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Gene polymorphisms of cellular senescence marker p21 and disease progression in non-alcohol-related fatty liver disease

Aloysious Aravinthan[1,†], George Mells[1,†], Michael Allison[1], Julian Leathart[2], Anna Kotronen[3], Hannele Yki-Jarvinen[3], Ann K Daly[2], Christopher P Day[2], Quentin M Anstee[2,‡,*], and Graeme Alexander[1,‡,*]

[1]Department of Medicine; University of Cambridge; Cambridge, UK; [2]Institute of Cellular Medicine; Newcastle University; Newcastle upon Tyne, UK; [3]Department of Medicine; University of Helsinki; Helsinki, Finland

[†]These authors contributed equally to this work.

[‡]These authors are joint senior authors.

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Non-alcohol-related fatty liver disease (NAFLD) encompasses a wide spectrum, ranging from steatosis alone to steatohepatitis and fibrosis. Presence of steatohepatitis and fibrosis are key hallmarks of disease progression. Previous studies have demonstrated an association between hepatocyte p21 expression and fibrosis stage in NAFLD. The aim of this study is to investigate the association between the variants of *CDKN1A*, which encodes p21, and disease progression in NAFLD. To this end, the relation between *CDKN1A* polymorphism and liver fibrosis was studied in 2 cohorts of biopsy-proven NAFLD patients from UK (n = 323) and Finland (n = 123). Genotyping was performed using DNA isolated from lymphocytes collected at the time of liver biopsy. The findings of the UK cohort were tested in the Finnish cohort. Both the UK and Finnish cohorts were significantly different from each other in basic demographics. In the UK cohort, rs762623, of the 6 SNPs across *CDKN1A* tested, was significantly associated with disease progression in NAFLD. This association was confirmed in the Finnish cohort. Despite the influence on fibrosis development, SNPs across *CDKN1A* did not affect the progression of liver fibrosis. In conclusion, *CDKN1A* variant rs762623 is associated with the development but not the propagation of progressive liver disease in NAFLD.

Introduction

Permanent cell cycle arrest is characteristic of cellular senescence, triggered by various mechanisms that cause cellular damage.[1] p21, a potent cell cycle inhibitor and a transcriptional target of p53, plays a vital role in the induction and maintenance of cellular senescence.[2,3] Both cell cycle arrest and cellular senescence have been demonstrated in a range of liver disorders and in diverse cell types, including hepatocytes,[4-6] cholangiocytes,[7,8] and stellate cells.[9] On one hand, senescence of hepatocytes is linked to fibrosis stage and disease progression in chronic liver disease.[4,6,10-12] On the other hand, senescence of hepatic stellate cells is associated with regression of liver fibrosis.[9,13,14] However, the mechanisms linking cellular senescence to liver disease progression are complex and remain to be clarified.

Non-alcohol-related fatty liver disease (NAFLD), a leading cause of liver-related morbidity and mortality worldwide, encompasses a wide spectrum of liver disease from simple steatosis through non-alcoholic steatohepatitis to fibrosis and cirrhosis.[15-18] Simple steatosis is often considered benign; only a proportion progress to steatohepatitis and fibrosis.[17,18] Established risk factors for disease progression in NAFLD include older age, presence of steatohepatitis, presence of fibrosis, and features of the metabolic syndrome, such as obesity, insulin resistance, and hypertension.[18-20] Genetic factors have also been shown to influence disease progression in NAFLD,[18,21] and such influence is considered the consequence of complex interplay between genetic variations and environmental factors.[21,22] The genetic factors identified so far can be grouped into 3 mechanistic groups:[18] those involved in the handling and metabolism of glucose, free fatty acids, or cholesterol;[23-27] those that influence cellular oxidative stress, endotoxin response, or cytokine and adipokine activity;[28,29] and those that might influence hepatic fibrogenesis.[30,31] The effect of variants in genes involved in cellular senescence on disease progression has not yet been explored.

The protein p21 is encoded by *CDKN1A*. Studies in diverse conditions showed that *CDKN1A* variants are associated with the rate of disease progression. For example, *CDKN1A* variants are

*Correspondence to: Graeme JM Alexander; Email: gja1000@doctors.org.uk; Quentin M Anstee; Email: quentin.anstee@newcastle.ac.uk
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associated with rapid progression of idiopathic pulmonary fibrosis,[32] increased risk of death in non-small cell lung cancer,[33] and joint involvement in systemic lupus erythematosus.[34] It is plausible that variants in *CDKN1A* might play a similar role in disease progression of NAFLD. This within-case association study of patients with NAFLD explores the relation of *CDKN1A* with steatohepatitis and fibrosis, using a cohort from the UK and an independent cohort from Finland.

Results

Demographics and genetic frequencies of p21 SNPs

There were 323 patients in the UK cohort and 123 patients in the Finnish cohort. The median age was 53 y (range 16–80) in the UK cohort and 46 y (range 24–71) in the Finnish cohort. There were more males in the UK cohort (59.2%), but the majority were females in the Finnish cohort (57.6). In the UK and Finnish cohorts, the median BMI was 34 (22.5–52.0) and 43.8 (15.6–62.5); 41.4% and 37.6% were diabetic; the median ALT (normal < 50 IU/l) was 62 IU/l (13–504) and 40 IU/l (8–299), respectively (**Table 1**).

Analysis of SNPs in the UK cohort

In univariate analysis of the UK data set, rs762623 ($P = 0.003$), rs2395655 ($P = 0.002$), and rs738409 ($P = 0.003$) were associated with fibrosis at the Bonferroni-corrected level of significance ($P < 0.0071$), and rs3176329 ($P = 0.025$) was associated with fibrosis at nominal level of significance ($P < 0.05$) (**Table 2A**). In logistic regression conditioned on rs762623, association with rs2395655 and rs3176329 did not remain significant, reflecting linkage disequilibrium (LD) between these SNPs (**Table S1A and B**). In addition, in logistic regression analysis of rs738409 conditioned on rs762623, the effect of rs762623 at *CDKN1A* was independent of rs738409 at *PNPLA3* (**Table S2A**).

Unlike in fibrosis, in univariate analysis of the UK cohort, only rs738409 ($P = 0.002$) was associated with steatohepatitis at the Bonferroni-corrected level of significance ($P < 0.0071$). However, the 3 SNPs across *CDKN1A* that were associated with fibrosis, rs762623 ($P = 0.009$), rs2395655 ($P = 0.016$), and rs3176329 ($P = 0.015$), were associated with steatohepatitis at nominal level of significance ($P < 0.05$) (**Table 2B**). Interestingly, there was a very close association noted between fibrosis and steatohepatitis (OR 24.233 (12.819–45.810; $P = 1.02 \times 10^{-22}$).

In univariate analysis, rs738409 ($P = 0.0012$) and rs12214686 ($P = 0.0057$) were associated with steatosis. None of the 3 SNPs across *CDKN1A*, which were associated with fibrosis and steatohepatitis, demonstrated any association with steatosis.

Table 1. Characteristics of patients in the UK and Finnish cohorts

Variable	UK (n = 323)	Finnish (n = 123)	*P* value
Male	197 (61.0%)	53 (43.1%)	9.76E-04
Female	126 (39.0%)	70 (56.9%)	
Diabetes mellitus	129 (40.8%)	47 (38.2%)	0.82
Age	52.0 (43.0–60.0)	46.0 (41.0–56.0)	1.44E-03
BMI	34.0 (30.1–37.0)	43.8 (34.0–49.0)	2.45E-15
ALT	62.0 (40.0–99.0)	40.5 (31.0–60.0)	5.97E-07
Fibrosis ≥ stage 1	210 (65.0%)	46 (37.4%)	2.42E-07
Steatohepatitis	175 (54.2%)	38 (30.9%)	0.00001

This table shows that in the UK vs. the Finnish cohort, there were significant differences by the chi-square test in the proportion of male patients; the proportion of patients with diabetes mellitus, and the proportion of patients with liver fibrosis ≥ stage 1. Furthermore, there were significant differences by the Wilcoxon summed-rank test in the distributions of age, body mass index (BMI) and the serum alanine transaminase (ALT).

Table 2.

A. Univariate analysis for association between SNPs and fibrosis in the UK cohort (n = 323)			
SNP-allele	**Reference allele**	**OR (L95–U95)**	***P* value**
rs12214686	G	0.755 (0.458–1.243)	0.269
rs762623	A	2.401 (1.330–4.336)	0.004
rs2395655	G	1.740 (1.210–2.502)	0.003
rs3176326	A	1.342 (0.883–2.039)	0.169
rs3176329	T	0.548 (0.324–0.926)	0.025
rs1801270	A	0.716 (0.372–1.378)	0.317
rs738409	G	1.705 (1.196–2.428)	0.003
B. Univariate analysis for association between SNPs and steatohepatitis in the UK cohort (n = 323)			
SNP-allele	**Reference allele**	**OR (L95–U95)**	***P* value**
rs12214686	G	0.733 (0.443–1.214)	0.228
rs762623	A	2.057 (1.194–3.543)	0.009
rs2395655	G	1.548 (1.086–2.207)	0.016
rs3176326	A	1.312 (0.856–2.011)	0.212
rs3176329	T	0.487 (0.272–0.871)	0.015
rs1801270	A	0.479 (0.242–0.947)	0.034
rs738409	G	1.755 (1.236–2.492)	0.002

Results of univariate logistic regression analysis for association between each SNP and steatohepatitis in the UK cohort. Allele refers to the minor allele as well as the reference allele in the analysis. The Bonferroni-corrected level of significance in this analysis was $P < 0.0071$. SNP, single nucleotide polymorphism; OR, odds ratio; L95, lower 95% confidence interval of the OR; U95, upper 95% confidence interval of the OR.

Association of rs762623 with fibrosis remained significant at $P < 0.05$ in multivariate analysis with the covariates, sex, age, BMI, and DM indicating that the effect of rs762623 is independent of all the clinical variables included in the model (**Table S3**). Of note, age, BMI, and DM were also associated with fibrosis in the multivariate analysis. The SNP rs762623 was taken forward for genotyping in the Finnish cohort.

Analysis of rs762623 and rs738409 in the Finnish cohort

In univariate analysis of the Finnish data set, rs762623 ($P = 0.02$) and rs738409 ($P = 0.005$) were associated with fibrosis at $P < 0.05$ (**Table 3**). Similar to the UK cohort, in the Finnish cohort, the effect of rs762623 at *CDKN1A* was independent of rs738409 at *PNPLA3* (**Table S2B**).

In univariate analysis for association with steatohepatitis, only rs738409 ($P = 0.002$), but not rs762623 ($P = 0.427$), remained significant. Neither rs738409 ($P = 0.059$) nor rs762623 ($P = 0.901$) were associated with steatosis.

In multivariate analysis of rs762623 with the covariates, sex, age, BMI, and DM, the association between fibrosis and rs762623 remained significant at $P < 0.05$ (**Table S4**). DM was also associated with presence of fibrosis in the multivariate analysis.

Analysis of combined UK and Finnish data sets

Inverse variance meta-analysis using a fixed effect model confirmed that rs762623 was associated with fibrosis in the combined UK and Finland cohort ($P = 0.0002$) (**Table 4**). Furthermore, a combined analysis of individual-level data from the UK and Finnish cohorts using logistic regression in which nationality was included as a covariate also demonstrated an independent association between rs762623 and fibrosis (**Table S5**).

Multinomial logistic regression analysis of rs762623 in the combined UK and Finnish data sets

A multinomial logistic regression analysis of rs762623 with nationality as a covariate was performed to investigate the risk conferred by the variant of developing different stages of fibrosis (**Table 5**). The risk of developing advanced fibrosis (e.g., stage 3 vs. no fibrosis, OR = 2.27, 95%CI 1.19–4.31) was comparable to the risk of developing early fibrosis (e.g., stage 1 vs. no fibrosis, OR = 2.15, 95%CI 1.28–3.59). Therefore the analysis was repeated, sequentially altering the reference stage of fibrosis to stages 1 through 4. For patients with early fibrosis, rs762623 did not confer significant risk of developing more advanced fibrosis (e.g., stage 3 vs. stage 1 fibrosis, OR = 1.06, $P = 0.866$). In contrast, multinomial logistic regression analysis of DM with nationality as a covariate showed that for patients with early fibrosis, DM confers significant risk of developing more advanced fibrosis (e.g., stage 3 vs. stage 1 fibrosis, OR = 2.44, $P = 0.009$; stage 4 vs. stage 1 fibrosis, OR = 3.24, $P = 0.007$) (**Table S6**).

Discussion

In this within-case association study, the candidate gene *CDKN1A* was studied for association with NAFLD-related liver disease progression in patient groups from the UK and Finland. Both cohorts were selected carefully to only include those with biopsy-proven NAFLD. Six SNPs across *CDKN1A* were studied in the UK cohort, and rs762623 was shown to be associated with liver fibrosis. Association with rs762623 was confirmed in a distinct second cohort from Finland and in combined analysis of the UK and Finnish data sets.

Only a minority of patients with NAFLD progress to steatohepatitis and fibrosis and the evolution of fibrosis is a critical hallmark of progressive disease. With continued injury, fibrosis, once present, progresses eventually to liver cirrhosis. This study examined the impact of *CDKN1A* variants on liver fibrosis by comparing NAFLD patients with no fibrosis to those with fibrosis thereby demonstrating a link between rs762623 at *CDKN1A* (p21) and fibrosis. Interestingly, though rs762623 seems to influence the development of fibrosis in NAFLD, it does not affect the progression once fibrosis has been initiated. One explanation is that the cohort is inadequately powered to observe the effects among fibrosis stage 1, 2, 3, and 4; however, an alternative explanation is that rs762623 influences development but not the progression

Table 3. Univariate analysis for association between SNPs and fibrosis in the Finnish cohort (n = 123)

SNP-Allele	OR (L95–U95)	*P* value
rs762623-A	2.251 (1.125–4.503)	0.022
rs2395655-G	1.133 (0.654–1.964)	0.655
rs738409-G	2.378 (1.292–4.379)	0.005

Allele refers to the minor allele, which was also the reference allele in the analysis. SNP, single nucleotide polymorphism; OR, odds ratio; L95, lower 96% confidence interval of the odds ratio; U95, upper 95% confidence interval of the OR.

Table 4. Meta-analysis of rs762623 and rs2395655

SNP	RR (L95–U95)		Meta-analysis: Fixed effect model		Test of heterogeneity		
	UK	Finnish	Meta-RR (L95–U95)	Meta-*P*	I^2 (%)	*Q*	*P*
rs762623-A	2.40 (1.33–4.34)	2.25 (1.13–4.50)	2.34 (1.49–3.66)	0.0002	0.0	0.02	0.890
rs2395655-G	1.74 (1.21–2.50)	1.13 (0.65–1.96)	1.53 (1.13–2.07)	0.0062	38.5	1.62	0.202

Inverse variance meta-analysis of summary statistics for rs762623 and rs2395655 from the UK and Finnish data sets. A fixed effect model was adopted because measures of heterogeneity were not significant. SNP, single nucleotide polymorphism; RR, relative risk; L95, lower 95% confidence interval of the relative risk; U95, upper 95% confidence interval of the relative risk; Meta-RR, the summary relative risk; Meta-P, the summary *P* value.

Table 5. Multinomial logistic regression analysis of rs762623 with nationality included as a covariate

Fibrosis stage	EST (SE)	OR (L95–U95)	*P* value
Stage 0 vs. stage 1	0.7632 (0.2631)	2.145 (1.281–3.593)	0.0037
Stage 0 vs. stage 2	1.1522 (0.3151)	3.165 (1.707–5.869)	0.0003
Stage 0 vs. stage 3	0.8175 (0.3279)	2.265 (1.191–4.307)	0.0127
Stage 0 vs. stage 4	0.6975 (0.4146)	2.009 (0.891–4.527)	0.0925
Stage 1 vs. stage 2	0.3890 (0.3066)	1.476 (0.809–2.691)	0.2045
Stage 1 vs. stage 3	0.05432 (0.3206)	1.056 (0.563–1.979)	0.8655
Stage 1 vs. stage 4	−0.0657 (0.4079)	0.936 (0.421–2.083)	0.8720
Stage 2 vs. stage 3	−0.3347 (0.3487)	0.716 (0.361–1.417)	0.3372
Stage 2 vs. stage 4	−0.4548 (0.4287)	0.635 (0.274–1.470)	0.2888
Stage 3 vs. stage 4	−0.1201 (0.4366)	0.887 (0.377–2.087)	0.7833

Multinomial logistic regression to determine the effect of rs762623 on the development of different stages of fibrosis in the combined UK and Finnish Cohort, with nationality included as a covariate. The reference level of fibrosis ranges from fibrosis stage 0 to 4. EST, estimate of the effect; SE, standard error of the estimate; OR odds ratio; L95 lower 95% confidence interval of the odds ratio; U95 upper 95% confidence interval of the OR.

of fibrosis. This suggests that the initiation and progression of fibrosis in NAFLD may have different underlying pathophysiology and highlights how genetic variations might play a role.

p21 plays a vital role in the induction and maintenance of cellular senescence. Senescence cells secrete a variety of biologically active factors that alter the microenvironment and exert effects on other cells in paracrine fashion.[35] Hepatocyte p21 expression was associated independently with fibrosis stage in NAFLD.[4,6,11] A positive paracrine effect from senescent hepatocytes on hepatic stellate cell activation is one explanation for this association and is consistent with a recent study.[36] However, stellate cell senescence leads to reduced liver fibrosis,[9] as senescent cells lose their function specific to that cell type during senescence.[37] A p21 SNP variant that leads to increased p21 expression is likely to cause increased hepatocyte p21 expression and activation of stellate cells to cause liver fibrosis, but may also induce increased stellate cell senescence, reducing liver fibrosis. Similarly, a p21 SNP that leads to reduced p21 expression may reduce activation of stellate cells through reduced hepatocyte p21 expression but increase fibrosis through reduced stellate cell senescence. Hence, the end result is the difference between these 2 opposing mechanisms. Interestingly, rs762623 is located in the promoter region of p21 and could affect p21 expression by modifying the promoter activity. Notably for rs762623, substitution of the G allele by the A allele has been shown to abolish the E2F binding site and reduce p21 expression.[34]

PNPLA3 locus (rs738409) has been shown to influence histological severity and disease progression in NAFLD.[25,27] Corroborating these findings, in this study, rs738409 was associated with steatohepatitis and fibrosis in both UK and Finnish cohorts and therefore validates these 2 cohorts. More interestingly, this study demonstrated the influence of rs762623 (*CDKN1A*) on fibrosis to be independent of this well-recognized rs738409 (*PNPLA3*).

In conclusion, this study demonstrates for the first time an association between rs762623, a p21 SNP located in the promoter region of p21, and development of fibrosis in NAFLD. However, this SNP did not influence the progression of liver fibrosis.

Materials and Methods

Patients

Patients with biopsy-proven NAFLD were prospectively recruited from the UK and Finland. The study had all necessary ethical approvals (REC reference: 11/NE/0356) in both countries and informed written consent from participants.

In these respective units, all new patients undergo liver biopsy where there is a clinical suspicion of NAFLD based on (1) persistently abnormal liver biochemistry; (2) negative chronic liver disease screen, i.e., no laboratory evidence of chronic viral hepatitis, autoimmune liver disease, hemochromatosis, α1-antitrypsin deficiency, or Wilson disease; (3) insubstantial intake of alcohol, i.e., alcohol intake ≤ 30 g/d for males and ≤ 20 g/day for females; and (4) fatty liver on ultrasound scan.

In this study, patients were prospectively recruited when they attended for liver biopsy to evaluate a potential diagnosis of NAFLD. Patients were excluded from analysis if the biopsy showed an additional or alternative cause of liver disease. A total of 323 patients from Newcastle upon Tyne or Cambridge (UK cohort) and 123 patients from Helsinki (Finnish cohort) across all stages of NAFLD were enrolled. Clinical and laboratory data were collected on the date a diagnostic liver biopsy was performed. These data included the age, sex, body mass index (BMI), and the presence of diabetes mellitus (DM).

Liver biopsy and interpretation of slides

Liver biopsy specimens were fixed in formalin and stained with hematoxylin and eosin, impregnated with silver for visualizing reticulin framework, and trichrome for visualizing collagen. These were reviewed by a single histopathologist at each participating center, blinded to the clinical or genetic information. The histological diagnosis of steatohepatitis was based on evidence of hepatocellular injury and inflammation. The stage of fibrosis was scored according to the well-validated NIDDK NASH CRN Kleiner score:[38] fibrosis stage 0 = no fibrosis; stage 1 = isolated perisinusoidal or portal fibrosis; stage 2 = perisinusoidal and portal/periportal fibrosis; stage 3 = septal or bridging fibrosis; and stage 4 = cirrhosis.

The aim of the study was to investigate the possible role of *CDKN1A* variants in the development and propagation of disease progression of NAFLD. Therefore, fibrosis stages 1 to 4 were grouped together and compared with NAFLD without fibrosis

(stage 0). Similarly, presence and absence of steatohepatitis was also compared.

DNA preparation

DNA was prepared from peripheral blood lymphocytes collected at the time of liver biopsy, as described previously.[39] Genotyping was performed by personnel unaware of clinical status or histology of patients.

Identification of tag-SNPs across *CDKN1A*

The National Center for Biotechnology Information (NCBI) Gene database was used to define the physical coordinates of *CDKN1A* at 6p21.2 as 36644237 to 36655116 on build 37.3 (GRCh37 assembly), corresponding to 36752215 to 36763094 on build 36.3 (reference assembly) (http://www.ncbi.nlm.nih.gov/gene/1026). Tagger in Haploview 4.2 was used to identify SNPs tagging *CDKN1A*, using the following specifications: HapMap download, version 3, release R2; analysis panel CEU+TSI; chromosome 6, start position 36750 kb, end position 36765 kb (build 36.3, reference assembly); Hardy–Weinberg *P* value cut-off 0.001; minimum minor allele frequency 0.05; pairwise tagging only, and r^2 threshold 0.8. The SNP rs1801270 was force-included. Six tag-SNPs were identified using this strategy: rs12214686, rs762623, rs2395655, rs3176326, rs3176329, and rs1801270 (**Table 6**).

Identification of SNPs at *PNPLA3*

To date, the only locus shown in more than one study to be associated with histological severity in NAFLD is the *PNPLA3* locus on chromosome 22.[25,27] We selected rs738409 at *PNPLA3* for genotyping in the UK and Finnish populations to (1) confirm association with this locus; and (2) validate the cohorts, themselves.

Genotyping

Genotyping was performed by Kbiosciences. The Kbiosciences competitive allele-specific PCR (KASP) genotyping system is a homogenous, fluorescent, endpoint genotyping system that uses 3 unlabelled primers and a universal reaction mix based on a proprietary method (www.kbioscience.co.uk). All assays were validated before use with a standard 96-well validation plate used by Kbiosciences. Quality control (QC) measures included negative (water only) controls and genotyping replicate DNA samples.

Genotype quality control and statistical analysis

Analysis of genotype data was performed using PLINK version 1.07 and R 2.13.2. In the UK data set, individuals or markers that failed the following QC criteria were excluded: missingness per individual (–mind in PLINK) < 0.2; minor allele frequency (–maf) > 0.05; missingness per marker (–geno) < 0.1; Hardy-Weinberg test (–hwe) $P < 0.001$. The same QC criteria were applied to the Finnish data set, apart from a threshold of 0.5 for missingness per individual, selected because only 2 markers were genotyped.

Table 6. Selected tag-SNPs across CDKN1A

CHR	SNP	BP	A1/A2	MAF (CEU)
6	rs12214686	36642551	G/A	0.156
6	rs762623	36645466	A/G	0.127
6	rs2395655	36645696	G/A	0.393
6	rs3176326	36647289	A/G	0.212
6	rs3176329	36647463	T/G	0.068
6	rs1801270	36651971	A/C	0.063

Tag-single nucleotide polymorphisms (SNPs) across CDKN1A, selected for genotyping in the UK ascertainment cohort. CHR, chromosome; BP, base pairs (build 36.3, reference assembly); A1, minor allele; A2, major allele; MAF, minor allele frequency in the HapMap CEU sample

Comparison of categorical variables was undertaken using the chi-square test. Comparison of continuous variables was undertaken using the Wilcoxon signed-rank test. To test the association of SNPs with steatohepatitis and fibrosis, univariate logistic regression analysis of each SNP in turn was undertaken. To confirm that SNPs were independently associated with fibrosis or steatohepatitis, multivariate logistic regression of each SNP in turn with the covariates, sex, age, BMI, and DM was undertaken. To test whether the SNPs themselves had independent effects, logistic regression was undertaken of one SNP with the second SNP included as a covariate.

In these analyses, the dependent variable (i.e., fibrosis or steatohepatitis) was binomial (i.e., "no fibrosis" [stage 0] vs. "any fibrosis" [stages 1–4] or steatohepatitis vs. no steatohepatitis). For combined analysis, inverse variance meta-analysis of summary statistics from the UK and Finnish analyses using the R package "meta" was undertaken. Binomial logistic regression of the combined UK and Finland genotype data set was also performed with nationality included as a covariate. To determine whether *CDKN1A* variants influence the stage of fibrosis, multinomial logistic regression was performed, in which the dependent variable (i.e., fibrosis) was multinomial (i.e., stage 0 vs. stages 1, 2, 3, or 4 fibrosis).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/cc/article/28471

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