Short Communication JHEP|Reports

Quantitative digital pathology reveals association of cell-specific *PNPLA3* transcription with NAFLD disease activity

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Highlights

- Relationships between *PNPLA3* transcription and the severity of human non-alcoholic fatty liver disease was unknown.
- *PNPLA3* transcript abundance is negatively associated with hepatic steatosis and non-alcoholic fatty liver disease activity.
- Nuclear-to-cytoplasmic translocation of *PNPLA3* is also negatively associated with non-alcoholic fatty liver disease severity.
- *PNPLA3* transcript abundance in activated myofibroblasts is inversely associated with the stage of liver fibrosis.

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Lay summary

A genetic variant in patatin-like phospholipase domain-containing protein 3 (or *PNPLA3*) is the most important genetic determinant of non-alcoholic fatty liver disease (NAFLD). However, it is not known how transcriptional regulation of the *PNPLA3* gene contributes to the disease characteristics of human NAFLD. Herein, we show that the mRNA levels of *PNPLA3*, particularly in the cytoplasm, are negatively associated with the severity of NAFLD in humans.

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Quantitative digital pathology reveals association of cell-specific *PNPLA3* transcription with NAFLD disease activity

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Background & Aims: The I148M variant (rs738409) in patatin-like phospholipase domain-containing protein 3 (PNPLA3) is by far the most important genetic determinant of non-alcoholic fatty liver disease (NAFLD). However, in the context of NAFLD, the transcriptional regulation of *PNPLA3* in human liver cells is not known. In this study, we aimed to define the relationship between *PNPLA3* transcription and disease characteristics of human NAFLD.

Methods: The abundance of *PNPLA3* and collagen 1α (*COL1α*) transcripts was quantified *in situ* at single-cell resolution using RNAscope® in 87 patients with NAFLD. We examined the association of *PNPLA3* and *COL1α* transcript levels with NAFLD disease severity, defined by histology.

Results: While the majority of *PNPLA3* transcripts were found in hepatocytes, approximately 7% of *PNPLA3*-positive cells co-express *COL1α*, representing activated myofibroblasts. There is no association between the rs738409 genotype and the level of *PNPLA3* transcript. The overall *PNPLA3* transcript abundance is lower in zone 1 hepatocytes, patients with higher body mass index, and those with advanced liver fibrosis. The negative association between the *PNPLA3* transcript levels and liver fibrosis is largely driven by *COL1α*-positive cells. A significant proportion of *PNPLA3* mRNA is seen in the nucleus. The cytoplasmic-to-nuclear *PNPLA3* mRNA ratio is inversely associated with NAFLD disease activity.

Conclusions: *PNPLA3* transcript abundance and nuclear-to-cytoplasmic translocation are negatively associated with hepatic steatosis and NAFLD disease activity, while its abundance in activated myofibroblasts is inversely associated with the stage of liver fibrosis.

Introduction

A genetic variant in patatin-like phospholipase domain-containing protein 3 (PNPLA3), encoding an I148M mutation (rs738409: G), is by far the most important genetic determinant of non-alcoholic fatty liver disease (NAFLD).[1,2] In addition to hepatic steatosis, the I148M variant is also associated with faster progression of liver fibrosis and higher risk of hepatocellular carcinoma.[3,4]

PNPLA3 is an acyltransferase expressed by both hepatocytes and hepatic stellate cells (HSCs).[5] It converts lysophosphatidic acid into phosphatidic acid, the activity of which is decreased in I148M.[6] Interestingly, neither genetic deletion nor overexpression of PNPLA3 impacts intrahepatic fat content.[7,8] *PNPLA3* is transcriptionally regulated by sterol regulatory element-binding protein-1c (SREBP-1c) and carbohydrate-responsive element-binding protein (ChREBP).[9,10] In murine models, a Western diet induces PNPLA3 expression.[11] The I148M variant leads to the retention of PNPLA3 on lipid droplets by evading ubiquitylation.[12] This has been proposed as a key mechanism leading to hepatic steatosis. Furthermore, the expression of PNPLA3 is increased during the activation of immortalized HSCs[13]. The PNPLA3 148I isoform induces a reduction in the secretion of profibrotic cytokines, whereas the 148M variant potentiates the profibrogenic transformation of HSC.[13,14]

Much of the mechanistic insight into PNPLA3 is derived from *in vitro* and animal studies, while in-depth data on human samples are scarce. Previous locus-wide expression quantitative trait loci (eQTL) mapping indicates that rs738409 does not impact *PNPLA3* transcription.[15] Herein, we aim to examine *PNPLA3* transcript levels using quantitative single-cell analysis to determine their relationship with NAFLD disease characteristics.

Patients and methods

Patient population

A total of 100 patients were identified from a prospective NAFLD registry at Beth Israel Deaconess Medical Center (BIDMC), which has been described elsewhere.[16] Data on patient demographics, medical history and physical examinations, as well as whole blood samples, were obtained at enrollment. The study has been approved by the BIDMC institutional review board.

Liver biopsy

An ultrasound-guided liver biopsy was performed within 3 months of enrollment. Biopsy results were interpreted by staff pathologists specialized in hepatopathology and reported in a

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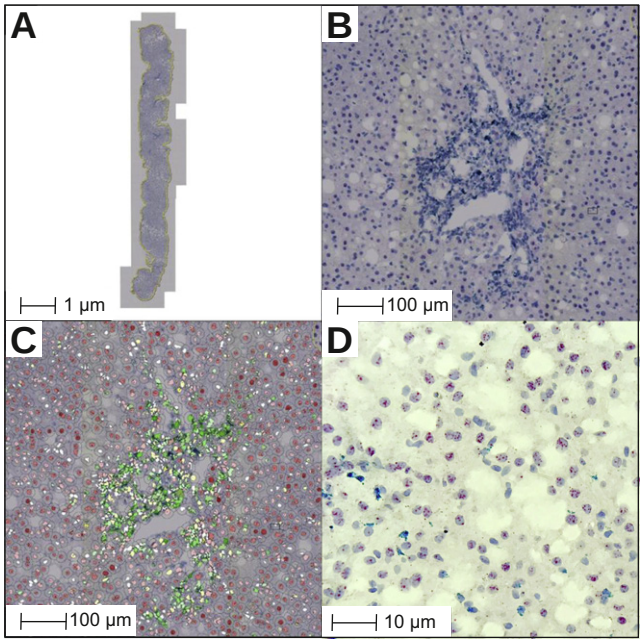

Fig. 1. RNAscope and *in silico* single-cell analysis of *PNPLA3* and *COL1α*. (A) Digitized whole section scan of FFPE liver biopsy. (B) A close-up view of a portal area demonstrating zone 1 macrovesicular steatosis. (C) *In situ* analysis of *PNPLA3* (red) and *COL1α* (green) transcript abundance using Halo. Darker color representing higher mRNA copy-number. (D) Cellular distribution of *PNPLA3* (magenta) and *COL1α* (cyan) mRNA. FFPE, formalin-fixed paraffin-embedded.

standardized fashion, including fibrosis stages (1-4) and NAFLD activity score (NAS, 0-8) calculated based on the degrees of hepatic steatosis (0-3), lobular inflammation (0-3) and ballooning degeneration (0-2).[17]

DNA extraction and SNP genotyping

Genomic DNA was extracted from stored human whole blood samples. Single nucleotide polymorphism (SNP) rs738409 was genotyped by TaqMan Allelic Discrimination using a predesigned TaqMan SNP genotyping assay (Applied Biosystems).[18]

RNAscope staining of *PNPLA3* and *COL1α*

The RNA probes for *PNPLA3* (Magenta) and *COL1α* (cyan) were customer-designed at ACDBio (Newark, CA, USA) using standard protocol and quality control criteria. RNA *in situ* hybridization was performed on 5 μm formalin-fixed paraffin-embedded (FFPE) sections using RNAscope® 2.5 HD Duplex Assay manual kit (ACDBio). Duplicate samples were simultaneously stained for house-keeping genes *PPIB* and *POLAR2A*. Samples with inadequate *PPIB/POLAR2A* staining were excluded in the analysis.

Imaging processing and analysis

The entire biopsy was scanned using a Zeiss Axio Scan Z1 microscope (Zeiss, Oberkochen, Germany) with automation. The single-cell analysis was performed on the entire section using Halo (Indica Labs, Corrales, NM, USA). Cell size criteria were refined to maximize the number of detected cells and a 5 μm perimeter from tissue-edge was excluded in analysis. Automated Image analysis was performed to determine *PNPLA3* or *COL1α* mRNA copy count per cell. We then compared the copies of *PNPLA3* or *COL1α* mRNA per cell across rs708409 genotype, body mass index (BMI), fibrosis stages, NAS, and the 3 individual components of NAS. We identified 25 randomly selected, well-defined hepatic lobules and measured copies of *PNPLA3* mRNA in 3 evenly divided concentric zones within the lobule, representing zone 1 to 3. The cytoplasmic-to-nuclear (C/N) mRNA ratio was calculated by dividing the total cytoplasmic copies of mRNA by the nuclear copy count. One-way ANOVA was used to compare the difference of *PNPLA3* transcription over rs708409 genotype and the hepatic zones. We performed linear regressions to determine the relationship between copies of *PNPLA3* mRNA, mRNA C/N ratio, and fibrosis stage, BMI, NAS, and its components.

For further details regarding the materials used, please refer to the CTAT table and supplementary information.

Results

Patient cohort and standardization of single-cell analysis via RNAscope

A total of 100 biopsy-proven cases were identified with relatively even distribution of *PNPLA3* rs738409 genotype and fibrosis stage. RNA *in situ* hybridization was performed on FFPE liver biopsies via RNAscope (Fig. 1A-D). *COL1α* was chosen as both a marker for activated myofibroblasts and internal control. Each copy of mRNA is discretely detected by a nucleotide probe that can be visualized and counted *in situ* (Fig. 1D). A total of 87 samples passed quality control criteria and were included in the study with the baseline characteristics summarized in Table S1. The mean age of the cohort was 49 years and the mean BMI was 33.5 ± 6.3; 38% of the cohort were female, 40% were GC and 39% were GG at rs738409. About 80% of patients had non-alcoholic steatohepatitis with a mean NAS of 4.5 ± 1.5, and fibrosis stages from 0-4: 32%, 16%, 26%, 14% and 12%, respectively. An average of 27,000 cells per sample were digitally analyzed.

Localization of *PNPLA3* mRNA on the liver biopsy

The *PNPLA3* mRNA was predominantly seen in hepatocytes (Fig. 1C, D). In comparison, *COL1α* mRNA was noted in cells congregating in the portal area, in keeping with the distribution of myofibroblasts (Fig. 1C). Approximately 53% of *COL1α*-positive cells carried at least 1 copy of *PNPLA3*, whereas only 7% of *PNPLA3*-positive cells carried at least 1 copy of *COL1α*. A significant proportion of *PNPLA3* mRNA was located in the nucleus, with a mean C/N ratio of 0.52 (95% CI 0.43–0.60), whereas *COL1α* had a C/N ratio of 3.33 (2.84–3.82).

Association between *PNPLA3* transcription and rs738409 genotype

No statistical difference was noted in the abundance of *PNPLA3* or *COL1α* mRNA across rs738409 genotypes ($p = 0.9$ and 0.5 respectively by ANOVA) (Fig. 2A). Limiting the analysis to *COL1α*-positive cells did not change this result, nor did the adjustment for BMI or fibrosis stages.

Association of *PNPLA3* transcription with histological characteristics of NAFLD

Macrovesicular steatosis typically occurs in zone 1 of the hepatic lobule. Compared to zone 2 and 3, *PNPLA3* transcript abundance was 0.35 standard deviations lower in zone 1, while no differences were seen between zone 2 and 3 hepatocytes (Fig. 2B). *PNPLA3* total transcript abundance was also inversely associated with BMI (p trend = 0.03) (Table S2).This association was driven by *COL1α*-negative cells, but not *COL1α*-positive cells. *PNPLA3* mRNA in either *COL1α*-positive or *COL1α*-negative cells had no significant association with steatosis, lobular inflammation, or ballooning degeneration scores. While *COL1α* demonstrated the

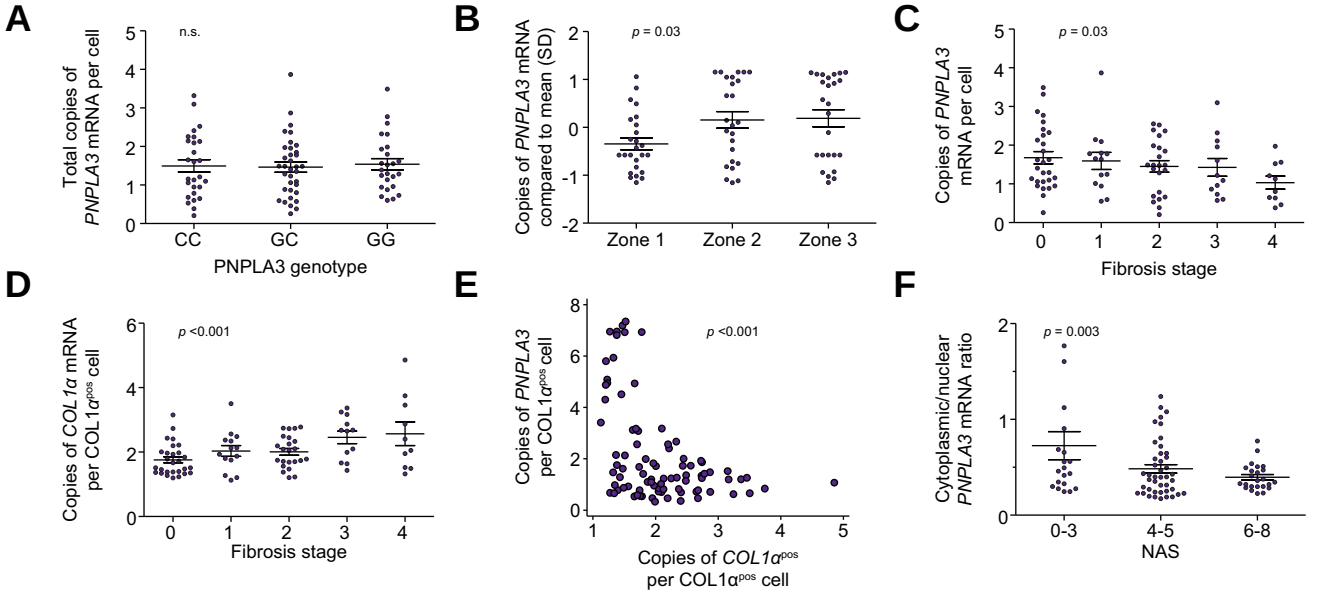

Fig. 2. *PNPLA3* transcription in relation to rs708409 genotype and NAFLD disease characteristics. (A) Comparison of *PNPLA3* transcription across rs708409 genotypes. (B) Comparison of *PNPLA3* transcription in zone 1, 2, and 3 hepatocytes expressed in standard deviation from the mean mRNA/cell in all zones. (C,D) Relationships between *PNPLA3* (C), *COL1α* (D) transcription and liver fibrosis. (E) Relationship between *PNPLA3* and *COL1α* transcription in *COL1α*-positive cells. (F) Relationship between *PNPLA3* mRNA C/N ratio and NAS. *P* values calculated by ANOVA in A and B, linear regression in C-F. C/N, cytoplasmic-to-nuclear; NAFLD, nonalcoholic fatty liver disease; NAS, NAFLD activity score.

expected robust association with the stage of liver fibrosis, *PNPLA3* mRNA levels were inversely associated with fibrosis, but not the histological diagnosis of non-alcoholic steatohepatitis ($p = 0.98$) (Fig. 2C, D). This inverse association with fibrosis was predominantly driven by *COL1α*-positive cells that only represented a minority of *PNPLA3*-positive cells, and it was not changed after adjustment for BMI. Furthermore, *PNPLA3* mRNA was inversely associated with *COL1α* mRNA in *COL1α*-positive cells ($\beta = -1.3$, 95% CI -1.8 to -0.7) (Fig. 2E). We found that a significant proportion of *PNPLA3* mRNA was in the nucleus rather than the cytoplasm. The C/N ratio of PNPLA3 was inversely associated with NAS (Fig. 2F) and its 3 components (Fig. S1A-C). In comparison, the C/N ratio of *COL1α* was positively associated with the stage of fibrosis (Fig. S1D) but had no relationship with NAFLD disease activity.

Discussion

Mechanistic investigation of PNPLA3 in NAFLD has predominantly relied on murine models and *in vitro* studies. The homology between mouse and human PNPLA3 is only 68%, with significant differences in expression.[12] This study demonstrates the feasibility of digital pathology and single-cell analysis to study the relationship between *PNPLA3* transcription and NAFLD disease characteristics in human liver biopsy.

Our study provides 3 important observations. First, we confirmed that the rs738409 genotype does not impact on *PNPLA3* transcript expression, as previously reported by eQTL mapping.[9,15] Secondly, our study suggests an inverse relationship between NAFLD disease activity and *PNPLA3* transcription on multiple levels. The *PNPLA3* transcript abundance was the lowest in zone 1 hepatocytes where steatosis is the most severe. This relationship with steatosis is in keeping with an inverse relationship with BMI in hepatocytes that are *COL1α* negative. It is plausible that insulin resistance is the common driver between these associations. Further, the *PNPLA3* mRNA C/N distribution was also lower in biopsies with increased disease activity. The export of mRNA from the nucleus to cytoplasm is a part of regulatory control of protein translation.[19] The regulation on nucleus exportation has not been described for PNPLA3, and warrants further investigation. Finally, we observed a co-localization of *PNPLA3* and *COL1α* mRNA transcripts indicating that both genes are transcribed in myofibroblasts. The abundance of *PNPLA3* transcript is inversely associated with the fibrosis stage and the abundance of *COL1α* mRNA in *COL1α*-positive cells, representing activated myofibroblasts. This supports the recent study by Bruschi and colleagues demonstrating that PNPLA3 may regulate the activation of HSCs.[13,14] However, our study did not detect a statistically significant impact of rs738409 genotype on the abundance of the *COL1α* transcript, which needs to be reconciled with published *in vitro* observations.

Our study demonstrates that digital pathology with single-cell *in situ* analysis can provide powerful mechanistic insights. In particularly, it allowed us to measure the C/N ratio, a potentially informative parameter of cellular state that has not been previously characterized. Several limitations of the current study are noted. First, liver biopsy is usually obtained under fasting conditions. Thus, this study does not capture nutritient-mediated regulation of *PNPLA3* mRNA transcription under SREBP-1c or ChREBP.[9] The current method only allows simultaneous staining of 2 genes, limiting the ability to precisely define cell type. Finally, future studies may need to validate the relationship between *PNPLA3* transcription and liver histology in other liver diseases.

In summary, our study demonstrated that basal *PNPLA3* transcript abundance and cytoplasmic translocation is negatively associated with hepatic steatosis, BMI, and NAFLD disease activity. In activated myofibroblasts, *PNPLA3* transcript abundance is inversely associated with the stage of liver fibrosis.

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Conflict of interest

The authors declare no conflict of interest relevant to this study.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

BS and ZGJ were involved in the study design and data analysis; MCPM, ST, EC, AZ, MK, MH, IN and ML provided significant material and technical support; all authors participated in manuscript preparation. All authors approved the final version of the manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jhepr.2019.05.007.

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