A Human REPIN1 Gene Variant: Genetic Risk Factor for the Development of Nonalcoholic Fatty Liver Disease

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OBJECTIVES: **We tested the hypothesis that a genetic deletion (Del) variant in the *REPIN1* gene is associated with the severity of nonalcoholic fatty liver disease (NAFLD) in humans.**

METHODS: **Sixty-three donors of liver biopsies from individuals with obesity and different degrees of NAFLD and fibrosis were screened for a Del REPIN1 gene variant and liver *REPIN1* mRNA expression.**

RESULTS: **In 8 homozygous Del carriers, we found significantly lower NAFLD activity and fibrosis scores compared with 55 wild-type allele carriers.**

DISCUSSION: **A Del variant of *REPIN1* may be associated with a lower risk of the development of NAFLD.**

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INTRODUCTION

Pathological accumulation of hepatic fat can lead to nonalcoholic fatty liver disease (NAFLD), including simple steatosis and nonalcoholic steatohepatitis (NASH), which may progress to cirrhosis and increase the risk to develop hepatocellular carcinoma (1). Although obesity is an important risk factor for NAFLD, not all patients with obesity develop steatosis hepatis (2). The mechanisms underlying hepatic steatosis and its progression to more severe stages are complex and involve nutritive, behavioral, genetic, epigenetic, as well as environmental factors. In this context, prospective twin studies estimated the heritable component of hepatic steatosis at ~50% (3). Recently, we found that a genetic variant, a 12 base pair (bp) deletion (Figure 1a), within the *REPIN1* gene causes a loss of function and is associated with alterations in glucose and lipid metabolism (4). Functional consequences of this variant were confirmed in HepG2 cells *in vitro* (4). In addition, studies in mice lacking hepatocellular Repin1 provided evidence that loss of Repin1 in the liver attenuates progression of NAFLD most likely by reducing fat accumulation and alleviating chronic tissue inflammation and injury (5). Moreover, Repin1-deficient mice exhibited lower NAFLD-related tumor incidence accompanied by a lower liver weight/body weight index (5). Beneficial effects of a liver-specific *REPIN1* small interfering RNA (siRNA) treatment confirmed the potential of *REPIN1* as a target gene for the prevention and therapy of NAFLD (5). These results prompted us to search for homozygous carriers of the 12 bp deletion in the *REPIN1* gene in a cohort of human liver biopsy donors (N = 63). In a cross-sectional study, we compared homozygous deletion carrier (Del) with wild-type carrier.

METHODS

Study population

The middle-aged cohort was recruited at the University Hospital of Leipzig, Germany, and includes 63 (men, $n = 21$; women, $n = 42$) subjects. We selected small liver biopsies in patients, who underwent abdominal surgery for Roux-en-Y gastric bypass, sleeve gastrectomy, or elective cholecystectomy (6). All participants gave their written informed consent before taking part in the study. All investigations were approved by the Ethics Committee of the University of Leipzig, Germany (363-10-13122010 and 017-12-230112), and performed in accordance with the Declaration of Helsinki.

Anthropometric measures and HbA1c levels

All patients underwent anthropomorphic measurements (weight and height) using standardized methods, and body mass index (BMI) was calculated as weight (kg)/height (m^2). HbA1c levels were measured in an automated clinical chemistry analyzer (Hitachi/Roche Diagnostics, Grenzach-Wyhlen, Germany) at the Institute of Laboratory Medicine, University Hospital Leipzig.

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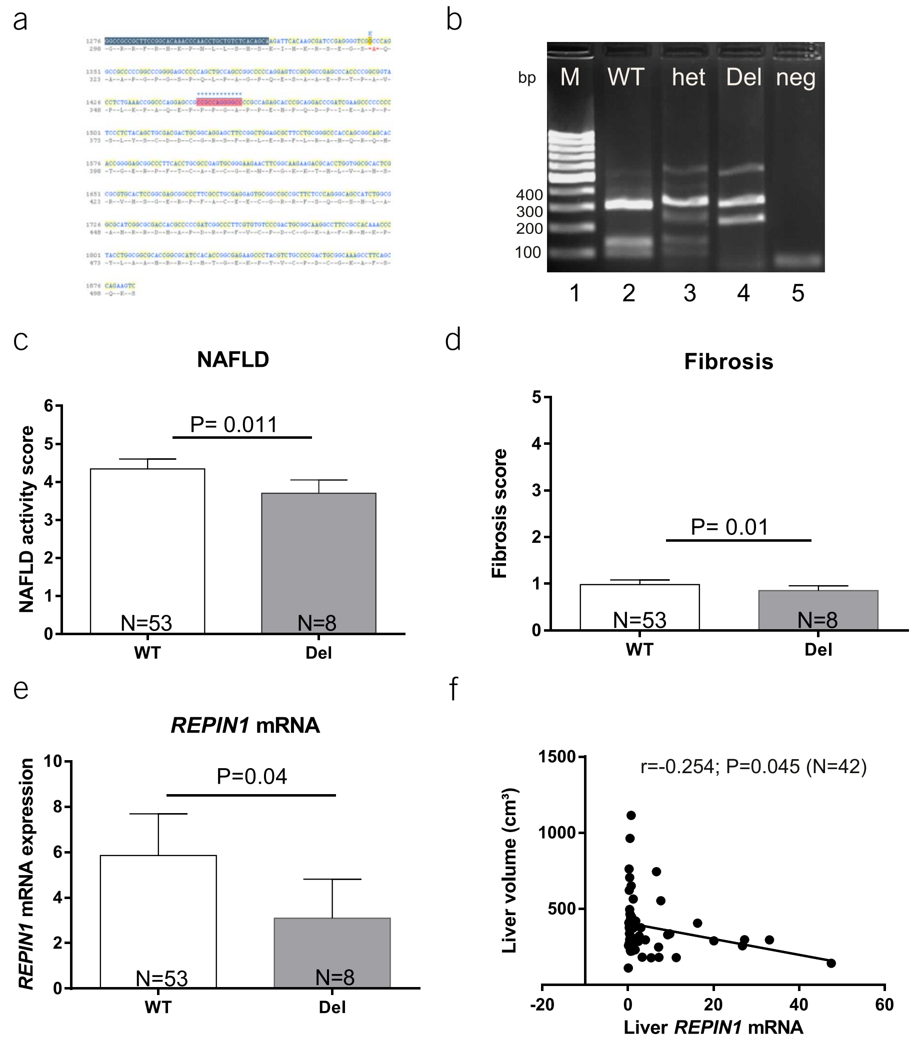

Figure 1. (**a**) Sequence of *REPIN1* region with the12 bp deletion marked in gray and (**b**) *REPIN1* genotyping by agarose gel after RFLP for the human genotyping. Lane 1 marker (M, 100bp marker), lane 2 WT, homozygote for wild-type (WT, 3 fragments, 99bp, 143bp, 322bp), lane 3 het, heterozygote (het, 4 fragments, 99 bp, 143 bp, 230 bp, 322 bp), lane 4 homozygote for 12 bp deletion (Del, 2 fragments, 230 bp, 322 bp), and lane 5 neg (negative control). (**c** and **d**) The NAFLD activity score and fibrosis score in subjects with *REPIN1* wildtype allele (WT, N = 53) and homozygous deletion (Del, N = 8) variant. (**c**) The NAFLD activity score and (**d**) fibrosis score are significantly reduced in subjects with *REPIN1* Del variant compared with wildtype allele carrier. (**e**) Significantly reduced *REPIN1* mRNA expression in liver biopsies in subjects with *REPIN1* Del variant compared with wildtype allele carrier. Results are expressed as means ± SE. (**f**) Liver volume (cm^3) correlation of all subjects with hepatic mRNA level of *REPIN1* (N = 42). bp, base pair; NAFLD, nonalcoholic fatty liver disease; RNA, ribonucleic acid; RFLP, restriction fragment length polymorphism.

Genotyping and *REPIN1* mRNA expression analysis

Screening for the 12 bp deletion was performed by restriction fragment length polymorphism as described recently (4). Briefly, genomic DNA was amplified by polymerase chain reaction (PCR), and the corresponding product (564 bp) was subsequently digested with the enzyme *Apa*I. The products were visualized using gel electrophoresis (Figure 1b).

A small liver biopsy was taken during the surgery, immediately snap frozen in liquid nitrogen, and stored at −80°C until further preparations. The hepatic expression of *REPIN1* mRNA has been measured by quantitative PCR using specific *REPIN1* probe (Hs00274221_s1) and calculated relative to *18S rRNA* (Hs99999901_s1; both Applied Biosystems, Warrington, GB). Specific mRNA expression was calculated relative to *18S rRNA* which was used as reference because of its resistance to glucose-dependent regulation (7). mRNA expression levels were quantified by using the second derivative maximum method.

NASH, NAFLD activity, and fibrosis scoring

The NASH score and fibrosis score were assessed on liver sections by a certified pathologist as described elsewhere (8,9). The NASH Clinical Research Network system for scoring activity and fibrosis in NAFLD was used to calculate the NAFLD Activity Score ranging 0–8 (10).The activity score is graded according to the intensity of necroinflammatory lesions (A0 = no activity, A1 = mild activity, A2 = moderate activity, and A3 = severe activity), and the fibrosis score is assessed on a five-point scale (F0 = no fibrosis, F1 = portal fibrosis without septa, F2 = few septa, F3 = numerous septa without cirrhosis, and F4 = cirrhosis) (11). Liver volume was quantified by magnetic resonance imaging (Achieva

LIVER

Table 1. Main characteristics of liver biopsy cohort

M/F	WT (19/36)	Del (2/6)	*P* value
Parameter			
Age, yr	49.4 ± 10.1	48.7 ± 9.8	0.854
BMI, kg/cm^2	45.5 ± 5.0	47.7 ± 3.9	0.238
HbA1C, %	5.8 ± 1.3	6.3 ± 2.0	0.348
Diabetes mellitus, %	49	50	0.934
Serum cholesterol, mmol/L	4.5 ± 0.8	4.7 ± 1.0	0.482
Serum trigycerides, mmol/L	1.4 ± 0.7	1.3 ± 0.5	0.824
HDL-cholesterol, mmol/L	1.1 ± 0.3	1.1 ± 0.2	0.937
LDL-cholesterol, mmol/L	2.6 ± 0.8	2.9 ± 0.8	0.491

Data are expressed as means ± SD. The unpaired 2-tailed Student *t* test was used between groups, and diabetes frequency was assessed with the χ^2 test. BMI, body mass index; HDL, high density lipoprotein; LDL, low denisty lipoprotein.

XR, Philips Healthcare, Best, the Netherlands; N = 42) and calculated by an adapted software package (Matlab; MathWorks, Natick, MA).

Statistical analysis

Statistical significance between the groups was evaluated using the unpaired 2-tailed Student *t* test. Differences were considered statistically significant at $P < 0.05$. Correlation between *REPIN1* mRNA expression in human liver and liver volume (cm^3) was assessed by the Spearman rank correlation analysis after the Kolmogorov-Smirnov test was performed to assess normality of the data. Statistical analysis of diabetes frequency was assessed with the χ^2 test. Logistic linear regression analysis was performed to estimate relationship between *REPIN1* genotype, BMI, age, diabetes frequency, and gender. All statistical analyses were performed using SPSS Statistics (v24; IBM Corp., Armonk, NY).

RESULTS

The study included 63 liver biopsy donors with mean age of 49 years. Among the 63 donors, we identified 8 homozygous carriers of the 12 bp deletion in the *REPIN1* gene (Figure 1a,b) and no heterozygous carriers.

Interestingly, we observed a significant lower NAFLD activity score as well as fibrosis score in liver biopsies of subjects with Del compared with wildtype subjects (Figure 1c,d) (Table 1). There were no significant differences between carriers of the 12 bp deletion and wildtype allele carriers in HbA1c levels, diabetes frequency, and BMI as shown in Table 1. Moreover, logistic linear regression analysis confirmed these findings (data not shown). Hence, we suggest that carriers of the Del variant are more protected from hepatic fat accumulation and progression to fibrosis than wildtype subjects. Furthermore, we found significant lower *REPIN1* mRNA level in human livers of Del carrier compared with wildtype allele carriers (Figure 1e). Interestingly, a negative correlation between hepatic *REPIN1* mRNA expression level and liver volume was found as well (Figure 1f).

DISCUSSION

Obesity is an important risk factor for NAFLD, but not all patients with obesity develop steatosis hepatis (2). The factors and mechanisms that cause progression from steatosis to hepatocellular carcinoma are not fully understood. Prospective twin studies estimated the heritable component of hepatic steatosis at ~50% (3). Previous findings suggested that *REPIN1* plays a significant role in lipid metabolism and glucose homeostasis (5,6,12,13). In the present cross-sectional study of middle-aged participants, we demonstrate in humans that the genetic 12 bp Del variant of *REPIN1* is associated with a lower severity of NAFLD despite obesity and independently from diabetes mellitus, gender, and age. Our findings are supported by the *in vivo* data in mice with progressive NAFLD that hepatic *REPIN1* deficiency attenuated NAFLD progression by alleviating systemic and hepatic lipid accumulation, chronic inflammation, and subsequently reducing liver injury (5). Consequently, and most strikingly, these mice exhibited lower NAFLD-related tumor incidence accompanied by a lower liver weight/body weight index (5). Moreover, *REPIN1* siRNA treatment confirmed the potential of *REPIN1* as a target gene for the prevention and therapy of NAFLD (5). Liver volume is known to be related to NAFLD and human obesity (14,15). However, not consistent is the negative correlation between hepatic *REPIN1* mRNA expression level and liver volume. As REPIN1 deficiency was observed to be accompanied with less fat accumulation in recent studies (6,13), and a correlation between the degree of steatosis and liver volume in NAFLD exists (16), it should be expected that changes in hepatic fat content in human livers of Del carrier are also accompanied by changes in liver volume. Thus, this fact needs further investigations in larger prospective studies with more homozygous Del carriers to interpret and validate our study results. In summary, we conclude that *REPIN1* might be an important genetic risk factor for the development of NAFLD and is an attractive therapeutic target for the treatment of NAFLD.

Study Highlights

WHAT IS KNOWN

✓ Studies in mice lacking hepatocellular Repin1 provided evidence that loss of Repin1 in the liver attenuates progression of NAFLD.

WHAT IS NEW HERE

✓ Genetic variant, a 12 bp deletion of Repin1 is relevant for NAFLD in humans.

TRANSLATIONAL IMPACT

✓ Mice to human studies indicate that REPIN 1 is an important genetic risk factor for the development of NAFLD in humans.

CONFLICTS OF INTEREST

Guarantor of the article: Nora Klöting, PhD.

Specific author contributions: N.K.: contributed to the initial concept, experimental data, data collection and interpretation of results, and manuscript writing. K.A., T.S.: contributed to protocol writing, submission, and manuscript review. A.D.: performed open abdominal surgery for Roux-en-Y bypass, sleeve gastrectomy, or cholecystectomy and took liver biopsies. C.W. performed hepatic scoring. C.B. performed genotyping. M.B., C.B., K.A., and M.S.: reviewed and contributed to the manuscript writing. All authors read and approved the final manuscript.

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Potential competing interests: None to report.

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