META-ANALYSIS

PNPLA3 polymorphism increases risk for and severity of chronic hepatitis C liver disease

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Abstract

AIM
To examine the association of *PNPLA3* polymorphisms in chronic hepatitis C patients and development of liver disease spectrum.

METHODS
Literature was searched systematically from PubMed/MEDLINE, EMBASE, and Cochrane search engines for full-length articles written in English that examined *PNPLA3* polymorphism in chronic hepatitis C (CHC) patients. Studies evaluating the association of *PNPLA3* polymorphism spectrum (fatty liver, steatohepatitis, cirrhosis, and hepatocellular carcinoma) of CHC were included. Pooled data are reported as OR with 95%CI. Our study endpoint was the risk of the entire liver disease spectrum including: Steatosis/fatty liver, cirrhosis, and hepatocellular carcinoma in CHC patients with *PNPLA3* polymorphisms.

RESULTS
Of 380 studies identified, a total of 53 studies were included for full-text review. Nineteen on chronic he-

patitis C were eligible for analysis. Pooled ORs for rs738409 GG compared to CC and CG among patients with fatty liver was 2.214 (95%CI: 1.719-2.853). ORs among advanced fibrosis/cirrhosis were 1.762 (95%CI: 1.258-2.468). Similar odds ratios among hepatocellular carcinoma patients were 2.002 (95%CI: 1.519-2.639). Pooled ORs for rs738409 GG and CG compared to CC among patients with fatty liver were 1.750 (95%CI: 1.542-1.986). Pooled ORs for advanced fibrosis/cirrhosis patients were 1.613 (95%CI: 1.211-2.147). All analyses were homogenous and without publication bias except one. The associations were maintained after adjusting for publication bias and heterogeneity.

CONCLUSION
PNPLA3 polymorphisms have strong association with increased risk and severity of the liver disease spectrum in CHC patients.

Key words: *PNPLA3* polymorphism; Cirrhosis; rs738409; Hepatitis C virus; Hepatocellular carcinoma

Core tip: *PNPLA3* polymorphisms (rs738409 CG and GG) are associated with increased risk of steatosis, advanced fibrosis, cirrhosis, and hepatocellular carcinoma in chronic hepatitis C patients.

INTRODUCTION

Hepatitis C virus (HCV) infection is one of the most important causes of chronic liver disease in the United States[1]. About 27% of cases of cirrhosis and 25% of hepatocellular carcinoma (HCC) worldwide are secondary to HCV infection[2]. Multiple genetic factors identified within the past few years have been shown to be associated with the predisposition to chronic liver disease and the progression to cirrhosis and HCC[3,4]. The single nucleotide polymorphism (SNP) rs738409 C>G (isoleucine to methionine substitution at position 148, I148M) in the *PNPLA3* gene has been strongly linked to progression of liver disease in multiple studies, and this association was confirmed in meta-analyses of the spectrum of alcoholic liver disease (ALD)[5] as well as non-alcoholic fatty liver disease (NAFLD)[6-9].

The frequency of hepatic steatosis varies with ethnicity where it was reported as 45%, 33% and 24% in Hispanics, Whites and Blacks respectively[10]. At the

same time the frequencies of the PNPLA3 rs738409[G] allele were 0.49, 0.23, and 0.17 in Hispanics, European Americans and African Americans[11]. In addition, the prevalence of the GG genotype in different races in fact correlates with the rate of NAFLD in each respective population, with nearly half of all Hispanics possessing the allele, who in turn are also most likely to have NAFLD. The same is true of the inverse, with less than one quarter of African Americans having the PNPLA3 rs738409[G] allele, and they are least likely to develop NAFLD compared to Hispanics and Caucasians[10,11].

Given that the association between *PNPLA3* polymorphism and liver disease spectrum in chronic hepatitis C (CHC) patients has not been consistent, especially for HCC[12,13] and cirrhosis[14,15], we performed this meta-analysis to further examine the association of *PNPLA3* polymorphisms with the predisposition to the entire spectrum of liver disease among patients with CHC.

MATERIALS AND METHODS

Literature search
Utilizing the Meta-analysis of Observational Studies in Epidemiology guidelines, literature was searched from PubMed/Medline, Embase, and Cochrane search engines for full-length articles written in English that examined *PNPLA3* polymorphism in CHC patients[16]. The initial medical subject headings search terms were: "Hepatitis C, Chronic" and "adiponutrin, human". The search was then expanded using the terms: "rs738409" and "patatin-like phospholipase domain-containing 3 protein". All databases were searched from their inception date through March 2015. Meeting abstracts from major gastroenterology conferences over the past 3 years were also searched to identify studies that were potentially overlooked in our database search. Articles were selected for full text review based on title and abstract.

Study selection
Three independent investigators (Masadeh M, Al Hanayneh M and Maslonka M) manually search the retrieved publications to ensure all appropriate articles were discovered and included. Two authors (HS and AKS) reviewed articles in question for possible inclusion. The following inclusion criteria were set for inclusion in this meta-analysis: (1) studies published as full-length articles which reported association of the *PNPLA3* variant (rs738409 C>G) among CHC patients; and (2) studies which analyzed patients with other liver diseases and reported separate data on *PNPLA3* polymorphisms for CHC.

The following exclusion criteria were set: (1) studies without available gene frequency data for analysis; and (2) studies including subjects with other liver diseases without separate data on CHC patients.

Definitions
HCV infection was diagnosed with both positive serum

anti-HCV antibodies and serum HCV ribonucleic acid (RNA). The disease spectrum was defined as the following: steatosis = fatty liver (FL) on imaging without evidence of cirrhosis or HCC; advanced fibrosis and cirrhosis = biopsy-proven bridging fibrosis, or clinical evaluation supported by hematological, biochemical, and radiologic imaging findings; and HCC = diagnostic imaging findings on triple phase magnetic resonance imaging or computed tomography, or using histological confirmation from liver tissue. Healthy controls were defined as subjects without liver disease and without HCV infection.

Data extraction
After determining eligibility for inclusion, two reviewers (Masadeh M and Al Hanayneh M) independently extracted data for (1) study characteristics: Author and year of publication, and study design (population based or not, using controls or not); (2) study population: Liver disease spectrum and sample size; (3) frequencies of *PNPLA3* polymorphism genotypes (rs738409 CC, CG, and GG); and (4) OR: For association of *PNPLA3* polymorphism and the spectrum of liver disease and for severity of liver disease. Any discrepancies amongst the reviewers were resolved by jointly reviewing the study in question. Among studies comparing diseased population with healthy controls, similar data were also extracted on healthy controls.

Endpoints and outcomes
Our study endpoint was the risk of the entire liver disease spectrum including: Steatosis/fatty liver, cirrhosis, and HCC in CHC patients with *PNPLA 3* polymorphisms.

Quality assessment
The quality of included studies was assessed independently by three authors (Masadeh M, Al Hanayneh M and Maslonka M) using the Newcastle-Ottawa Quality Assessment Scale for case-control studies[17]. This scale has one instrument for assessing case-control studies and another one for cohort studies. Each of these instruments includes measures of quality in selection, comparability, and exposure domains. While one point is granted for each of the areas measured within the selection and exposure domains, a maximum of two points can be assigned within the comparability domain with highest possible total score of nine. Previous studies have reported that a score of seven or greater denotes a high-quality study[18]. Any discrepancies between the three coauthors were addressed by a joint reevaluation of the original article.

Statistical analysis
The strength of the association between rs738409 and CHC liver disease spectrum prevalence was expressed by OR and their corresponding 95%CI. The Random effects model was used for analyzing pooled data for all the analyses[19]. Heterogeneity was measured using I^2 statistics for inter-study variance, with the χ^2 test

used for statistical analysis. Heterogeneity was defined with $I^2 \geqslant 50\%$ or $\chi^2 P < 0.10$[20]. At least two studies are needed to examine and report heterogeneity. To examine the heterogeneous data and source of heterogeneity, sensitivity analyses were performed in a stepwise fashion for (1) study quality; and (2) excluding studies with the highest and lowest OR. Publication bias was assessed using Egger regression and the Begg-Mazumdar rank correlation tests[21-23]. Egger test is a regression method checking for association between effect sizes and standard error and uses actual effect size for each study[23]. Begg-Mazumdar is a rank correlation test examining the potential association between effect estimates (taken as a rank and not exact effect size) and sampling variance (or standard error)[22]. At least three studies are needed for examining and reporting publication bias. For analyses with publication bias, the analyses were repeated either by performing sensitivity analysis or using the Duval and Tweedie Trim and Fill method, a nonparametric (rank-based) data augmentation technique[24]. The method can be used to estimate the number of studies missing from a meta-analysis resulting in a skew of the data due to the suppression of the most extreme results on one side of the funnel plot. The method then amplifies the observed data so that the funnel plot is more symmetric and re-computes the summary estimate based on the comprehensive data. The method should not be regarded as a way of yielding a more "valid" estimate of the overall effect or outcome, but as a means of examining the sensitivity of the results to one particular selection mechanism[25]. All statistical analyses were performed using R (Foundation for Statistical Computing) utilizing the metaphor package, or Comprehensive Meta-analysis (Biostat, Englewood, NJ). Singal AK from University of Alabama, Birmingham, reviewed the statistical methods of this study.

RESULTS

Baseline study characteristics
A total of 380 citations were retrieved on initial search. After reviewing article titles and abstracts, a total of 53 studies were included for full-text review (Figure 1). Of these, twenty articles were excluded because they did not have sufficient data for our analysis. Nine studies were excluded for including subjects with liver disease not caused by HCV[11,26-33], and three studies were excluded for including subjects co-infected with human immunodeficiency virus and/or hepatitis B virus infection[34-36]. One duplicate study[37] and one study that looked at treatment response[38] were excluded. Nineteen studies evaluating 9093 patients (57.6% males, mean body mass index 25.1 kg/m^2) on association of *PNPLA3* polymorphisms in CHC[12-15,39-53] were included for the analysis. Data on study design, ethnicity and genotype frequency are summarized in Table 1.

Table 1 Baseline characteristics of patients from studies included in the analysis

Ref.	Study design	Controls (n)	n	Ethnicity	M%	Mean age	Mean BMI	rs 738409 genotype count (CC:CG:GG)[4] FL	Hepatitis	Cirrhosis	HCC
Cai et al[39] (2011)	R	-	626	C	61.8	44.7	23.7	62:28[1]	-	-	-
Valenti et al[40] (2011)	R	179	819	C + NA	56.4	57.4	24.8	269:219:73	-	119:172:229	17:21:12
Trépo et al[41] (2011)	R	-	537	C	63	49.4	25.5	136:106:31	-	108:85:23	-
Corradini et al[42] (2011)	P[6]	-	221	C	63	58	-	-	-	-	-
Nischalke et al[13] (2011)	P	190	162	C	57	56	28.4	-	-	45:31:05	40:33:08
Valenti et al[43] (2012)	P	-	567	NS	-	-	-	-	-	-	-
Valenti et al[15] (2012)	P[6]	-	602	NS	51	51	25.1	364:42[2]	-	158:21[2]	-
Guyot et al[12] (2013)	P	-	253	NS	54.2	56.7	27.3	-	-	140:75:38	54:26:13
Ezzikouri et al[44] (2013)	P	132	230	NA	45.2	63.63	-	-	47:71:11	-	43:35:23
Stättermayer et al[45] (2014)	R	-	478	NS	65.7	44.9	25.6	190:23[2]	-	101:57[2]	-
Ampuero et al[46] (2014)	P[6]	-	474	M	64.8	43.4	25.7	94:126[3]	-	-	-
Sato et al[47] (2014)	R	-	358	A	55.9	69.76	-	41:20[2]	-	112:37[2]	100:176:82
Yasui et al[48] (2014)	P[6]	-	276	A	40.6	58.2	23	23:75:39	45:66:38	20:31:21	-
Petta et al[14] (2015)	P	-	434	C	53.9	51.7	-	-	40:35:12	71:36:13	-
Nakaoka et al[49] (2015)	P	-	231	A	44.6	62.9	22.5	-	-	90:27[2]	12:22:14
Tamaki et al[50] (2015)	R	-	176	A	39.8	56.5	22.9	-	-	52:87:37	-
Huang et al[51] (2015)	R	-	1018	A	56.6	51.8	24.9	175:205:75	-	-	-
Petta et al[52] (2016)	P[6]	-	694	C	53	54	26.5	151:151:45[5]	-	-	-
Ali et al[53] (2016)	P	-	937	M	70.1	49.5	-	-	-	172:212[3]	-
Summary		501	9093		57.6	52.7	25.1				

[1]C allele: G allele; [2]CC + CG: GG; [3]CC: CG + GG; [4]Genotype counts were reported as ratios (CC wild genotype: CG heterozygote genotype: GG homozygote genotype) unless indicated by star(s); [5]Calculated from percentages in the original article; [6]Population based studies. A: Asian; BMI: Body mass index; C: Caucasian; FL: Fatty liver; HCC: Hepatocellular carcinoma; M: Mixed (Caucasians and non-Caucasians[46] and White/Black/Hispanic[53]); P: Prospective; R: Retrospective; M%: Males percentage; N: Number of cases; NA: North African; NS: Not specified in the original manuscripts (although all 4 studies included European referral centers only).
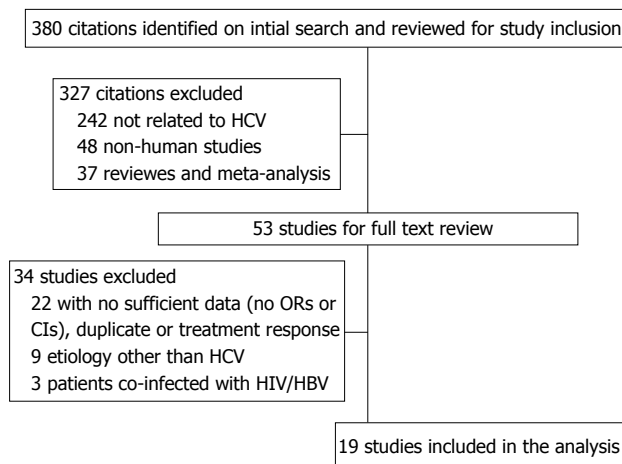

Figure 1 Attrition on literature search and study inclusion. HCV: Hepatitis C virus; HBV: Hepatitis B virus; HIV: Human immunodeficiency virus.

Study quality assessment

Based on the Newcastle-Ottawa Scale, nine studies were of "high quality" with a score of seven or more, and the remaining ten studies had a score of six or below (Table 2).

Association between PNPLA3 polymorphism and liver disease spectrum (GG vs CG and CC analysis)

Association of *PNPLA3* polymorphisms with FL in CHC patients: Among six studies on 3310 patients, the pooled OR for rs738409 GG genotype compared to CC and CG genotypes in CHC was 2.214 (95%CI: 1.719-2.853) (Figure 2A). The data was homogeneous (I^2 = 9.4%, P = 0.36) and without publication bias as assessed by Egger test (P = 0.08) and Begg-Mazumdar test (P = 0.14).

Association of *PNPLA3* polymorphisms with advanced fibrosis and cirrhosis in CHC patients: Among seven studies on 3377 patients, the pooled OR for rs738409 GG genotype compared to CC and CG genotypes in CHC was 1.762 (CI: 1.258-2.469) (Figure 2B). The data was heterogeneous (I^2 = 65.9%, P = 0.081), with evidence of publication bias as assessed by Begg-Mazumdar test (P = 0.036) and tendency for publication bias as assessed by Egger test (P = 0.059). Sensitivity analysis after excluding studies with lowest[14] and highest[49] OR revealed similar effect size: 1.82 (95%CI: 1.41-2.34) with I^2 = 18.8%, P = 0.30. Additionally, when Dual and Tweedie trim and fall test was used to assess publication bias, 3 studies were trimmed with no change in effect size (OR = 1.39, 95%CI: 1.01-1.92).

Association of PNPLA3 polymorphisms and HCC in CHC patients: Among seven studies on 2274 patients, the pooled OR for rs738409 GG genotype compared to CC and CG genotypes in CHC was 2.002 (95%CI: 1.519-2.639) (Figure 2C). The data was homogenous (I^2 = 30%, P = 0.19), without publication bias as assessed by Egger test (P = 0.91) and Begg-Mazumdar test (P = 0.99).

Table 2 Newcastle - Ottawa Scale on quality score of the included studies

Ref.	Selection				Comparability	Exposure			Total
	Independent validation	Case representation	Controls selection	Controls definition	Case and control design/analysis	Ascertainment of exposure	Same method of ascertainment	Same response rate	
Cai *et al*[39] (2011)	1	1			2	1	1	1	7
Valenti *et al*[40] (2011)	1	1	1	1	2	1	1	1	9
Trépo *et al*[41] (2011)	1	1			2	1			5
Corradini *et al*[42] (2011)	1	1			2	1			5
Nischalke *et al*[13] (2011)	1	1	1	1	2	1	1	1	9
Valenti *et al*[43] (2012)	1	1			2	1	1		6
Valenti *et al*[15] (2012)	1	1			2	1	1		6
Guyot *et al*[12] (2013)	1	1			2	1			5
Ezzikouri *et al*[44] (2013)	1	1	1	1	2	1	1	1	9
Stättermayer *et al*[45] (2014)	1	1			2	1			5
Ampuero *et al*[46] (2014)	1	1			2	1	1	1	7
Sato *et al*[47] (2014)	1	1			2	1			5
Yasui *et al*[48] (2014)	1	1			2	1			5
Petta *et al*[14] (2015)	1	1			2	1			5
Nakaoka *et al*[49] (2015)	1	1			2	1			5
Tamaki *et al*[50] (2015)	1	1			2	1	1	1	7
Huang *et al*[51] (2015)	1	1			2	1	1	1	7
Petta *et al*[52] (2016)	1	1			2	1	1	1	7
Ali *et al*[53] (2016)	1	1			2	1	1	1	7

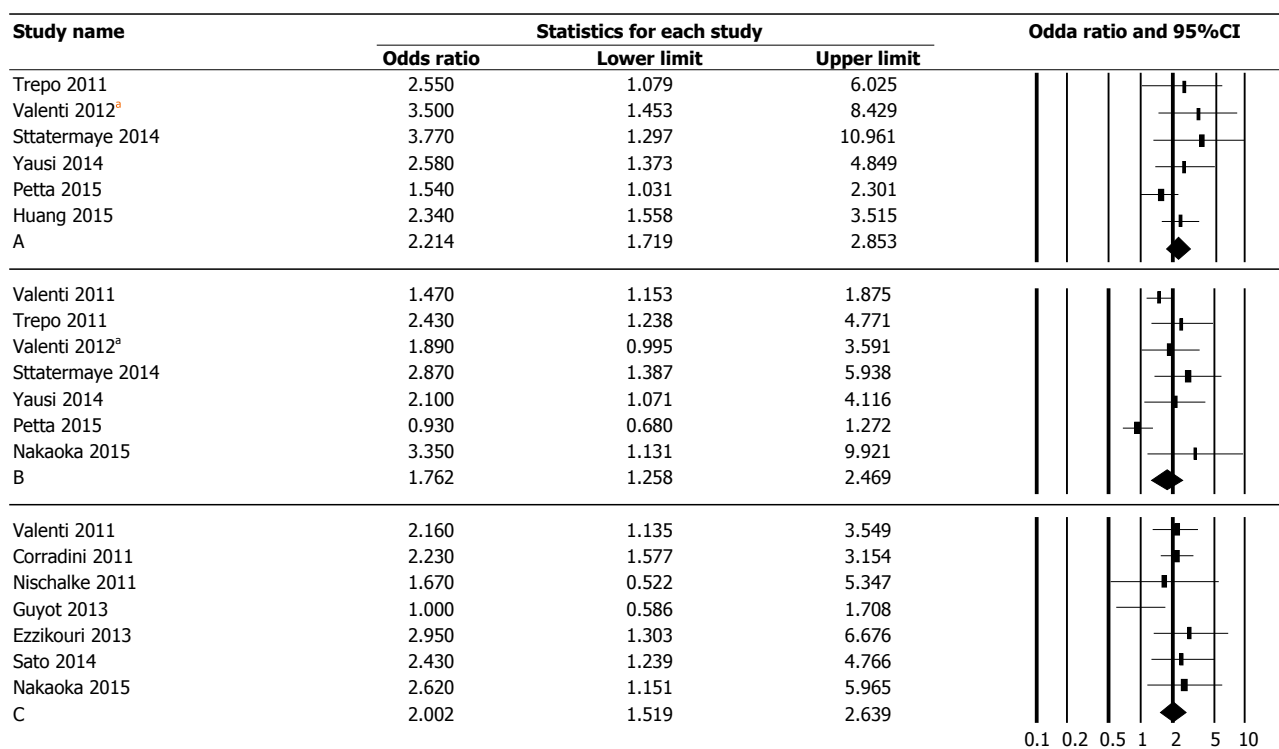| Study name	Statistics for each study			Odda ratio and 95%CI
	Odds ratio	Lower limit	Upper limit	
Trepo 2011	2.550	1.079	6.025	
Valenti 2012[a]	3.500	1.453	8.429	
Sttatermaye 2014	3.770	1.297	10.961	
Yausi 2014	2.580	1.373	4.849	
Petta 2015	1.540	1.031	2.301	
Huang 2015	2.340	1.558	3.515	
A	2.214	1.719	2.853	
Valenti 2011	1.470	1.153	1.875	
Trepo 2011	2.430	1.238	4.771	
Valenti 2012[a]	1.890	0.995	3.591	
Sttatermaye 2014	2.870	1.387	5.938	
Yausi 2014	2.100	1.071	4.116	
Petta 2015	0.930	0.680	1.272	
Nakaoka 2015	3.350	1.131	9.921	
B	1.762	1.258	2.469	
Valenti 2011	2.160	1.135	3.549	
Corradini 2011	2.230	1.577	3.154	
Nischalke 2011	1.670	0.522	5.347	
Guyot 2013	1.000	0.586	1.708	
Ezzikouri 2013	2.950	1.303	6.676	
Sato 2014	2.430	1.239	4.766	
Nakaoka 2015	2.620	1.151	5.965	
C	2.002	1.519	2.639	

0.1 0.2 0.5 1 2 5 10

Figure 2 Forest plots for analysis of chronic hepatitis C studies on the association of *PNPLA3* polymorphisms GG *vs* CG and CC with fatty liver in (A), cirrhosis in (B), and hepatocellular carcinoma in (C). The effect size is reported as odds ratio with 95%CI. The bottom line in "the statistics for each study" heading is the pooled effect size analyzed using the random effects model. OR greater than 1 denotes risk for the respective outcome or positive association, and OR less than 1 indicates a protective effect or negative association. The 95%CI not crossing 1 indicates a significant association. Valenti 2012[a] in panel (A) refers to reference[43], and in panel (B) refers to reference[14].

Association between PNPLA3 polymorphism and liver disease spectrum (GG and CG vs CC analysis)

Association of *PNPLA3* polymorphisms with FL in CHC patients: Among three studies on 1794 patients, the pooled OR for rs738409 GG and CG genotypes compared to CC genotype in CHC was 1.750 (95%CI: 1.542-1.986) (Figure 3A). The data was homogeneous

(I^2 = 0.0%, P = 0.84), without publication bias as assessed by Egger test (P = 0.57) and Begg-Mazumdar test (P = 0.99).

Association of *PNPLA3* polymorphisms with advanced fibrosis, and cirrhosis in CHC patients: Among three studies on 2131 patients, the pooled OR
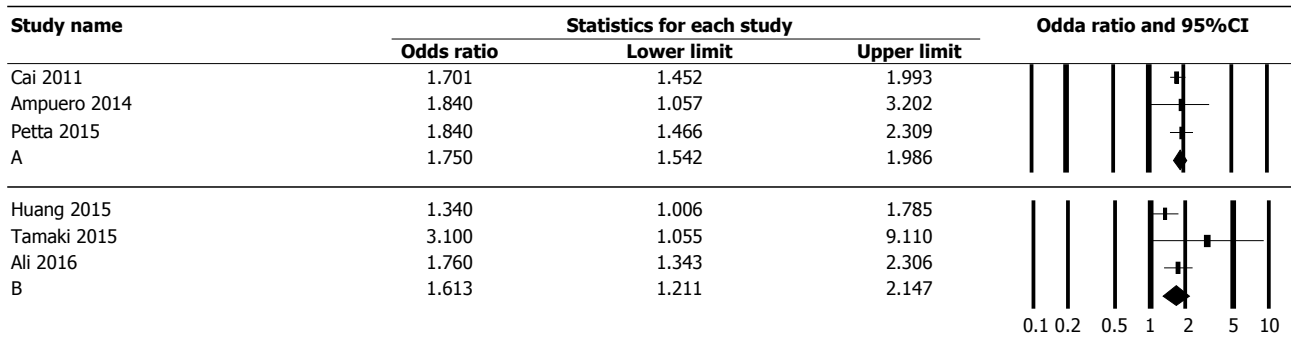
Study name	Statistics for each study			Odds ratio and 95%CI
	Odds ratio	Lower limit	Upper limit	
Cai 2011	1.701	1.452	1.993	
Ampuero 2014	1.840	1.057	3.202	
Petta 2015	1.840	1.466	2.309	
A	1.750	1.542	1.986	
Huang 2015	1.340	1.006	1.785	
Tamaki 2015	3.100	1.055	9.110	
Ali 2016	1.760	1.343	2.306	
B	1.613	1.211	2.147	

0.1 0.2 0.5 1 2 5 10

Figure 3 Forest plots for analysis of chronic hepatitis C studies on the association of *PNPLA3* polymorphisms GG and CG *vs* CC with fatty liver in (A), and cirrhosis in (B). The effect size is reported as odds ratio with 95%CI. The bottom line in the "statistics for each study" heading is the pooled effect size analyzed using the random effects model. OR greater than 1 denotes risk for the respective outcome or positive association, and OR less than 1 indicates a protective effect or negative association. The 95%CI not crossing 1 indicates a significant association.

for rs738409 GG and CG genotypes compared to CC genotype in CHC was 1.613 (95%CI: 1.211-2.147) (Figure 3B). The data was homogeneous (I^2 = 41%, P = 0.18), without publication bias as assessed by Egger test (P = 0.056) and Begg-Mazumdar test (P = 0.99).

DISCUSSION

We have previously described that *PNPLA3* polymorphism is a modifier in the natural history of ALD[5] and NAFLD[6-8]. In this meta-analysis, we found a clear association between *PNPLA3* polymorphisms and the entire spectrum (steatosis/fatty liver, cirrhosis, and HCC) of liver disease in CHC patients.

It was previously reported that *PNPLA3* polymorphisms were an independent predictor of more rapid fibrosis progression in patients with chronic hepatitis C[50]. The mechanism whereby rs738409 influences the development of fatty liver likely involves a decreased ability of the 148M *PNPLA3* variant to regulate hepatic lipid metabolism[54]. It is not known whether the rs738409 SNP influences the steatogenic effect of HCV and the progression of CHC. However, if steatosis causes fibrosis progression in CHC, then it may be assumed the rs738409 SNP should also be associated with advanced fibrosis and HCC[40].

Like any other meta-analysis, our study had to face the possibility of publication bias. In order to minimize this possibility, and the subsequent overestimation of the true effect size due to negative study identification failure[55], we combined searches from PubMed/Medline, Embase and Cochrane with manual searches. Although we used procedures in agreement with current guidelines, we cannot formally rule out the possibility that we overlooked studies that were not accessible[55]. Another limitation of this meta-analysis is the inclusion of case-control studies in which the potential for biases (*e.g.,* selection and reporting) is higher when compared to randomized trials, and they are more inherent to confounding factors. In contrary to the previous meta-analysis on *PNPLA3* polymorphisms in alcoholic and non-alcoholic liver diseases that compared different genotypes[5-8], our current analysis used the recessive model when comparing GG genotype *vs* CC and CG genotypes, and the dominant model when comparing GG and CG genotypes related to the CC genotype. Finally, no pooled data were provided on steatohepatitis in chronic HCV patients as only one study had reported such an association[14], while the studies by Ezzikouri *et al*[44] and Yasui *et al*[48] either did not have biopsies performed or reported "necroinflammatory changes". Lack of standard definition amongst these studies prevented pooling them together.

The *PNPLA3* GG genotype was negatively associated with sustained virological response and early viral kinetics in patients receiving peginterferon and ribavirin[15]. Also, in patients with chronic hepatitis C who failed to achieve sustained virologic response following interferon-based therapy, IL28B and *PNPLA3* were independent predictors of rapid fibrosis progression[50]. Tamaki *et al*[50] developed a fibrosis progression-score by combining IL28B and *PNPLA3* genotypes and ALT values, which stratified patients into low, intermediate, and high-risk groups for fibrosis progression. However, this fibrosis progression score needs external validation. In the era of direct-acting antiviral therapy, the question that remains unanswered is whether or not *PNPLA3* polymorphisms identify high-risk CHC patients that are responsive to new treatment regimens. In summary, this meta-analysis provides strong evidence for the association of *PNPLA3* polymorphisms and the spectrum of liver disease in patients with CHC, beginning with fatty liver disease and extending as far as cirrhosis and even HCC in patients with CHC. Further studies on treatment response are needed in this group of patients who carry a higher risk for more rapidly progressive liver disease.

COMMENTS

Background
Hepatitis C virus (HCV) infection is one of the most important causes of chronic liver disease in the United States. About 27% of cases of cirrhosis and 25% of hepatocellular carcinoma (HCC) worldwide are secondary to HCV infection.

Research frontiers
Given that the association between *PNPLA3* polymorphism and liver disease

spectrum in chronic hepatitis C (CHC) patients has not been consistent, especially for HCC and cirrhosis, the authors performed this meta-analysis to further examine the association of *PNPLA3* polymorphisms with the predisposition to the entire spectrum of liver disease among patients with CHC.

Innovations and breakthroughs

In this meta-analysis, they found a clear association between *PNPLA3* polymorphisms and the entire spectrum (steatosis/fatty liver, cirrhosis, and HCC) of liver disease in CHC patients.

Peer-review

This manuscript is very well designed; the authors did a great effort in selecting the articles to be included in the meta-analysis with a proper quality scoring of selected articles.

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