Metastasis-Associated Lung Adenocarcinoma Transcript 1 as a Common Molecular Driver in the Pathogenesis of Nonalcoholic Steatohepatitis and Chronic Immune-Mediated Liver Damage

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Long noncoding RNAs (lncRNAs) are functional molecules that orchestrate gene expression. To identify lncRNAs involved in nonalcoholic fatty liver disease (NAFLD) severity, we performed a multiscale study that included: (a) systems biology modeling that indicated metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) as a candidate lncRNA for exploring disease-related associations, (b) translational exploration in the clinical setting, and (c) mechanistic modeling. *MALAT1* liver profiling was performed in three consecutive phases, including an exploratory stage (liver samples from patients with NAFLD who were morbidly obese [n = 47] and from 13 individuals with normal liver histology); a replication stage (patients with NAFLD and metabolic syndrome [n =49]); and a hypothesis-driven stage (patients with chronic hepatitis C and autoimmune liver diseases, [n = 65]). Liver abundance of *MALAT1* was associated with NAFLD severity ($P = 1 \times 10^{-6}$); *MALAT1* expression levels were up-regulated 1.75-fold ($P = 0.029$) and 3.6-fold ($P = 0.012$) in patients with nonalcoholic steatohepatitis compared to those diagnosed with simple steatosis (discovery and replication set, respectively; analysis of covariance adjusted by age, homeostasis model assessment, and body mass index). Quantification of liver vascular endothelial growth factor A messenger RNA, a target of *MALAT1*, revealed a significant correlation between the two RNAs (R, 0.58; $P = 5 \times 10^{-8}$). Increased levels of *MALAT1* were also associated with autoimmune liver diseases. Interactome assessment uncovered significant biological pathways, including Janus kinase-signal transducers and activators of transcription and response to interferon-γ. *Conclusion:* Deregulated expression of *MALAT1* stratifies patients into the histologic phenotypes associated with NAFLD severity. *MALAT1* up-regulation seems to be a common molecular mechanism in immune-mediated chronic inflammatory liver damage. This suggests that convergent pathophenotypes (inflammation and fibrosis) share similar molecular mediators. (*Hepatology Communications* 2018;2:654-665)

Nonalcoholic fatty liver disease (NAFLD) is a chronic liver disorder that exhibits complex phenotypic diversity.[1] The scope of the histologic disease severity varies, ranging from a relatively benign and mild condition known as simple (bland) steatosis or nonalcoholic fatty liver (NAFL) to a more severe histologic picture characterized by liver cell injury, a mixed inflammatory lobular infiltrate, and variable fibrosis, referred to as nonalcoholic steatohepatitis (NASH).[2] These main histologic phenotypes

Abbreviations: BMI, body mass index; GEO, Gene Expression Omnibus; GO, gene ontology; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; JAK-STAT, Janus kinase-signal transducers and activators of transcription; lncRNA, long noncoding RNA; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; MetS, metabolic syndrome; mRNA, messenger RNA; NAFL, nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NRF, nuclear respiratory factor; PCR, polymerase chain reaction; TF, transcription factor; VEGFA, vascular endothelial growth factor A.

(NAFL and NASH) display distinctive degrees of severity.[2]

Irrespective of whether NAFL and NASH should be considered as having different long-term clinical impact, it is clear that the progression of NASH into more aggressive phenotypes, including NASH fibrosis and NASH cirrhosis and eventually hepatocellular carcinoma (HCC), imposes a tremendous public health problem of epidemic proportions.[1,3]

While the molecular mechanisms that drive the severity and progression of NAFLD and NASH are an important subject of a large body of scientific research, transcriptome analysis of liver tissue has provided the most compelling information of deregulated signatures operating at the gene level that modulate the natural history of the disease.[4,5] Nevertheless, with the exception of recent reports,[6,7] most findings yielded by previous studies indicated aberrant patterns of liver expression of messenger RNAs (mRNAs).

Virtually 60% of the human transcriptome is represented by long RNAs (with length exceeding 200 nucleotides) that lack protein-coding capacity and are thus referred to as long noncoding RNAs (lncRNAs).[8] LncRNAs play a remarkable role not only in regulating the entire transcriptome by interacting with multiple mRNAs and modulating epigenetic mechanisms but also in posttranslational regulation and direct interference with protein activity.[9] Ultimately, lncRNAs are involved in the orchestration of cell-to-cell signaling and cell functioning.[9] Consequently, it is plausible to hypothesize that lncRNAs may be involved not only in NAFLD pathogenesis[10] but also in determining the fate of the disease course and severity.

Patients and Methods

STUDY DESIGN AND PATIENT SELECTION CRITERIA

To identify lncRNAs involved in NAFLD severity, we performed a multidimensional study that included

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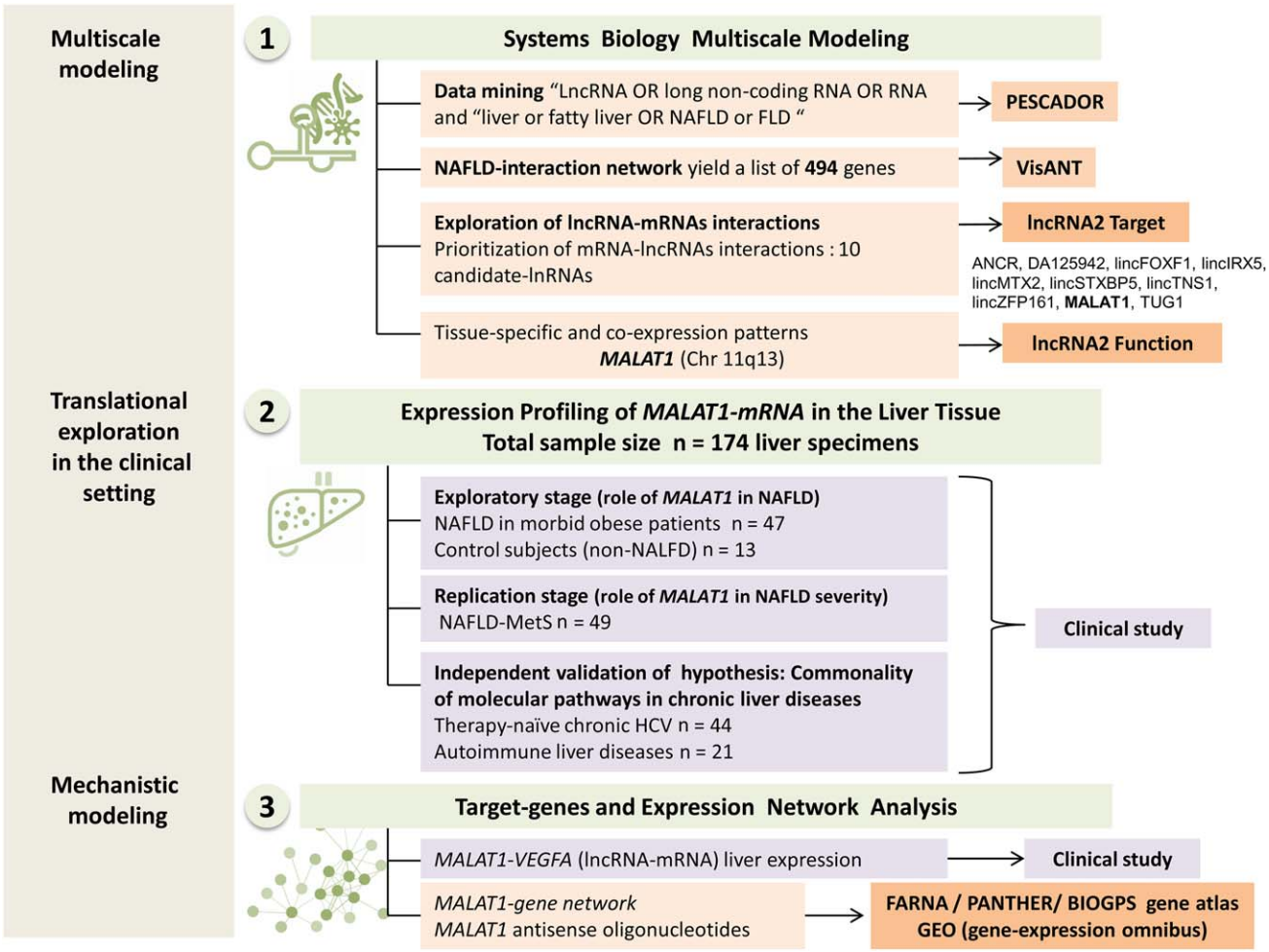

FIG. 1. Flow chart of work undertaken. Literature mining was performed using the PESCADOR tool, a web resource that allows exploring interactions between genes and proteins by identifying the co-occurrences of their terms in data extracted from the National Center for Biotechnology Information's PubMed database. The NAFLD interaction network was modeled using the resource VisANT. LncRNA2Target[40] (http://bio-annotation.cn/lncrna2target/) and LncRNA2Function[40] (http://bio-annotation.cn/lncrna2-target/) were used to explore and prioritize lncRNA−mRNA interactions. LncRNA2Function identifies protein-coding genes that are significantly co-expressed with one or more lncRNAs across 19 normal human tissues; target genes of an lncRNA are defined as the differentially expressed genes after knocking down or overexpressing the lncRNA. The function of the candidate lncRNA *MALAT1* was explored using the FARNA tool, a knowledge base of inferred functions of 10,289 human noncoding RNA transcripts (comprising 2,734 microRNAs and 7,555 lncRNAs) in 119 human tissues and 177 primary cells. Pathway analysis was performed using the PANTHER tool; normal *MALAT1* cell expression levels were extracted from the BIOPGS gene-expression atlas, while profiling was performed using Affymetrix tiling arrays (U133 Affymetrix chip).

the following: a core of multiscale systems biology modeling in four hierarchical dimensions (data mining of biological terms, building of a NAFLD interaction network, and searching and prioritization of lncRNA–mRNA interactions); translational exploration in the clinical setting (expression profiling of a candidate lncRNA in the liver tissue of affected patients); and mechanistic modeling (analysis of co-expression interactions). A detailed workflow depicting all study stages is shown in Fig. 1.

This strategy indicated metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*; alias names LINC00047, *NEAT2*, NCRNA00047, ENSG00000251562) as a candidate lncRNA for exploring disease-related associations. Liver profiling of *MALAT1* expression levels was performed in three consecutive study phases involving 174 unrelated patients. Specifically, 47 patients with NAFLD and morbid obesity (n = 47) took part in the exploratory stage; 49 individuals with NAFLD and metabolic

syndrome (MetS; n = 49) were involved in the replication stage; and 65 patients diagnosed with chronic liver disease of diverse etiology participated in the subsequent hypothesis-driven stage, 44 of whom were therapy-naive subjects with chronic hepatitis C virus (HCV) infection (n = 44), while the remaining 21 had autoimmune liver diseases (n = 21). Details can be found in the Supporting Material.

All investigations performed as part of the present study were conducted in accordance with the guidelines of the 1975 Declaration of Helsinki. Written consent from the participating individuals was obtained in accordance with the procedures approved by the ethical committee of our institution (protocol numbers 104/HGAZ/09, 89/100, and 1204/2012).

PHYSICAL, ANTHROPOMETRIC, AND BIOCHEMICAL EVALUATION

Details of the physical, anthropometric, and biochemical evaluations can be found in the Supporting Material.

HISTOLOGIC EVALUATION

NAFLD disease severity was assessed by liver biopsy (performed before any intervention) with ultrasound guidance and a modified 1.4-mm-diameter Menghini needle (Hepafix, Braun, Germany) under local anesthesia on an outpatient basis or during bariatric surgery in which surgically excised samples from the left lobe were immediately collected after the abdomen was opened and before organs were manipulated.

A portion of each liver biopsy specimen was routinely fixed in 40 g/L formaldehyde (pH 7.4), embedded in paraffin, and stained with hematoxylin and eosin, Masson trichrome, and silver impregnation for reticular fibers. All biopsies were at least 3 cm in length and contained a minimum of eight portal tracts. Degree of steatosis was assessed according to the system developed by Kleiner et al.[11] based on the percentage of hepatocytes containing macrovesicular fat droplets. NASH and NAFLD activity score were defined as reported[11,12]; NASH was defined as steatosis plus mixed inflammatory cell infiltration, hepatocyte ballooning and necrosis, Mallory's hyaline, and any stage of fibrosis, including absent fibrosis.[11,12] In patients with chronic HCV, liver histopathology was scored according to Ishak's fibrosis grading and staging system.[13]

RNA PREPARATION AND REAL-TIME REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION FOR QUANTITATIVE ASSESSMENT OF lncRNA AND mRNA EXPRESSION

Total RNA was prepared from liver tissue using the phenol extraction step method, with an additional deoxyribonuclease digestion. After extraction, RNA quantity was measured using the ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). For reverse-transcription polymerase chain reaction (PCR), 1-3 μg of total RNA was reverse transcribed using high capacity complementary DNA reverse transcriptase (Life Technologies, Camarillo, CA).

Real-time PCR was performed for quantitative assessment of lncRNA and mRNA expression in a Step One Plus Real-Time PCR System (Applied Biosystems, Buenos Aires, Argentina).

MALAT1 RNA liver expression was assessed by a TaqMan noncoding RNA gene expression assay (Assay ID, Hs01910177_s1) according to the specifications of the manufacturer (Life Technologies, Buenos Aires, Argentina). RNA or mRNA abundance of target genes was normalized to the amount of a housekeeping gene (ribosomal protein L19 [*RPL19*]) to carry out comparisons between groups. The selection of the housekeeping gene was based on the exploration of the most stable reference gene for testing liver mRNA expression among other housekeeping genes tested before starting this experiment. The geNorm program[14] was used to identify the appropriate reference control in our samples. Primer sequences are shown in Supporting Table S1. All real-time PCR reactions were run in duplicate. The lncRNA or mRNA levels were expressed as the ratio of the estimated amount of target gene relative to *RPL19* mRNA levels, using fluorescence threshold cycle values calculated for each sample; the estimated efficiency of the PCR for each product was expressed as the average of all sample efficiency values obtained.

STATISTICAL ANALYSIS

Quantitative data were expressed as mean ± SE unless otherwise indicated. Because significant differences in variance were observed between groups for most variables and the distribution was significantly skewed in most cases, we chose to be conservative and assessed the differences between groups by using the nonparametric

Mann-Whitney U test. As indicated in some comparisons, log-transformed variables were compared by analysis of variance for mean differences. Univariate correlations were obtained with the Spearman's rank correlation test. For logistic analysis or analysis of covariance, we adjusted for co-variables that were not normally distributed through log-transformation. Significance was assessed using a nominal P value of 0.05. The CSS/Statistica program package version 6.0 (StatSoft, Tulsa, OK) was used in the analyses.

Results

SYSTEMS BIOLOGY MODELING INDICATED *MALAT1* AS A CANDIDATE lncRNA IN THE BIOLOGY OF NAFLD

We followed the multistage system biology strategy (Fig. 1) to identify a significant lncRNA potentially involved in the biology of NAFLD. Modeling included several hierarchical levels of biological abstraction. First, we identified relevant biological terms by data mining using the terms "LncRNA OR long non-coding RNA OR RNA" and "fatty liver OR NAFLD OR FLD" and Medical Subject Heading terms. In our search, we used the PESCADOR platform for exploration of significant concepts associated with relationship co-occurrences.

Second, we built the NAFLD interaction network using the platform visANT, which consisted of different levels of gene/protein interactions, co-expression patterns, and gene ontology (GO) terms. Hierarchical exploration revealed metanodes that resulted from annotation enrichment analysis (Supporting Fig. S1). Third, we explored the presence of lncRNA−mRNA interactions using the platform LncRNATarget (http://bio-annotation.cn/lncrna2target/), which is based on information of validated targets and/or targets differentially expressed after knockdown or overexpression of lncRNAs. Prioritization of candidate lncRNAs was further analyzed with the LncRNA2Function resource (http://bio-annotation.cn/lncrna2target/), which identifies gene co-expressed patterns among lncRNA–mRNA pairs along with GO terms and pathway enrichment based on RNA sequencing data. LncRNAs known to be expressed in the liver tissue or were predicted to have interactions with genes/proteins involved in metabolic pathways were selected.

The NAFLD interaction network yielded a list of 494 genes. Subsequent prioritization of mRNA–lncRNA interactions revealed 10 candidate lnRNAs potentially involved in gene expression regulation (Fig. 1). Based on assessment of tissue-specific and co-expression patterns, we selected the lncRNA *MALAT1* for further exploration. *MALAT1* is a ∼8.7-kb gene located in chromosome 11q13 that is highly conserved across species.[15] This gene produces a precursor transcript from which an lncRNA is derived by ribonuclease P cleavage of a transfer RNA-like small noncoding RNA (known as *MALAT1*-associated small cytoplasmic RNA) from its 3′ end; *MALAT1* has pleiotropic abundance and localizes to the nucleus.[15]

UP-REGULATION OF LIVER *MALAT1* LEVELS STRATIFIES PATIENTS INTO THE HISTOLOGIC PHENOTYPES ASSOCIATED WITH DISEASE SEVERITY

Clinical, biochemical, and histologic features of NAFLD patients and controls are presented in Table 1. We found that *MALAT1* is constitutively expressed in the liver. However, *MALAT1* abundance in the liver was significantly associated with NAFLD severity ($P = 1 \times 10^{-6}$). Specifically, analyses performed on the exploratory and replication sets revealed that liver *MALAT1* expression was up-regulated 1.75-fold ($P = 0.029$) and 3.6-fold ($P = 0.012$) in patients with NASH compared with those diagnosed with simple steatosis (NAFL), respectively (analysis of covariance adjusted by age, homeostasis model assessment, and body mass index [BMI]) (Fig. 2A). Differences between *MALAT1* expression levels in control and NAFL liver samples were not statistically significant (exploratory set, $P = 0.9$).

In addition, by grouping patients according to the severity of their NAFLD histologic features, we observed a significant association with the level of *MALAT1* expression in the liver. Specifically, greater abundance of *MALAT1* was observed in patients with higher scores of ballooning degeneration ($P = 0.0001$), lobular inflammation ($P = 0.0025$), and the presence of fibrosis ($P = 1 \times 10^{-7}$) (Fig. 2B).

Likewise, *MALAT1* abundance in the liver was significantly and positively correlated with the results yielded by biochemistry tests, including serum levels of alanine aminotransferase (Spearman R, 0.30; $P = 0.016$), aspartate aminotransferase (R, 0.29; $P = 0.008$), and alkaline

TABLE 1. CLINICAL AND BIOCHEMICAL CHARACTERISTICS OF THE WHOLE STUDY SAMPLE ACCORDING TO DISEASE STATUS

	Exploratory Stage			Replication Stage	
Variable (mean ± SD)	Controls	NAFL	NASH	NAFL	NASH
Number of subjects	13	32	15	15	34
Female (%)	60	65	66	60	56
Age, years	40 ± 9.6	40.6 ± 10	44 ± 12	49.7 ± 10.5	49 ± 11
BMI, kg/m^2	58.5 ± 14	53 ± 12.4	46 ± 7.5	30 ± 3.5	33.4 ± 7
Waist circumference, cm	-	-	-	104.8 ± 6.5	112 ± 15
Waist:hip ratio	-	-	-	1.0 ± 0.03	1.0 ± 0.08
Arterial hypertension (%)	20	37.5	54	45	53
Type 2 diabetes (%)	15	32	73	21	47
Fasting plasma glucose, mg/dL	102 ± 20.5	104.5 ± 31	134 ± 71	100 ± 16	123 ± 51
Fasting plasma insulin, mg/dL	11.3 ± 6	14 ± 7	30 ± 52	15 ± 11	17 ± 10.4
HOMA-IR index	2.7 ± 1.5	3.5 ± 2	17 ± 48	3.6 ± 2.4	5 ± 4.8
Hb1C	6.5 ± 1.4	6.4 ± 1.4	6.8 ± 2	5.9 ± 0.7	7.5 ± 2.7
Total cholesterol, mg/dL	180 ± 25	183 ± 40	177 ± 46	196 ± 47	209 ± 42
HDL-cholesterol, mg/dL	42 ± 10	47 ± 9.5	40 ± 8.5	54 ± 13	50 ± 14
LDL-cholesterol, mg/dL	115 ± 22	128 ± 27	127 ± 38	119 ± 48.5	126 ± 32
Triglycerides, mg/dL	116 ± 46	143 ± 53	155 ± 53	183 ± 95	175 ± 95
ALT, U/L	20.5 ± 9	32.5 ± 21.5	44 ± 21	55 ± 30.5	80 ± 41
AST, U/L	20.4 ± 13	24 ± 14	34 ± 19	37.5 ± 15	54 ± 26
AP, U/L	86 ± 16	76 ± 20	83 ± 27	198 ± 100	169 ± 88
Degree of steatosis, %	0 ± 0	32 ± 25	46 ± 24	44 ± 32	64.5 ± 21
Lobular inflammation (0-3)	0 ± 0	0.3 ± 0.6	1.4 ± 0.9	0.4 ± 0.5	1 ± 0.58
Portal inflammation (0-2)	0 ± 0	0.4 ± 1	1.06 ± 1	0.0 ± 0.0	0.2 ± 0.5
Hepatocellular ballooning (0-2)	0 ± 0	0.15 ± 0.3	1.07 ± 0.7	0.0 ± 0.0	1 ± 0.6
Fibrosis stage (1-4)	0 ± 0	0.03 ± 0.17	1.7 ± 1.2	0.0 ± 0.0	1.6 ± 1.1
NAS	0 ± 0	2 ± 2	4.6 ± 1	2.4 ± 1.5	4.6 ± 1.4

Abbreviations: ALT alanine aminotransferase; AP, alkaline phosphatase; AST, aspartate aminotransferase; Hb1C, hemoglobin A1c; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; LDL, low-density lipoprotein; NAS, NAFLD activity score.

phosphatase (R, 0.37; $P = 0.0009$) as well as with the presence of surrogate biomarkers of hepatocellular apoptosis (cytokeratin-18 [R, 0.52; $P = 0.04$]).

In contrast, liver *MALAT1* expression was not associated with the presence of type 2 diabetes or cardiovascular disease or with any metabolic parameters examined, including peripheral insulin resistance (homeostasis model assessment of insulin resistance), glucose-related parameters, or lipid traits.

COMMONALITY IN DISEASE PATHOGENIC PATHWAYS OF CHRONIC LIVER DISEASES: *MALAT1* UP-REGULATION IS A SHARED MOLECULAR EVENT IN IMMUNE-MEDIATED CHRONIC INFLAMMATORY DAMAGE

We further postulated that chronic inflammatory liver diseases that involve both viral and sterile autoimmune-mediated processes might share common pathogenic/signaling pathways that are independent of the underlying event that triggered liver injury. Moreover, we hypothesized that liver *MALAT1* up-regulation could be a common molecular mechanism in the pathogenesis of chronic liver damage. To test this hypothesis, we profiled liver expression levels of *MALAT1* in two independent samples of patients comprising individuals with chronic HCV infection and those diagnosed with autoimmune diseases. Relative to NAFL, the expression levels of liver *MALAT1* were significantly higher ($P = 1 \times 10^{-7}$, one-way analysis of variance) in all patients diagnosed with the remaining chronic liver diseases (NASH, autoimmune, and chronic HCV) examined in this study (Fig. 3A). Specifically, liver *MALAT1* abundance was significantly up-regulated in NASH (3.58-fold increase, $P = 0.0008$; nonparametric Mann-Whitney U test), autoimmune liver diseases (4.47-fold increase, $P = 0.03$), and chronic HCV (2-fold increase, $P = 0.04$), as shown in Fig. 3A. Notably, the differences between the levels of *MALAT1* expression in NASH patients and those diagnosed with autoimmune liver diseases were not statistically significant (Fig. 3A). Clinical features of patients with chronic HCV and

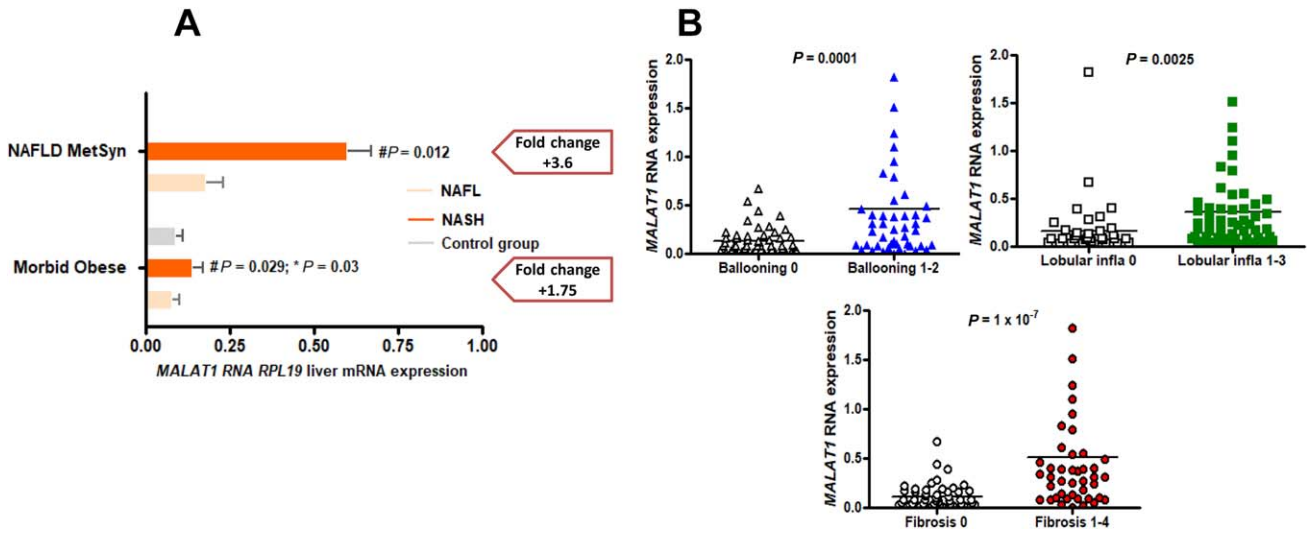

FIG. 2. Up-regulation of liver *MALAT1* levels stratifies patients into the histologic phenotypes associated with disease severity. (A) Liver abundance of *MALAT1* is significantly associated with NAFLD histologic severity. Each bar represents mean ± SE values. In each sample, liver abundance of *MALAT1* was expressed as normalized by the liver expression levels of a housekeeping gene (*RPL19* mRNA). Fold change pertains to the increase in NASH with respect to NAFL. Human expression analysis was conducted in two stages. Discovery set (NAFLD morbidly obese, sample size n = 47 [NAFL n = 32, NASH n = 15]) and control group (n = 13); and replication set (NAFLD-MetS, sample size n = 49, NAFL n = 15, NASH n = 34). *P* value denotes statistical significance ascertained with analysis of variance adjusted by age, homeostasis model assessment of insulin resistance, and BMI; #*P*, NASH versus NAFL within groups; **P*, NASH versus control subjects in morbidly obese category; nonparametric Mann-Whitney U test. (B) Liver *MALAT1* expression levels are significantly associated with the full spectrum of NAFLD histologic severity. The severity of histologic features was graded according to scores described by Brunt et al.[12] and Kleiner et al.[11] as indicated in the Methods section. Horizontal lines refer to mean value. Abbreviations: Lob infla, lobular inflammation; *RPL19*, ribosomal protein L19.

autoimmune liver diseases are shown in Supporting Table S2.

MALAT1 TARGET GENE (lncRNA–mRNA) INTERACTION: LIVER EXPRESSION OF *MALAT1* SIGNIFICANTLY CORRELATES WITH LIVER VASCULAR ENDOTHELIAL GROWTH FACTOR A mRNA LEVELS

MALAT1 has proangiogenic properties that are mediated by induction of vascular endothelial growth factor A (*VEGFA*) expression. The biological interaction between *MALAT1* and *VEGFA* has been robustly validated in *in vitro* experiments[16]; however, whether this association is also observed in liver tissue remains unknown. Therefore, we measured the liver abundance of *VEGFA* in all patients with NAFLD included in the study (NAFL and NASH in the bariatric cohort as well as patients with MetS). We observed that the levels of the two RNAs (*MALAT1*, *VEGFA*) were significantly and positively correlated (Spearman *R*, 0.59; $P = 5 \times 10^{-8}$; Fig. 3B). Moreover, we found that liver *MALAT1* abundance was significantly and positively associated with circulating lactic acid levels (Spearman *R*, 0.50; $P = 0.01$).

MECHANISTIC MODELING: ANALYSIS OF THE *MALAT1* NETWORK OF EXPRESSION INTERACTIONS

To gain insight into the role of *MALAT1* in chronic liver damage, we performed mechanistic modeling. To this end, we first explored the *MALAT1*-associated transcriptional network using the FARNA tool. The search was narrowed to human liver tissue by applying appropriate filters. FARNA predicted 106 transcription factors (TFs), including GO biological process, pathways, and reactome (Supporting Fig. S2); details are fully disclosed in Supporting Tables S3-S5.

Integration of predicted *MALAT1*-associated TFs into pathways and biological processes is shown in

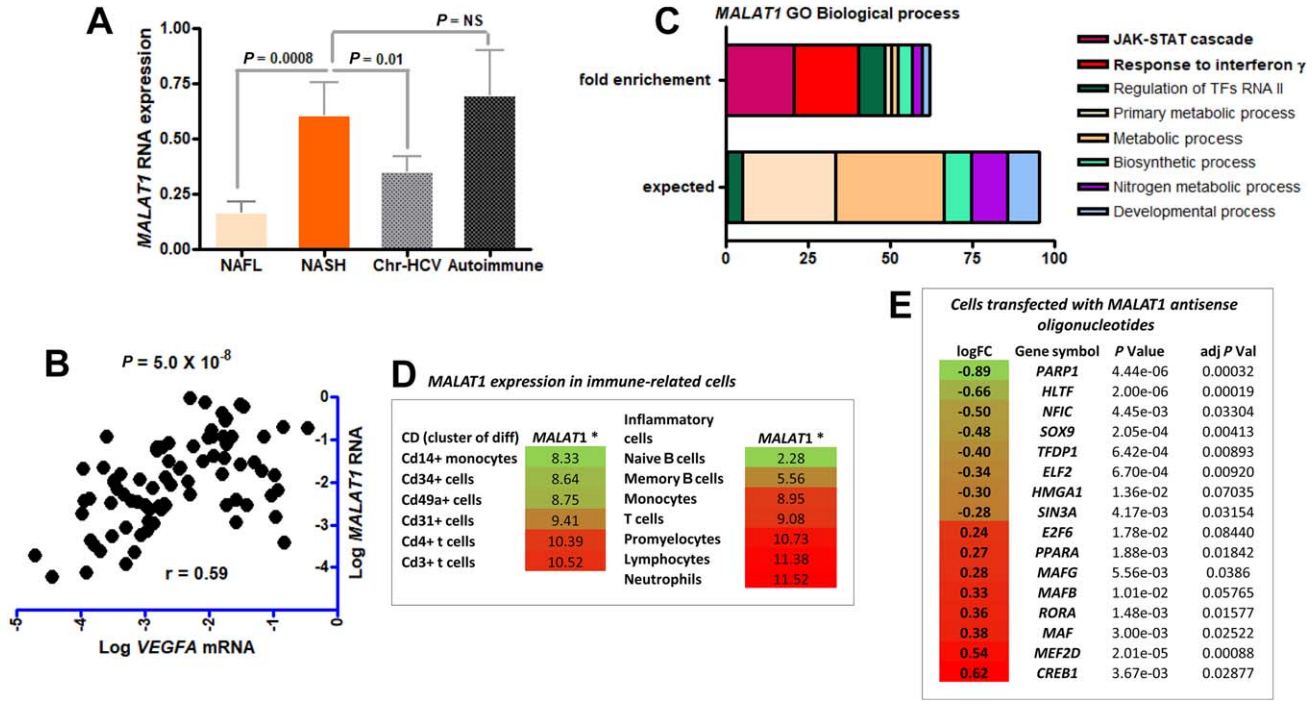

FIG. 3. *MALAT1* up-regulation is a common molecular event in immune response-mediated chronic inflammatory liver damage. (A) *MALAT1* expression in chronic liver diseases. *MALAT1* expression in the liver of patients with NAFLD and MetS (NAFL and NASH, n = 49), therapy naive subjects with chronic HCV infection (n = 44), and patients with autoimmune liver diseases (primary biliary cholangitis and autoimmune hepatitis, n = 21). Each bar represents mean ± SE values. In each sample, liver abundance of *MALAT1* was expressed as normalized by the liver expression levels of a housekeeping gene (*RPL19* mRNA). The *P* value is the statistical significance indicated by the nonparametric Mann-Whitney U test. (B) *MALAT1* liver expression significantly correlates with *VEGFA* mRNA levels. Correlation between log-transformed liver *MALAT1* RNA and log-transformed *VEGFA* mRNA expression levels. Liver abundance of both transcripts is expressed as normalized by the liver expression levels of a housekeeping gene (*RPL19* mRNA). The *P* value stands for Spearman *R* for nontransformed variables. (C) *MALAT1* gene network pathways. Pathway was predicted by the resource PANTHER version 12.0, released on July 10, 2017, based on the list of liver TFs predicted by the FARNA tool. Bars represent the results yielded by the overrepresentation test ($P < 0.05$, adjusted by Bonferroni correction for multiple testing) after contrasting the list of predicted TFs associated with the *MALAT1* gene transcription network with the whole human genome transcriptome (n = 21,002 genes); PANTHER Overrepresentation Test (release 20170413). STAT proteins and JAK involve intracellular signal transduction. Regulation of TFs by RNA II polymerase is the process that modulates the frequency, rate, or extent of transcription from an RNA polymerase II promoter. Primary metabolic processes are the normal anabolic and catabolic processes (carbohydrate, cellular amino acid, lipid, nucleobase-containing compound, and protein metabolic process as well as tricarboxylic acid cycle). Metabolic processes are chemical reactions and pathways, including anabolism and catabolism, by which living organisms transform chemical substances; these processes include macromolecular processes, such as DNA repair and replication and protein synthesis and degradation. Biosynthetic processes are the chemical reactions and pathways resulting in the formation of substances; this is typically the energy-requiring part of metabolism in which simpler substances are transformed into more complex ones. Nitrogen compound metabolic processes are pathways involving organic or inorganic compounds that contain nitrogen. Developmental processes occur at the structural level, such as a subcellular structure, cell, tissue, or organ, or organism, and modify the pertinent structure over time, transforming it from an initial condition to a later condition. (D) *MALAT1* expression levels in inflammatory-related cells. Data were retrieved from the integrated data set of human gene expression patterns (BioGPS).[17] Expression was measured using the U133 Affymetrix chip; *MALAT1 abundance is expressed as gcrma-normalized expression data relative to fluorescence intensity. Grna: Because there are multiple probes for each transcript on the microarray, the intensity values were summarized using various data processing algorithms. (E) *MALAT1* blockade by antisense oligonucleotides: analysis of gene expression network. The heat map illustrates gene expression levels (mRNAs) of transcription factors predicted by the FARNA tool in a mechanistic experiment that involved *MALAT1* blockade by antisense nucleotides. Values represent log-transformed up/down fold changes of differentially expressed mRNAs in cells (human diploid fibroblasts) transfected with *MALAT1* antisense oligonucleotides relative to the level of untreated cells. Data retrieved from the Gene Expression Omnibus, accession number GDS5352. Abbreviations: Adj. P Val, Bonferroni correction for multiple testing of nominal *P* values; CD, cluster of differentiation; chr-HCV, chronic HCV; gcrma, analysis package used for microarray data in R/Bioconductor; logFC, log-transformed up/down fold change; NS, not significant; *RPL19*, ribosomal protein L19.

Fig. 3C. Test analysis showed that both the Janus kinase-signal transducers and activators of transcription (JAK-STAT) cascade ($P_e = 1.15 \times 10^{-2}$) and the response to interferon-γ ($P_e = 1.79 \times 10^{-4}$) were statistically overrepresented (P_e denotes an empirical P value after applying Bonferroni correction for multiple testing).

Given that the infiltration of liver tissue by inflammatory cells is regarded as a hallmark feature of chronic liver damage, we aimed to ascertain whether *MALAT1* is expressed in the cellular milieu that is commonly involved in mediating the immune response. Hence, we retrieved gene expression data pertaining to *MALAT1* abundance in inflammatory response-related cells from the integrated data set of human gene expression patterns (BioGPS).[17] The analysis indicated that *MALAT1* is highly expressed in a large number of immune-related cells, including lymphocyte and leukocyte subsets, although the relative abundance seems to be different in B and T cells, with higher levels of expression noted in the latter (Fig. 3D).

Finally, to determine the functional relevance of the *MALAT1* gene expression network, we examined differential mRNA levels of predicted TFs in cells transfected with *MALAT1* antisense oligonucleotides (blockade of gene expression) relative to the level observed in untreated cells. To this end, we used the Gene Expression Omnibus (GEO) to retrieve raw data sets (accession number GDS5352) in which normal human diploid fibroblasts (WI38) were depleted of *MALAT1*.[18] Transcriptome analysis at the genome-wide level was assessed by Illumina Human HT-12 V4.0 expression beadchip. We specifically focused on the level of predicted TFs, and our analyses were performed using the FARNA tool, as described above.

After Bonferroni correction for multiple testing, we found that *MALAT1* depletion was significantly associated with 16 of the 106 predicted TFs, the levels of which were either down- or up-regulated (Fig. 3E). Among TFs significantly associated with *MALAT1* knockdown expression, we found nuclear receptors that are critically involved in NAFLD pathogenesis (peroxisome proliferator activated receptor α [*PPARα*] and nuclear receptor subfamily 1 group F member 1 [*RORα*]); master transcriptional regulators (MAF BZIP transcription factor [*MAF*]) that control the T helper type 2 differentiation pathway; transcription factor Dp-1 (*TFDP1*), which controls the cell cycle and is involved in cell proliferation and apoptosis; SIN3 transcription regulator family member A (*SIN3A*) involved in transcriptional repression of circadian target genes; cyclic adenosine monophosphate response element-binding protein 1 (*CREB1*), which modulates mitochondrial function; and high mobility group AT-Hook 1 (*HMGA1*), a TF involved in metastatic progression of cancer cells.

Interestingly, *VEGFA* was shown to be differentially down-regulated after *MALAT1* depletion (0.44-fold decrease, nominal $P = 0.0019$, adjusted $P = 0.02$; GEO data set accession number GSE43830)[18] (http://bio-annotation.cn/lncrna2target/).

Discussion

In this work, we explored the role of lncRNAs in the severity of NAFLD. We integrated knowledge derived from high-throughput technologies that was obtained by employing multiscale systems biology modeling methods that included literature mining, analysis of NAFLD-interaction network at the gene/protein level, and prediction and prioritization of lncRNA−mRNA interactions from different cellular and tissue types. This strategy resulted in the selection of *MALAT1* as a candidate lncRNA with a potentially relevant function in liver tissue. We next translated this knowledge into the clinical setting and examined a large collection (n = 174) of unique liver tissue specimens linked to clinical, biochemical, and histologic records of patients. We found that, relative to their expression in the control tissue and samples obtained from patients diagnosed with simple steatosis (NAFL), the levels of *MALAT1* were significantly higher in the NASH subsamples. Liver *MALAT1* abundance was significantly associated with the full spectrum of histologic severity, including ballooning degeneration, lobular inflammation, and fibrosis. Of note, these associations were unrelated to any metabolic parameter, such as insulin resistance or lipid traits, or any underlying associated comorbidity.

We further hypothesized that deregulated *MALAT1* expression could be a common perturbed molecular feature of chronic liver diseases, regardless of whether the diseases are triggered by infectious or sterile inflammatory causes. To this end, we extended the quantification of *MALAT1* abundance to the liver of untreated chronic HCV patients and patients diagnosed with autoimmune liver diseases. Interestingly, we observed that *MALAT1* up-regulation occurred not only in NASH cases but also in immune-mediated chronic liver diseases.

Mechanistic exploration that involved quantification of *VEGFA* mRNA levels (a validated target of *MALAT1*) as well as analysis of the *MALAT1* gene regulatory network retrieved from the transcript data stored in the GEO database, yielded clinically relevant results. For example, interactome assessment uncovered significant biological pathways, including JAK-STAT and response to interferon-γ, for which perturbation or dysfunction could be linked to chronic liver injury of any cause rather than a particular disease phenotype. Furthermore, gene co-expression network analysis of *MALAT1* blockade by antisense oligonucleotides revealed a significant role of this lncRNA in metabolic processes as well as in immune-response modulation, circadian rhythm control, and lipid metabolism. In line with our results, a recent report demonstrated that *MALAT1* may promote hepatic steatosis by increasing sterol regulatory element binding transcription factor 1 stability through the inhibition of its ubiquitination.[19]

Why is the lncRNA *MALAT1* relevant to the progression of NAFLD? The first and perhaps the most obvious reason for its relevance is its ability to modify an aggressive phenotype. In our investigation, *MALAT1* abundance discriminated patients with different NAFLD histologic outcomes. Specifically, while our data do not suggest that *MALAT1* expression levels are able to discriminate patients into different fibrosis scores, they are robust in differentiating simple steatosis versus NASH. Of note, *MALAT1* upregulation was independent of the underlying metabolic status, which suggests that this lncRNA plays a significant role in triggering and perpetuating the human NASH phenotype rather than merely mediating the accumulation of lipids in the liver. This line of reasoning is in agreement with work that involved both transcriptome analysis and epigenetic profiling of the liver tissue in NAFLD. Authors of those studies, including us, demonstrated that the transition from normal liver to fatty liver is associated with perturbed metabolic pathways involving master genes that control glucose and lipid metabolism.[4,5,20] Conversely, the progression into a more severe phenotype redirects the transcriptome toward pathways biologically related with mitochondrial dysfunction[21,22] and malignancy.[4,5,10] In this respect, *MALAT1* represents a remarkable functional and versatile molecule that modulates a myriad of signaling pathways, including cell cycle control,[18] immune balance,[23] and endothelial cell function and vessel growth.[24] These phenomena, when present concurrently, escalate in complexity to entail cell proliferation and migration,[25] apoptosis, fibrosis,[26] and malignancy.[27]

The exact mechanism(s) by which liver *MALAT1* expression is regulated are still unknown; however, critical pathways, including modification of the chromatin state, as well as modulation of proteosome machinery are promising candidates. Functional experiments involving knockdown of *MALAT1* expression by antisense oligonucleotides in human multiple myeloma cell lines resulted in alterations of proteasome subunit of nuclear respiratory factor 1 and 2 (*NRF1*, *NRF2*) genes as well as in endoplasmic reticulum stress. In turn, *NRF1* seems to promote *MALAT1* expression, providing a positive feedback loop.[28] Together, the evidence suggests that liver *MALAT1* expression could be regulated by epigenetic mechanisms.

Second, exploration of liver *VEGFA* mRNA, a well-known driver of tumor angiogenesis, showed that levels of the two RNAs (*MALAT1*, *VEGFA*) are significantly and positively correlated. Likewise, liver *MALAT1* abundance correlated with serum lactate concentration. These findings support the notion that the progression of NAFL to NASH involves not only inflammation and fibrogenesis but also metabolic reprogramming into a cancer-related transcriptional signature. Alternatively, angiogenesis may be regarded as a wound healing response and thus being part of the normal response of the liver to injury or hypoxia.

Third, in terms of NASH phenotypic plasticity, the pattern of liver *MALAT1* expression seems to segregate the disease spectrum into the two well-known clusters of NAFLD histologic lesions, one of which is observed in individuals who are nonmorbidly obese while the other is noted in those who are morbidly obese.[29-31] Specifically, while we found that *MALAT1* levels were significantly higher in liver tissue samples taken from NASH patients compared to controls or those in the NAFL group, the magnitude of the effect was significantly higher in patients with NAFLD and MetS than in patients with NAFLD and individuals who were morbidly obese ($P = 0.0001$). While we could not find any biological explanation for this result, we observed that liver *MALAT1* expression was significantly and negatively correlated with the BMI of all patients included in the study; this was obvious because the difference between the BMI values of the two groups of patients was highly significant (BMI in patients who were morbidly obese was 50.92 ± 11.48 kg/m^2 compared to 32.48 ± 6.01 kg/m^2 measured for patients with MetS; $P < 0.0001$). Further studies will be required to elucidate whether the observed differences in

the pattern of *MALAT1* expression can explain the distinctive histologic picture of NAFLD observed in patients who are morbidly obese.[29-31] It could be argued, however, that RNA expression levels in liver specimens isolated by surgical procedures behave differently from liver tissue obtained by percutaneous liver biopsy.[32] This is a robust reason that supports the importance of a replication stage that was included in the present study.

Fourth, in terms of gene network-associated pathways, *MALAT1* involvement overlaps common mechanisms of liver injury. More explicitly, we found that *MALAT1* overexpression was a common molecular feature in chronic liver diseases, including NASH and autoimmune diseases. This observation could provide a novel framework for understanding the pathogenesis of chronic liver damage, which suggests that convergent pathophenotypes (inflammation and fibrosis) share similar molecular mediators. Therefore, the natural history of chronic liver damage, regardless of the leading cause, ultimately results in cirrhosis and/or eventually HCC.

Last, owing to the complexity of liver tissue, particularly during NASH development in which an intricate cellular milieu coexists, we cannot rule out the possibility that *MALAT1* abundance is derived from multicellular sources. In fact, we showed that *MALAT1* expression is particularly abundant in cells of the immune system. Together, whether originating from hepatocytes or tissue-resident macrophages, neutrophils, natural killer cells, or lymphocytes that colonized the liver from the circulating compartment, all of the above-mentioned observations support the notion that *MALAT1* participates in a positive feedback loop of inflammation →tissue damage ↔ tissue repair → fibrogenesis→ cancer.

A potential limitation of our study stems from its observational cross-sectional nature, which prevented us from proving causality. We have initiated the longitudinal assessment of paired liver biopsies taken at least 5 years apart from patients with NAFLD who had progressed to an advanced disease stage. Remarkably, measurements of liver *MALAT1* abundance showed a dramatic 8-fold increase in a patient who progressed from NAFL to NASH fibrosis and a 29-fold increase in a NASH patient who progressed from fibrosis 0 to fibrosis 3. This observation is particularly significant as fibrosis stage is known to be associated with long-term outcomes in patients with NAFLD.[33,34] Certainly, a more comprehensive study is needed but will require time and an adequate sample size.

Our findings may have clinical and therapeutic implications. First, the notion that *MALAT1* is involved in the regulation of JAK-STAT signaling suggests an interesting clue for the mechanism by which NAFL progresses into NASH and eventually to cirrhosis and/or HCC.[35] Min and coworkers[36] observed that the STAT3 pathway is activated in patients with NAFLD. In addition, we have reported an association between *STAT3* polymorphisms in the susceptibility to NAFLD and disease severity.[37]

Finally, the fact that deregulated expression of liver *MALAT1* was seen at convergent pathophenotypes, which include inflammation, fibrosis, and immune-response related mechanisms, supports the use of systems biology to predict novel therapeutic agents that target pathophenotypes rather than specific diseases. An interesting example of this strategy is the use of a farnesoid X receptor (*FXR*, formally *NR1H4*) agonist (obeticolic acid) for the treatment of both primary biliary cholangitis[38] and NASH.[39]

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