

The *ATG16L1* gene variant rs2241880 (p.T300A) is associated with susceptibility to HCC in patients with cirrhosis

Philipp A. Reuken¹ | Philipp Lutz^{2,3} | Markus Casper⁴ | Eihab Al-Herwi¹ |
Sven Stengel¹ | Ulrich Spengler^{2,3} | Andreas Stallmach¹ | Frank Lammert⁴ |
Hans Dieter Nischalke^{2,3} | Tony Bruns^{1,5}

¹Department of Internal Medicine IV (Gastroenterology, Hepatology and Infectious Diseases), Jena University Hospital, Jena, Germany

²Department of Internal Medicine I, University of Bonn, Bonn, Germany

³German Center for Infection Research, Bonn, Germany

⁴Department of Medicine II, Saarland University Medical Center, Saarland University, Homburg, Germany

⁵Medical Department III, University Hospital of Aachen, Aachen, Germany

Correspondence

Philipp A. Reuken, Department of Internal Medicine IV, Jena University Hospital, Am Klinikum 1, 07747 Jena, Germany.
Email: philipp.reuken@med.uni-jena.de

Funding information

PAR received funding from IZKF at the Jena University Hospital. TB received funding from the German Research Foundation (TB 4182/3-1). HDN and US were funded by the Deutsche Krebsstiftung (70112169).

Handling Editor: Alexander Thompson

Abstract

Background and aims: Protein and organelle turnover by autophagy is a key component to maintain cellular homeostasis. Loss of the autophagy protein *ATG16L1* is associated with reduced bacterial killing and aberrant interleukin-1 β production, perpetuating inflammation and carcinogenesis. Here we hypothesized that the functional p.T300A gene variant in *ATG16L1* is associated with an increased risk for hepatocellular carcinoma (HCC) in cirrhosis.

Methods: A case-control study was performed using a prospective derivation cohort (107 patients with HCC and 101 controls) and an independent validation cohort (124 patients with HCC and 108 controls) of patients with cirrhosis of any aetiology. *ATG16L1* p.T300A (rs2241880) and *PNPLA3* p.I148M (rs738409) variants were determined by real-time PCR.

Results: The G allele of the *ATG16L1* p.T300A variant was more frequent in patients with HCC compared to controls without HCC in the derivation cohort (0.62 vs. 0.51, $P = .022$) and in the validation cohort (0.59 vs. 0.50, $P = .045$). In combined analysis, the odds ratios (OR) were 1.76 (95% CI: 1.07-2.88) for G allele positivity and 2.43 (95% CI: 1.37-4.31) for p.T300A G allele homozygosity. This association was independent from the presence of a *PNPLA3* variant, which was also associated with HCC (OR 2.10; 95% CI: 1.20-3.66), and it remained significant after adjustment for male sex, age and aetiology in multivariate analysis.

Conclusion: The common germ-line *ATG16L1* gene variant is a risk factor for HCC in patients with cirrhosis. Personalized strategies employing the genetic risk conferred by *ATG16L1* and *PNPLA3* may be used for risk-based surveillance in cirrhosis.

KEYWORDS

ATG16L1, autophagy, cirrhosis, genetic polymorphisms, hepatocellular carcinoma

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BCLC, Barcelona clinic liver cancer; GWAS, Genome Wide Association Study; HCC, hepatocellular carcinoma; IL, interleukin; INR, international normalized ratio; MELD, model for end-stage liver disease; NOD2, nucleotide-binding oligomerization domain-containing 2; OR, odds ratio; PCR, polymerase chain reaction; SNP, single-nucleotide polymorphism; TLR, toll-like receptor.

Hans Dieter Nischalke and Tony Bruns are contributed equally to this work.

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2019 The Authors. *Liver International* published by John Wiley & Sons Ltd.

1 | INTRODUCTION

The development of hepatocellular carcinoma (HCC) is a frequent complication in cirrhosis, accounting for up to 8% of all cancers in males and up to 6% in females.¹ The incidence of HCC has been increasing affecting currently up to 4.8 per 100 000 inhabitants who have a poor overall survival.² Curative treatment options, including surgical resection, curative radiofrequency ablation and liver transplantation, are only available to <30% of patients due to advanced tumour stage at diagnosis.³ Therefore, the early detection of HCC in surveillance programmes is crucial for successful treatment. Currently, ultrasound surveillance at six month intervals is recommended for all patients at high risk, but patients who might benefit most from intensified surveillance programmes are insufficiently characterized.⁴

The identification of genetic and environmental risk factors for HCC may improve such surveillance strategies. Whereas genome-wide association studies (GWAS) have identified genetic risk loci for HCC in large Asian populations with predominantly viral hepatitis,⁵⁻⁷ genetic risk factors for non-viral HCC are less well characterized.⁸ Given the close relation between inflammation and hepatic carcinogenesis, variants in genes encoding for chemokines and cytokines have often been the subject of candidate gene association studies on HCC.⁹⁻¹² In a recent large candidate-gene study, Stickel et al¹³ confirmed an increased risk for HCC in patients with alcohol-related cirrhosis carrying genetic variants of Patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) and Transmembrane 6 superfamily 2 (*TM6SF2*)—genes related to hepatic lipid and fatty acid turnover.¹³

Autophagy is a central mechanism for maintaining homeostasis, metabolism and survival,¹⁴ thereby stabilizing the cell against metabolic stress and apoptosis.¹⁵ In carcinogenesis, autophagy plays a dual role: although maintaining autophagy is important for the survival of already established tumours, autophagy can also suppress the initiation and development of malignant tumours and decrease the susceptibility to carcinogens, especially under inflammatory conditions.^{16,17} In addition there is a role for autophagy in limiting immune activation and inflammation.¹⁸ Absence of autophagy-related protein 16-1 (*ATG16L1*) or a loss of function mutation promotes TLR4- and NOD2-dependent inflammation resulting in an increased release of IL-1 β by innate immune cells.¹⁹⁻²² In addition, the coding *ATG16L1* gene variant p.T300A affects intestinal barrier function and impairs antibacterial autophagy.²³

We hypothesized a role of *ATG16L1* in hepatic carcinogenesis and investigated the association of the *ATG16L1* gene variant p.T300A with HCC in a case-control study including 440 patients with cirrhosis from three German academic centres.

2 | MATERIALS AND METHODS

2.1 | Study design

For this candidate gene case-control study, we recruited two patient cohorts. The derivation cohort consisted of 208 patients

Key points

- This case-control study identifies a common *ATG16L1* p.T300A polymorphism as a substantial risk factor for hepatocellular carcinoma (HCC) in patients with cirrhosis of any aetiology
- The *ATG16L1*-attributed HCC risk in this study is more pronounced in women, older patients and in patients with viral hepatitis
- *ATG16L1* p.T300A remains associated with HCC after adjusting for confounding factors including the *PNPLA3* p.I148M genotype
- *ATG16L1* and *PNPLA3* genotyping may help to personalize HCC surveillance in patients with cirrhosis.

with cirrhosis (107 cases and 101 controls) that were consecutively recruited between January 2011 and January 2016 at the Jena University Hospital. Patients were stratified for HCC according to current guidelines either by histological criteria or contrast-enhanced imaging technologies (CT, MRI, contrast-enhanced ultrasound). Patients included as cases with HCC were eligible for this study at the time of diagnosis and or referral to our centre. 54 (50%) patients with HCC were participating in HCC surveillance programmes for a median of 4 years prior to diagnosis (range: 0.5 to 23), whereas 53 presented with HCC outside of surveillance programmes (Figure S1). Patients included as cirrhotic controls without HCC underwent surveillance in the majority of cases (95/101; 94%) for a median of 24 months (range: 1 to 96 months) with a median total imaging procedures of 2 (range: 1 to 15) before inclusion and a subsequent median 36 months (range 0 to 92) of HCC surveillance with a median of total imaging procedures of 3 (range 0 to 17) in order to exclude HCC. One patient, who was originally assigned to the control group, developed an HCC in follow-up after 84 months and was analysed as HCC case for final analysis.

The independent validation cohort consisted of 232 patients (124 cases and 108 controls) from three centres: Jena University Hospital (recruited between January 2016 and February 2017), Bonn University Hospital and Saarland Medical Center Homburg.

Cirrhosis was defined by histological criteria or by a combination of clinical, imaging and laboratory data. Staging, comorbidities, medication and laboratory values including the model for end-stage liver disease (MELD) and the Child-Pugh-Score at baseline were extracted using medical records including patients' files, electronic health records and nursing documentation. Survival and transplantation status within one year after inclusion were assessed by reviewing medical records and interviewing the patients or the general practitioner. The study was approved by the local ethics committees ('Ethikkommission der Friedrich-Schiller Universität Jena an der Medizinischen Fakultät' 3150-06/11,

'Ethikkommission bei der Ärztekammer des Saarlandes' Homburg 79/12 and 'Ethikkommission an der Medizinischen Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn' 362/15) and written informed consent was given by all patients prior to study inclusion.

2.2 | Genotyping

Genomic DNA was extracted from whole blood using the QIAmp DNA mini kit (Qiagen, Hilden, Germany). The DNA concentration was measured with a NanoDrop ND-1000 (PepLab, Erlangen, Germany) and calibrated to 20 ng/μl. Genotyping for the *ATG16L1* variant rs2241880 and the *PNPLA3* variant rs738409 was performed by real-time PCR using the TaqMan Universal Master Mix II and TaqMan SNP Genotyping Assays (C__9095577_20 and C__7241_10) (ThermoFisher Scientific, Germany). Determination of the *PNPLA3* rs738409 polymorphism in the Bonn cohort was performed via LightCycler real-time PCR (Roche, Mannheim, Germany) using LightSNiP (SimpleProbe) assays from TIB-MolBiol (Berlin, Germany). A subset of patients in the validation cohort has been reported in a previous case-control study.¹³

To validate the rs2241880 genotyping assay, Sanger sequencing of 15 randomly selected patient samples was performed (five homozygous wild type, five homozygous mutant and five heterozygous for rs2241880). *ATG16L1* genomic DNA was amplified using Platinum Taq DNA Polymerase (ThermoFisherScientific, Germany) as described by the manufacturer and amplification primers FW: ACAGGTTAGTGTGCAGGAGA and REV: CAGTCAGCTCTGCCATTACA (95°C 2 minutes, 33 cycles of 95°C 30 seconds–60°C 20 seconds–72°C 30 seconds). The PCR product

was cleaned using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Germany) (NTI-Buffer-Dilution 1:7). Sanger sequencing was performed by Macrogen (Macrogen Europe, Netherlands) using the sequencing primer TGCAGGAGAGTAAGGCATGT (Figure 1).

2.3 | Statistical analysis

Statistical analysis was performed using SPSS 16 and SPSS 23 (IBM, Armonk, NY). Baseline characteristics are presented as median and range for continuous variables or as frequency for discrete variables. Statistical differences were analysed using the Mann-Whitney U test for continuous variables and Fisher's Exact Test for discrete variables. Allele frequency for SNP-analysis were tested for deviation from the Hardy-Weinberg equilibrium and compared to detected statistical differences using the Cochran Armitage trend test (<https://ihg.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl>). Univariate and multivariate binary logistic regression analyses were performed with HCC as the dependent variable after adjusting sex, age and aetiology (Multivariate model 1) or adjusting for sex, age, aetiology and *PNPLA3* genotype (Multivariate model 2). Survival was analysed using Kaplan-Meier analysis and log-rank test. $P < 0.05$ in two-sided tests were regarded significant.

3 | RESULTS

3.1 | Patient characteristics

In both cohorts, patients with HCC were more frequently male (83.2% vs. 70.3%, $P = .003$; 80.6% vs. 66.7%, $P = .015$) and were older (median 66 vs. 59 years, $P < .001$; 65 vs. 57 years, $P < .001$) as

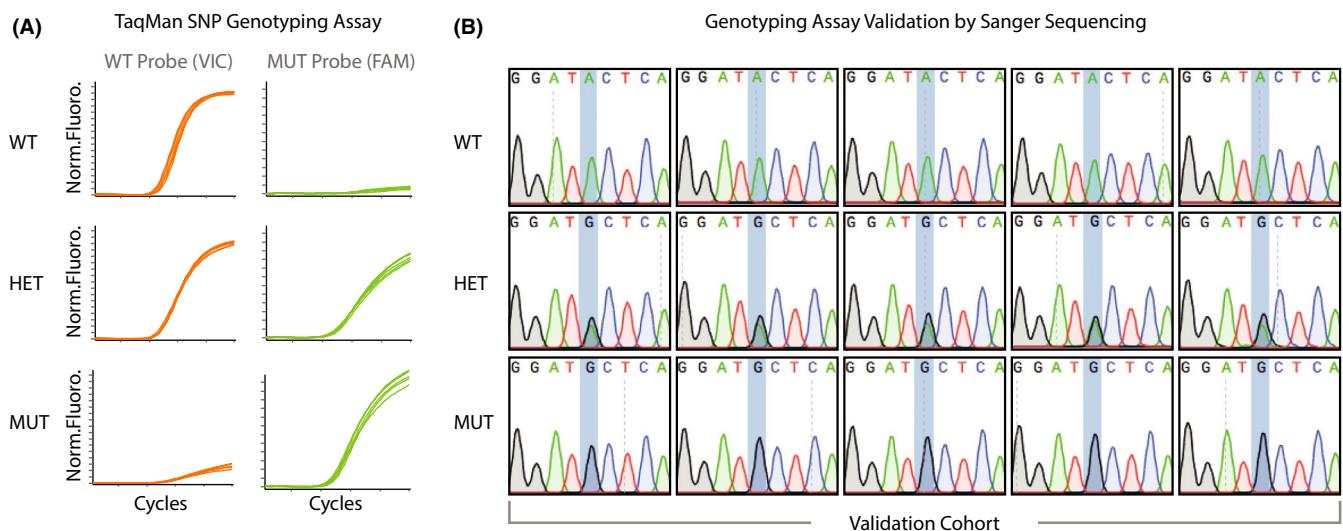


FIGURE 1 *ATG16L1* rs2241880 Genotyping. A, Principle of end-point quantitative TaqMan PCR-based genotyping assay. TaqMan polymerase chain reaction (PCR) was performed using wild-type and mutant-specific TaqMan-probes (VIC and FAM fluorescent dye). Normalized fluorescent signals were plotted against PCR cycle number and genotypes were called dependent on amplification signals (WT: VIC-signal but no FAM-signal, HET: VIC-signal and FAM-signal, MUT: no VIC signal but FAM-signal). B, Genotyping Assay Validation by Sanger Sequencing. *ATG16L1* deoxyribonucleic acid (DNA) from five randomly selected patients was amplified by PCR using *ATG16L1*-specific primers. DNA sequence was revealed by Sanger Sequencing (rs2241880 highlighted in light blue) and compared to genotypes identified by TaqMan quantitative PCR

TABLE 1 Demographic characteristics of the study population

	Derivation cohort			Validation cohort		
	HCC in cirrhosis	Cirrhosis	P value	HCC in cirrhosis	Cirrhosis	P value
Numbers of patients	107	101	N/A	124	108	N/A
Male sex	89 (83.2%)	71 (70.3%)	0.033	100 (80.6%)	72 (66.7%)	0.015
Age (years)	66 (58; 71)	59 (52; 69)	<0.001	65 (59; 72)	57 (49; 67)	<0.001
Aetiology						
Alcoholic	70 (65.4%)	65 (64.4%)	0.10	77 (62.1%)	94 (87.0%)	<0.001
Viral	15 (14.0%)	12 (11.9%)		35 (28.2%)	14 (13.0%)	
Autoimmune/ cholestatic	3 (2.8%)	10 (9.9%)		0	0	
Hemochromatosis	8 (7.5%)	2 (2.0%)		1 (0.8%)	0	
Cryptogenic/other	11 (10.3%)	12 (11.9%)		11 (8.9%)	0	
MELD ^a	13 (9; 19)	12 (7; 19)	0.11	16 (12; 21)	15 (11; 20)	0.49
Child-Pugh stage						
Child A	21 (19.6%)	30 (29.7%)	0.24	33 (30.0%)	7 (6.5%)	<0.001
Child B	36 (33.6%)	29 (28.7%)		48 (43.6%)	50 (46.7%)	
Child C	50 (46.7%)	42 (41.6%)		29 (26.3%)	50 (46.7%)	
Bilirubin ($\mu\text{mol/l}$) ^a	32 (15; 58)	25 (14; 56)	0.21	34 (16; 58)	38 (16; 55)	0.93
INR	1.3 (1.1; 1.5)	1.2 (1.1; 1.5)	0.13	N/A	N/A	N/A
ALT ($\mu\text{mol/l} \times \text{s}$) ^a	0.85 (0.57; 1.33)	0.55 (0.41; 0.89)	0.001	0.67 (0.38; 1.31)	0.37 (0.25; 0.60)	0.001
AST ($\mu\text{mol/l} \times \text{s}$) ^a	1.13 (0.76; 2.25)	0.81 (0.55; 1.52)	0.001	1.17 (0.58; 2.18)	0.62 (0.40; 0.98)	<0.001
Platelets ($\times 10^3/\mu\text{l}$) ^a	134 (88; 194)	137 (86; 199)	0.72	126 (89; 164)	130 (90; 178)	0.57

Abbreviation: HCC, hepatocellular carcinoma.

^aData for MELD, ALT, AST, platelets and bilirubin were available from 392, 431, 399, 407 and 435 patients respectively.

compared to cirrhotic controls without HCC (Table 1). Aetiology of cirrhosis was predominantly alcoholic liver disease accounting for 69.5% across both cohorts. Cases and controls were well balanced for MELD score and platelet count at study inclusion. Patients with HCC had higher levels of aspartate aminotransferases (1.13 vs. 0.81 $\mu\text{mol/l} \times \text{s}$, $P = .001$ in the validation cohort; 1.17 vs. 0.62 $\mu\text{mol/l} \times \text{s}$, $P < .001$ in the derivation cohort) and alanine aminotransferases (0.85 vs. 0.55 $\mu\text{mol/l} \times \text{s}$ in the derivation cohort and 0.67 vs. 0.37 $\mu\text{mol/l} \times \text{s}$ in the validation cohort, $P < .001$ in both cohorts) than controls without HCC. Self-reported alcohol consumption within 30 days prior to inclusion was more frequent in patients without HCC (48.1% vs. 27.1%, $P = .002$).

There were no differences in the Child-Pugh stage between cases with HCC and controls without HCC at study inclusion in the derivation cohort. In the validation cohort, however, controls without HCC presented with a more advanced stage of liver disease as compared to patients with HCC (Table 1).

3.2 | Allele frequencies of ATG16L1 p.T300A (rs2241880)

In both cohorts, the genotypic frequencies for rs2241880 did not deviate from the Hardy-Weinberg equilibrium in cases and controls

($P > .10$). The frequencies of the rs2241880 G allele in patients with cirrhosis without HCC were 0.51 in the derivation cohort and 0.50 in the validation cohort (Table 2), therefore almost identical to healthy controls in Northern Germany (0.52) as reported by Hampe et al.²⁴

In contrast, patients with HCC presented with a significantly higher G allele frequency as compared to patients without HCC. The G allele frequency was enriched in the derivation cohort (0.62 vs. 0.51, $P = .022$) without a difference between patients undergoing HCC surveillance or not (0.63 vs. 0.61; $P = .80$) as well as in the validation cohort (0.59 vs. 0.50, $P = .045$).

In the combined analysis comprising 231 cases with HCC and 209 controls without HCC, the association between carriage of the G allele in rs2241880 and HCC was confirmed (Table 2). The odds ratios (OR) were 1.51 (95% confidence interval [CI]: 1.16-1.97) for allele frequency difference, 1.76 (95% CI: 1.07-2.88) for G allele positivity and 2.43 (95% CI: 1.37-4.31) for G allele homozygosity. The association of the ATG16L1 genotype with HCC remained significant after adjustment for male sex, age and aetiology in multivariate analysis 1 (Table 3). The association of HCC with the homozygous G allele was pronounced in women (OR 11.6, 95% CI 2.85-47.4), in patients with viral cirrhosis (OR 6.4, 95% CI 1.35-30.14) and in patients older than 65 years of age (OR 4.37, 95% CI 1.63-11.73).

TABLE 2 Case-control genotypes and allele frequencies of *ATG16L1* p.T300A (rs2241880)

Phenotype (n)	ATG16L1 p.T300A (rs2241880)			Freq. (G)	Allele frequency difference		Linear-by-linear association	
	AA	AG	GG		Odds ratio	P value	χ^2	P value
Derivation cohort								
Cirrhosis (101)	21 (20.8%)	57 (56.4%)	23 (22.8%)	51.0%	1.58 (1.07; 2.33)	0.022	6.07	0.014
HCC (107)	11 (10.3%)	59 (55.1%)	37 (34.6%)	62.1%				
Validation cohort								
Cirrhosis (108)	25 (23.1%)	58 (53.7%)	25 (23.1%)	50.0%	1.46 (1.01; 2.10)	0.045	4.01	0.048
HCC (124)	21 (16.9%)	59 (47.6%)	44 (35.5%)	59.3%				
Combined analysis								
Cirrhosis (209)	46 (22.0%)	115 (55.0%)	48 (23.0%)	50.5%	1.51 (1.16; 1.97)	0.003	9.83	0.002
HCC (231)	32 (13.9%)	118 (51.1%)	81 (35.1%)	60.6%				

Abbreviation: HCC, hepatocellular carcinoma.

TABLE 3 Logistic regression analysis of risk factors for hepatocellular carcinoma (HCC)

	Univariate analysis		Multivariate analysis 1		Multivariate analysis 2	
	Odds ratio (95% confidence interval)	P value	Odds ratio (95% confidence interval)	P value	Odds ratio (95% confidence interval)	P value
<i>ATG16L1</i>						
p.T300A Genotype	1.00 (reference)	0.008	1.00 (reference)	0.006	1.00 (reference)	0.005
AA	1.48 (0.88; 2.48)		1.53 (0.86; 2.70)		1.62 (0.91; 2.90)	
AG	2.43 (1.37; 4.31)		2.69 (1.43; 5.07)		2.89 (1.49; 5.46)	
GG						
Male sex	2.08 (1.33; 3.24)	0.001	2.74 (1.65; 4.54)	<0.001	2.79 (1.67; 4.65)	<0.001
Age (per 1-year increase)	1.07 (1.05; 1.09)	<0.001	1.07 (1.05; 1.10)	<0.001	1.07 (1.05; 1.10)	<0.001
Alcoholic liver disease	0.55 (0.36; 0.83)	0.005	0.44 (0.28; 0.71)	0.005	0.39 (0.24; 0.64)	<0.001
<i>PNPLA3</i>						
p.I148M Genotype	1.00 (reference)	0.03	Not included	Not included	1.00 (reference)	0.03
CC	1.33 (0.87; 2.01)				1.24 (0.78; 1.98)	
CG	2.10 (1.20; 3.66)				2.34 (1.25; 4.37)	
GG						

Note: Odds ratios and 95% confidence intervals from univariate and multivariate binary logistic regression with HCC as the dependent variable in the combined cohort are shown. Reference categories are indicated. Multivariate models include the indicated significant variables from univariate analysis with (model 2) and without (model 1) *PNPLA3* p.I148M genotype as a covariate.

3.3 | HCC phenotypes

To analyse the impact of *ATG16L1* genotypes on the HCC phenotypes, the 107 HCC cases from the derivation cohort were stratified according to their p.T300A genotypes, resulting in 11 patients with genotype AA, 59 with AG and 37 with GG. There were no significant differences in age at diagnosis and disease stage as determined by BCLC or Okuda scores (Table S1). In total, 15 patients received potentially curative treatments, including ethanol injection, radiofrequency ablation, resection or transplantation. The actuarial 1-year survival after diagnosis of HCC was the lowest in patients with AA genotype (25.0% $\pm$ 15.3%) as compared to patients with AG (40.0% $\pm$ 7.7%) or GG (40.8% $\pm$ 10.0%) genotypes ($P = .31$ in log-rank test for trend). When 1-year survival was assessed based on the day of inclusion into the study, survival was significantly associated with

the *ATG16L1* p.T300A genotype: 0.0% in AA, 20.6% $\pm$ 6.5% in AG and 23.5% $\pm$ 8.9% in GG carriers ($P = .02$ in log-rank test for trend).

3.4 | Allele frequencies of *PNPLA3* p.I148M (rs738409)

In order to investigate, whether the association of p.T300A was independent from the genetic risk of complications of hepatic steatosis, we determined the *PNPLA3* p.I148M variant (rs738409) in both cohorts. The frequencies of the *PNPLA3* rs738409 G allele were 0.36 in patients with cirrhosis without HCC and 0.45 in patients with HCC in cirrhosis. Again, there was no difference in the G allele frequencies in patients with HCC when stratified for participating in a HCC surveillance programme or not (0.42 vs. 0.45; $P = .58$). As recently reported,¹³ the *PNPLA3* p.I148M variant was associated

with HCC (Table S2). The odds ratios were 1.45 (95% confidence interval [CI]: 1.10-1.90) for allele frequency difference, 1.50 (95% CI: 1.02-2.22) for G allele positivity and 2.10 (95% CI: 1.20-3.66) for G allele homozygosity.

The association of *ATG16L1* p.T300A with HCC remained independent of *PNPLA3* p.I148M in multivariate logistic regression (Table 3). Patients carrying 3 or more of the investigated risk alleles in *ATG16L1* and *PNPLA3* were at a significantly increased risk for HCC as compared to patients carrying both wild-type alleles (Figure 2).

4 | DISCUSSION

We herein report that the frequent genetic polymorphism *ATG16L1* p.T300A (rs2241880) was associated with the development of HCC in two independent cohorts of patients with cirrhosis, and remained significant after adjusting for potential confounders like age, sex, aetiology of cirrhosis and the *PNPLA3* genotype. As autophagy is an important mechanism for maintaining cell homeostasis and metabolism, it plays a role in stabilizing non-malignant as well as tumour cells against apoptosis, metabolic and inflammatory stress.^{15,25} The *ATG16L1* gene, located on chromosome 2, is a key regulator of autophagy. It is involved in the initiation of autophagy²⁶ and forms a large complex with *ATG12* and *ATG5*.²⁷ *ATG16L1*-deficiency or carriage of the investigated loss-of-function mutation p.T300A results in decreased antibacterial function and enhanced IL-1 β production by myeloid cells in response to pathogen-associated bacterial patterns.¹⁹⁻²²

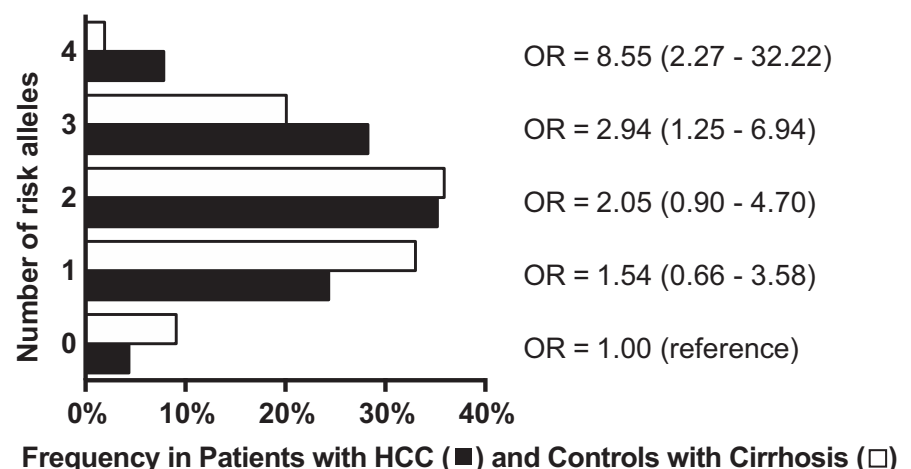
An association of *ATG16L1* polymorphisms with cancer has been observed in thyroid carcinoma,²⁸ gastric cancer^{29,30} and oral squamous cell carcinoma³¹ and affects the outcome in colorectal cancer³² and lung cancer.³³ Notably, the loss of function mutation p.T300A was protective in some but deleterious in other studies underlining that intact autophagy facilitates and suppresses tumorigenesis depending on the context. Concerning hepatic carcinogenesis, increased levels of proinflammatory cytokines and gene polymorphisms in the IL1- β gene have been shown to be associated

with HCC in patients with viral hepatitis.^{34,35} As autophagy can limit excessive inflammation, autophagy has been proposed as a protective mechanism in the inflammation-related HCC development.¹⁷ The development and growth of HCC are associated with proinflammatory mediators in the tumour microenvironment,³⁶ stage and aetiology of liver disease as well as on the genetic background of the patient. Underlining the role of intact autophagy to limit hepatic carcinogenesis, Okada *et al* reported that increased *ATG16L1* activation was required to prevent the progression of steatohepatitis and the development of HCC in a mouse model and the overexpression of *Atg16L1* inhibited NF- κ B and IL6 signalling in that study.³⁷

Although there have been some encouraging reports on the utilization of surrogate parameters of inflammation and immune activation to predict the susceptibility to develop HCC,³⁸ their application is limited due to high variability as their level is modulated by concurrent bacterial infections, the stage of liver disease and the degree of portal hypertension.³⁹ Therefore, it seems to be difficult to define high-risk groups for HCC-development according to inflammatory parameters alone, which are measured at a random time point in the course of disease in such a highly dynamic system. In addition to clinical and demographic features, genetic variants, such as the identified genetic variants in *ATG16L1* and *PNPLA3*,¹³ may be taken into account when performing risk-based HCC surveillance in cirrhosis.

Despite the significant association of HCC with *ATG16L1* p.T300A in two independent cohorts, our study has some limitations. First, the observed association of HCC with the G-Allele could result from a higher mortality of patients with the A-Allele before developing HCC. However, we did not detect an age difference between patients carrying the different genotypes. Second, as this was an unmatched case control study, which stratified patients according to the presence of HCC at baseline, we could not exclude cirrhotic controls, who will develop HCC at a later time point. However, patients with cirrhosis who were selected as controls underwent HCC screening at a high percentage for a median of 6 years. Third, only 50% of cirrhotic patients with HCC underwent surveillance, which may have impact the survival of HCC as these patients are diagnosed at an earlier stage, with smaller tumours more amenable to curative therapy. However, the SNP allele

FIGURE 2 Risk alleles in *ATG16L1* and *PNPLA3* and attributed hepatocellular carcinoma (HCC) risk. Distribution of risk alleles (rs2241880 and rs738409) among cases with HCC (n = 230) and controls with cirrhosis without HCC (n = 209). Odds ratios (OR) for HCCs are shown for 1 to 4 risk alleles in comparison to patients carrying the *ATG16L1* and *PNPLA3* wild-type alleles



frequencies were identical in patients with and without participation arguing against surveillance as a biasing factor in our analysis.

We herein demonstrate an independent association of a frequent ATG16L1 germ-line mutation with the risk of developing HCC. The multicentre setting and the composition of aetiologies is likely to be representative also for other Central European countries. Taken together, our data suggest that the ATG16L1 genotype can be employed in addition to clinical data and other genetic risk factors to develop personalized strategies to at risk of HCC, which might benefit from undergoing more intensive tumour surveillance.

CONFLICT OF INTEREST

The authors thank Kathrin Schulze for technical assistance. All authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTIONS

PAR wrote the study protocol, recruited patients, performed statistical analysis, analysed and interpreted the results, conducted literature search and drafted the manuscript. PL, HDN, US, MC and FL recruited patients, collected clinical data and performed laboratory analysis, interpreted the results and revised the manuscript. SS and EA performed SNP analysis, interpreted the results and revised the manuscript. AS, FL, HDN and TB participated in the design of the study, supervised the work of co-authors, interpreted the results and revised the manuscript. TB performed statistical analysis, analysed and interpreted the results, conducted literature search and drafted and revised the manuscript. All authors read and approved the final version of the manuscript.

ORCID

Philipp A. Reuken  <https://orcid.org/0000-0002-7696-475X>

Frank Lammert  <https://orcid.org/0000-0003-4450-7201>

Tony Bruns  <https://orcid.org/0000-0002-5576-6914>

REFERENCES

- El-Serag HB. Epidemiology of viral hepatitis and hepatocellular carcinoma. *Gastroenterology*. 2012;142(1264-1273):e1.
- Mittal S, El-Serag HB. Epidemiology of HCC: consider the population. *J Clin Gastroenterol*. 2013;47:S2-S6.
- Bruix J, Llovet JM. Prognostic prediction and treatment strategy in hepatocellular carcinoma. *Hepatology*. 2002;35:519-524. <https://doi.org/10.1053/jhep.2002.32089>.
- European Association for the Study of the Liver. EASL clinical practice guidelines: management of hepatocellular carcinoma. *J Hepatol*. 2018;69:182-236. <https://doi.org/10.1016/j.jhep.2018.03.019>.
- Zhang H, Zhai Y, Hu Z, et al. Genome-wide association study identifies 1p36.22 as a new susceptibility locus for hepatocellular carcinoma in chronic hepatitis B virus carriers. *Nat Genet*. 2010;42:755-758.
- Chan K-K, Wong C-M, Kwan J-H, et al. Genome-wide association study of hepatocellular carcinoma in Southern Chinese patients with chronic hepatitis B virus infection. *PLoS ONE*. 2011;6(12):e28798.
- Kumar V, Kato N, Urabe Y, et al. Genome-wide association study identifies a susceptibility locus for HCV-induced hepatocellular carcinoma. *Nat Genet*. 2011;43:455-458.
- Nahon P, Zucman-Rossi J. Single nucleotide polymorphisms and risk of hepatocellular carcinoma in cirrhosis. *J Hepatol*. 2012;57:663-674.
- Wei Y, Liu F, Li BO, et al. Polymorphisms of tumor necrosis factor- α and hepatocellular carcinoma risk: a HuGE systematic review and meta-analysis. *Dig Dis Sci*. 2011;56:2227-2236.
- Yang Y, Luo C, Feng R, Bi S. The TNF- α , IL-1B and IL-10 polymorphisms and risk for hepatocellular carcinoma: a meta-analysis. *J Cancer Res Clin Oncol*. 2011;137:947-952.
- Migita K, Miyazoe S, Maeda Y, et al. Cytokine gene polymorphisms in Japanese patients with hepatitis B virus infection—association between TGF- β 1 polymorphisms and hepatocellular carcinoma. *J Hepatol*. 2005;42:505-510.
- Charni F, Sutton A, Rufat P, et al. Chemokine RANTES promoter dimorphisms and hepatocellular carcinoma occurrence in patients with alcoholic or hepatitis C virus-related cirrhosis. *Cancer Epidemiol Biomark Prev*. 2011;20:1439-1446. <https://doi.org/10.1158/1055-9965.EPI-11-0341>
- Stickel F, Buch S, Nischalke HD, et al. Genetic variants in PNPLA3 and TM6SF2 predispose to the development of hepatocellular carcinoma in individuals with alcohol-related cirrhosis. *Am J Gastroenterol*. 2018;113(10):1475-1483. <https://doi.org/10.1038/s41395-018-0041-8>
- Levine B, Klionsky DJ. Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell*. 2004;6:463-477. [https://doi.org/10.1016/S1534-5807\(04\)00099-1](https://doi.org/10.1016/S1534-5807(04)00099-1)
- Mathew R, Karantza-Wadsworth V, White E. Role of autophagy in cancer. *Nat Rev Cancer*. 2007;7:961-967.
- Mariño G, Salvador-Montoliu N, Fueyo A, Knecht E, Mizushima N, López-Otín C. Tissue-specific autophagy alterations and increased tumorigenesis in mice deficient in Atg4C/autophagin-3. *J Biol Chem*. 2007;282:18573-18583.
- Yu S, Wang YU, Jing LI, et al. Autophagy in the "inflammation-carcinogenesis" pathway of liver and HCC immunotherapy. *Cancer Lett*. 2017;411:82-89.
- Levine B, Mizushima N, Virgin HW. Autophagy in immunity and inflammation. *Nature*. 2011;469:323-335.
- Saitoh T, Fujita N, Jang MH, et al. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1 β production. *Nature*. 2008;456:264-268.
- Plantinga TS, Crisan TO, Oosting M, et al. Crohn's disease-associated ATG16L1 polymorphism modulates pro-inflammatory cytokine responses selectively upon activation of NOD2. *Gut*. 2011;60:1229-1235.
- Travassos LH, Carneiro L, Ramjeet M, et al. Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. *Nat Immunol*. 2010;11:55-62.
- Cooney R, Baker J, Brain O, et al. NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. *Nat Med*. 2010;16:90-97.
- Lassen KG, Kuballa P, Conway KL, et al. Atg16L1 T300A variant decreases selective autophagy resulting in altered cytokine signaling and decreased antibacterial defense. *Proc Natl Acad Sci*. 2014;111:7741-7746.
- Hampe J, Franke A, Rosenstiel P, et al. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. *Nat Genet*. 2007;39:207-211.
- Kung C-P, Budina A, Balaburski G, Bergenstock MK, Murphy M. Autophagy in tumor suppression and cancer therapy. *Crit Rev Eukaryot Gene Expr*. 2011;21:71-100.
- Zavodszky E, Vicinanza M, Rubinsztein DC. Biology and trafficking of ATG9 and ATG16L1, two proteins that regulate autophagosome formation. *FEBS Lett*. 2013;587:1988-1996.

27. Mizushima N, Kuma A, Kobayashi Y, et al. Mouse Apg16L, a novel WD-repeat protein, targets to the autophagic isolation membrane with the Apg12-Apg5 conjugate. *J Cell Sci.* 2003;116:1679-1688.
28. Huijbers A, Plantinga TS, Joosten L, et al. The effect of the ATG16L1 Thr300Ala polymorphism on susceptibility and outcome of patients with epithelial cell-derived thyroid carcinoma. *Endocr Relat Cancer.* 2012;19:L15-L18.
29. Burada F, Ciurea ME, Nicoli R, et al. ATG16L1 T300A polymorphism is correlated with gastric cancer susceptibility. *Pathol Oncol Res POR.* 2016;22:317-322.
30. Castaño-Rodríguez N, Kaakoush NO, Goh K-L, Fock KM, Mitchell HM. The NOD-like receptor signalling pathway in *Helicobacter pylori* infection and related gastric cancer: a case-control study and gene expression analyses. *PLoS ONE.* 2015;10:e0117870.
31. Fernández-Mateos J, Seijas-Tamayo R, Klain J, et al. Analysis of autophagy gene polymorphisms in Spanish patients with head and neck squamous cell carcinoma. *Sci Rep.* 2017;7:6887.
32. Grimm WA, Messer JS, Murphy SF, et al. The Thr300Ala variant in ATG16L1 is associated with improved survival in human colorectal cancer and enhanced production of type I interferon. *Gut.* 2016;65:456-464.
33. Li Q-X, Zhou X, Huang T-T, et al. The Thr300Ala variant of ATG16L1 is associated with decreased risk of brain metastasis in patients with non-small cell lung cancer. *Autophagy.* 2017;13:1053-1063.
34. Tanaka Y, Furuta T, Suzuki S, et al. Impact of interleukin-1beta genetic polymorphisms on the development of hepatitis C virus-related hepatocellular carcinoma in Japan. *J Infect Dis.* 2003;187:1822-1825.
35. Wang Y, Kato N, Hoshida Y, et al. Interleukin-1beta gene polymorphisms associated with hepatocellular carcinoma in hepatitis C virus infection. *Hepatology.* 2003;37:65-71. <https://doi.org/10.1053/jhep.2003.50017>
36. Hernandez-Gea V, Toffanin S, Friedman SL, Llovet JM. Role of the microenvironment in the pathogenesis and treatment of hepatocellular carcinoma. *Gastroenterology.* 2013;144:512-527.
37. Okada H, Takabatake R, Honda M, et al. Peretinoin, an acyclic retinoid, suppresses steatohepatitis and tumorigenesis by activating autophagy in mice fed an atherogenic high-fat diet. *Oncotarget.* 2017;8:39978-39993.
38. Chounta A, Ellinas C, Tzanetakou V, et al. Serum soluble urokinase plasminogen activator receptor as a screening test for the early diagnosis of hepatocellular carcinoma. *Liver Int.* 2015;35:601-607. <https://doi.org/10.1111/liv.12705>
39. Zimmermann HW, Reuken PA, Koch A, et al. Soluble urokinase plasminogen activator receptor is compartmentally regulated in decompensated cirrhosis and indicates immune activation and short-term mortality. *J Intern Med.* 2013;274:86-100.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Reuken PA, Lutz P, Casper M, et al. The ATG16L1 gene variant rs2241880 (p.T300A) is associated with susceptibility to HCC in patients with cirrhosis. *Liver Int.* 2019;39:2360-2367. <https://doi.org/10.1111/liv.14239>