine the prevalence of PNPLA3 gene polymorphism in HCV/HIV coinfecting persons we (a) developed and validated a unique assay based upon high resolution melting point (HRM) analysis, and (b) conducted a retrospective analysis on the participants of a completed, nation-wide multicenter treatment trial in the NIAID AIDS Clinical Trials Group (ACTG) network, and (c) evaluated the relationship of the gene polymorphisms to measures of hepatic fibrosis.

Methods

Gene Polymorphism Analysis

We developed a unique method for determination of PNPLA3 gene polymorphisms. Briefly, genomic DNA was extracted from whole blood, buffy coat, or PBMCs, using a Qiagen QIAamp DNA Blood mini kit, according to the manufacturer's protocol. Realtime qPCR was then performed on a BioRad CFX96 thermal cycler using the extracted genomic DNA, utilizing a primer pair modified from Enache, et al., which flanks the SNP region of interest, at the corresponding human genomic region 43928824–43928869 on chromosome 22.⁷ The forward primer (5'-GCCTTGGTATGTTCTGCTTC-3') was utilized with an adaptation of the reverse primer to include a degenerate base near the 3' end, to accommodate a variant (rs738408 A/G) at this location, (5'-GGATAAGGCCACTGTAGAARG-3'). Following qPCR amplification, a high resolution melt curve was generated by heating the qPCR products from 65–80° in 0.2° increments (10 seconds/step). Then derived plots were normalized and temperature-shifted to facilitate melt curve differential comparison. Using specific post-melt analysis parameters for both the shape and size of the different melting curve profiles, the rs738409 genotypes (CC, CG, or GG) can be clearly discriminated. All

samples were run in triplicate and all runs included controls for each possible variant along with a notemplate control. Results were confirmed by direct nucleotide sequencing to determine PNPLA3 genotypes, aligning sequence data with reference sequences from NCBI, and visualizing actual sequence traces to detect the alleles present.

Study Population

The assay development samples were obtained from a liver research repository at the University of Cincinnati. They were collected from patients seen in the University Clinics, under a protocol allowing research use of samples and associated clinical data, which was approved by the Institutional Review Board.

The study population consisted of the participants of ACTG 5294, a large, multicenter, prospective Phase 3 trial examining the treatment of HCV in HCV/HIV coinfecting individuals with boceprevir/pegylated interferon alfa/ribavirin.⁸ Participants were both HCV treatment naïve and treatment experienced patients in 42 sites of the clinical trials network. Key inclusion criteria for the A5294 study included HCV RNA greater than 10,000 IU/mL and HCV genotype 1. To determine whether or not cirrhosis was present, all participants underwent either liver biopsy or non-invasive biomarker (FibroSure[®]) testing. FIB-4 scores were calculated using a combination of age, platelet count, ALT, and AST to assess liver fibrosis. For those participants on an approved antiretroviral regimen, the HIV RNA viral level was required to be less than 50 copies/mL, or less than 50000 copies/mL for those not on antiretroviral therapy. Among the exclusion criteria were mixed HCV genotypes, prior use of polymerase or protease inhibitors, and those with HBV, HAV, alpha-1 antitrypsin deficiency, or hemochromatosis.⁸ Overall, 257 subjects (135 HCV treatment naïve and 122 HCV treatment-experienced) were enrolled. De-identified repository samples and demographic/clinical data were utilized. All enrolled patients provided informed consent, which included collection and use of stored samples and data for research purposes. The study was approved by the Institutional Review Boards of all enrolling ACTG sites.

Statistical Analysis

Data analysis was performed by the ACTG Statistical and Data Center at the Harvard TH Chan School of Public Health. Dichotomized variables were estimated with two-sided 95% Wilson confidence intervals. Fisher's exact tests were used to compare associations between two categorical variables; specifically, to assess the association between APRI dichotomized as ≤ 1.5 and >1.5 and the dichotomized PNPLA3. A Fisher's exact test was also used to assess association between each of the treatment response measures and PNPLA3 dichotomized as CC vs non-CC.

Associations between FIB-4 and PNPLA3 were conducted in similar ways, where FIB-4 was analyzed as a continuous measure and dichotomized as ≤ 3.25 and >3.25 . The association between HOMA-IR and PNPLA3 was conducted similarly, where HOMA-IR was analyzed as a continuous measure and also as a binary variable dichotomized as ≤ 3 and >3 .

Wilcoxon rank sum tests were used to compare categorical with continuous variables; for example, to assess the association between APRI as a continuous measure and PNPLA3

dichotomized as CC and non-CC. A Cochran-Armitage trend test was used to assess associations between ordered and binary variables, and a Jonckheere-Terpstra test was used when ordered categories were compared to a continuous variable. All testing was performed as a two-sided analysis with a 5% type 1 error rate without adjustment for multiple testing.

Results

The HRM genotyping assay was developed and optimized on 24 repository samples. Figure 1 demonstrates the difference curves showing the relative distribution of samples by the rs738409 genotypes. The results were 100% concordant with the results obtained by direct Sanger sequencing of the amplicons from the region of interest.

There were 257 participants with HCV/HIV coinfection in the completed clinical trial. All participants in the analysis initiated the A5294 parent study HCV treatment. All participants except for 8 were on ART regimens, which included EFV-, RAL-, LPV-, ATV- and DRV-based regimens. 19% were on PI-based regimens (21% in the HCV treatment naïve group and 16% in the HCV treatment experienced group,) which are known to be associated with higher lipid levels.

The median age was 52 years and 79% were male. 44.7% were white and 49% were Black/African-American. Ethnicity was identified as Hispanic/Latino in 17.5%. The median BMI was 26.9 kg/m², and median body weight was 82.6 kg. Overall, 33.2% of the study population had a BMI<25 and 25.8% had a BMI ≥ 30 (obese). The remaining participants were classified as overweight. The baseline FIB-4 of the study cohort had a median score of 1.9 among 249 participants with available data, but 20.1% were >3.25, consistent with the presence of advanced fibrosis/cirrhosis.⁹ Indeed, pre-randomization characterization on all 257 trial participants by the site investigators indicated that 21.8% were cirrhotic, with cirrhosis status determined by liver histology in 63.4% participants and by FibroSure in the remainder.

Appropriate genetic samples were available for PNPLA3 genotyping on 241 of the 257 participants (93.7%) enrolled in the parent study. Among the tested participants, 66.0% (C.I. 59.8%–71.7%) had the wildtype allele (CC) and the remainder had the aberrant PNPLA3 gene polymorphism in the homozygotic (GG; 2.9%, C.I. 1.4–5.9%) or heterozygotic (CG; 31.1%, C.I. 25.6–37.2%) form. Figure 2 demonstrates the proportions of each genotype among tested individuals.

Baseline demographic and clinical variables by PNPLA3 genotype, categorized as CC vs non-CC are shown in Table 1. Since there were only 7 participants with GG, CG and GG participants were combined as non-CC.

Race and ethnicity were associated with PNPLA3 polymorphisms. Blacks were more likely to have CC allele while those with the non-CC alleles were more likely to be Hispanic/Latino (p<0.001). Proportions of CC vs. non-CC by race are shown in Figure 3

There was no observed association with PNPLA3 polymorphisms and the degree of hepatic fibrosis in this cohort. Similarly, there was no association with measured lipid parameters

including total and HDL cholesterol, triglycerides, or calculated LDL levels. Additionally, we collected and analyzed data from the parent study related to use of (1) lipid-lowering medications and (2) diabetes medications. There was no association found between PNPLA3 and either medication variable.

The HOMA-IR (Homeostatic Model Assessment of Insulin Resistance) was also evaluated relative to the PNPLA3 polymorphism and there was no association with either HOMA-IR or the clinical diagnosis of diabetes.

HCV treatment response was evaluated using week 12 response to HCV antiviral therapy as was HCV sustained viral response. Neither outcome seemed affected by the presence or absence of PNPLA3 gene polymorphisms.

Discussion

There is increased recognition of the association between fatty liver and adverse liver-related outcomes of patients with or without HIV infection. In recent years, it has been recognized that a subset of patients with fatty liver (NAFLD) may have increased hepatic inflammation, progressive hepatic fibrosis and more rapid progression to cirrhosis and HCC. Genome wide association studies (GWAS) have identified PNPLA3 as a key marker of progression associated with the presence of NASH.¹⁰ Interestingly, this gene polymorphism may also be a marker of alcoholic liver disease.¹¹ Laboratory studies suggest that the mutational variants of PNPLA3 affect triglyceride lipase function leading to accumulation of hepatic fat.⁴ In this study we sought to determine the prevalence of PNPLA3 gene polymorphisms (rs738409) in a nation-wide treatment cohort in the United States that was dually infected with HCV and HIV. The selection of this cohort was based upon several key observations. First, there are limited studies that have characterized the distribution and associations of PNPLA3 polymorphisms in those with HIV in the U.S. population which is ethnically and racially different from studies in European populations.^{12,13} Second, some investigators have speculated that disease progression and injury would be greater in those with risk for NASH combined with HCV infection. Indeed, a recent meta-analysis suggested that the GG allele was highly associated with the risk of advanced liver fibrosis and steatosis in Caucasian populations with chronic HCV infection.¹⁴ Finally, patients with HIV are thought to be at increased risk of NASH due to the presence of persistent immune activation and oxidative stress in the liver.¹ Therefore, the most susceptible group to complications of NASH which include diabetes, lipid disorders and ongoing liver injury should be those who possess variant PNPLA3 gene function.

In an effort to better characterize PNPLA3 gene polymorphisms we first sought to develop a rapid and inexpensive but reliable assay that did not rely upon sequence analysis. To this end we developed and optimized a high resolution melting (HRM) point assay and validated its functionality and reproducibility. The assay was 100% concordant with sequence-derived results. Enache and colleagues also developed an HRM method which differs from ours in terms of the primer set and concentrations, reaction mix, and cycling conditions, but which also yielded 100% concordance in classification when compared to traditional sequencing methods.⁷ Regardless of protocol, this methodology allows for rapid and relatively

inexpensive characterization of PNPLA3 gene polymorphisms without need for expensive sequence analysis.

Price and colleagues studied a large cohort of HIV-infected persons in the MACS cohort focused on the presence or absence of fatty liver as determined by non-contrast CT imaging. In this cohort they determined frequency of PNPLA3 gene polymorphisms and reported that CC (wildtype) was present in 63%, CG in 33% and GG in 4% of those without fatty liver, but that those with fatty liver were enriched for the non-CC genotype which accounted for 56% of that group ($p = 0.001$).⁶ The proportions seen in those without fatty liver were remarkably similar to that observed in our cohort. Though we are unable to directly comment on the association with fat in our cohort, it is interesting that we found no direct association with diabetes, lipid disorders, or insulin resistance as determined by HOMA-IR.¹⁵ Furthermore, we did not find any association with advanced fibrosis/cirrhosis which has been noted in other studies.¹⁶ Our findings are in agreement with Scheiner et al, who reported no association between PNPLA3 polymorphisms and fibrosis stage. Similarly, they also reported no clear link between PNPLA3 and effect on virologic response following pegylated interferon/ribavirin therapy.¹⁷

Limitations of our study include the lack of direct characterization of fat content of the patient livers, either by histology or by a controlled-attenuation parameter, which was not widely available when this study was performed. Baseline characteristics were assessed to explore possible confounding by factors associated with both the PNPLA3 genotype and the outcome measures of interest. While there was no evidence of potential confounding, this assessment was limited by the small numbers of participants in many subgroups of the potential confounder. The literature suggests that GG genotype may be associated with poorer outcomes of HCV treatment; that is, better outcomes are expected with CC genotype or non-GG genotype. In this analysis, we sought to assess the PNPLA3 genotype with categorization as CC vs non-CC and as GG vs non-GG. However, the evaluation of GG vs non-GG was limited due to only seven participants with PNPLA3 GG genotype. Some investigators have suggested that an additive model which includes other genetic polymorphisms that could contribute to the phenotypic effect be utilized.¹⁸ However, these genetic markers were not tested in the context of this analysis.

The overall prevalence of NAFLD/NASH in those with HIV remains uncertain with wide ranges reported for each. There is considerable overlap with reported ranges in non-HIV infected persons raising the possibility that HIV-infected may or may not be at increased risk. Overall, BMI ranges of our cohort trend lower than that seen in the general population. However, the patients with HIV have more reasons than the general population to have NAFLD/NASH including persistent systemic immune activation associated with gut leakiness, use of medications that may promote fat deposition, and oxidative stress increases risk of liver injury.¹ Further studies are needed to determine if a subset of patients with HIV can be identified who are at the highest risk of fibrotic progression due to the presence of non-wildtype PNPLA3 allelic variants that can be targeted for interventional strategies to reduce liver disease.

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Figure 1:
HRM genotyping assay result in assay validation set depicting the relative distribution among difference curves

Figure 2:
Pie chart showing relative proportions (%) of PNPLA3 alleles

Figure 3:
Distribution of PNPLA3 genotype by race. Percentage of subjects is shown on left axis

Table 1:

Baseline Demographic Variables by PNPLA3 Genotype, Categorized as CC vs non-CC

		Total (N=241)	CC (N=159)	non-CC (N=82)	P-Value
Age (years)	Mean (s.d.)	51.0 (8.4)	51.9 (8.2)	49.4 (8.6)	
Sex	Male	191 (79.3%)	129 (81.1%)	62 (75.6%)	0.320 **
	Female	50 (20.7%)	30 (18.9%)	20 (24.4%)	
Race	Asian	4 (1.7%)	2 (1.3%)	2 (2.4%)	0.013 **
	Black or African American	119 (49.4%)	89 (56.0%)	30 (36.6%)	
	White	108 (44.8%)	61 (38.4%)	47 (57.3%)	
	American Indian	1 (0.4%)	0 (0.0%)	1 (1.2%)	
	More than One Race	3 (1.2%)	3 (1.9%)	0 (0.0%)	
	Unknown	6 (2.5%)	4 (2.5%)	2 (2.4%)	
Ethnicity	Hispanic or Latino	42 (17.4%)	17 (10.7%)	25 (30.5%)	<0.001 **
	Not Hispanic or Latino	197 (81.7%)	140 (88.1%)	57 (69.5%)	
	Unknown	2 (0.8%)	2 (1.3%)	0 (0.0%)	
Race/ethnicity	White Non-Hispanic	76 (31.5%)	49 (30.8%)	27 (32.9%)	<0.001 **
	Black Non-Hispanic	117 (48.5%)	89 (56.0%)	28 (34.1%)	
	Hispanic (Regardless of Race)	42 (17.4%)	17 (10.7%)	25 (30.5%)	
	Asian, Pacific Islander	4 (1.7%)	2 (1.3%)	2 (2.4%)	
	More than one race	2 (0.8%)	2 (1.3%)	0 (0.0%)	
Cirrhosis	Yes	50 (20.7%)	34 (21.4%)	16 (19.5%)	0.867 *
	No	191 (79.3%)	125 (78.6%)	66 (80.5%)	
BMI (kg/m2)	Mean (s.d.)	27.4 (4.8)	27.2 (4.7)	27.6 (5.0)	0.494 *
APRI	Mean (s.d.)	1.0 (1.0)	1.0 (1.0)	1.0 (0.9)	0.745 **
APRI, categorized	1.5	194 (82.9%)	128 (82.6%)	66 (83.5%)	1.00 *
	>1.5	40 (17.1%)	27 (17.4%)	13 (16.5%)	
	Missing	7	4	3	
FIB-4	Mean (s.d.)	2.4 (1.8)	2.5 (2.0)	2.3 (1.5)	0.349 **
FIB-4, categorized	3.25	189 (80.8%)	123 (79.4%)	66 (83.5%)	0.487 *
	>3.25	45 (19.2%)	32 (20.6%)	13 (16.5%)	
	Missing	7	4	3	

* Fisher's Exact Test

** Wilcoxon Test