Trajectories of Liver Fibrosis and Gene Expression Profiles in Nonalcoholic Fatty Liver Disease Associated With Diabetes

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Understanding the mechanisms linking steatosis to fibrosis is needed to establish a promising therapy against nonalcoholic fatty liver disease (NAFLD). The aim of this study was to clarify clinical features and hepatic gene expression signatures that predict and contribute to liver fibrosis development during the long-term real-world histological course of NAFLD in subjects with and without diabetes. A pathologist scored 342 serial liver biopsy samples from 118 subjects clinically diagnosed with NAFLD during a 3.8-year (SD 3.45 years, maximum 15 years) course of clinical treatment. At the initial biopsy, 26 subjects had simple fatty liver, and 92 had nonalcoholic steatohepatitis (NASH). In the trend analysis, the fibrosis-4 index ($P < 0.001$) and its components at baseline predicted the future fibrosis progression. In the generalized linear mixed model, an increase in HbA_{1c}, but not BMI, was significantly associated with fibrosis progression (standardized coefficient 0.17 [95% CI 0.009–0.326]; $P = 0.038$) for subjects with NAFLD and diabetes. In gene set enrichment analyses, the pathways involved in zone 3 hepatocytes, central liver sinusoidal endothelial cells (LSECs), stellate cells, and plasma cells were coordinately altered in association with fibrosis progression and HbA_{1c} elevation. Therefore, in subjects with NAFLD and diabetes, HbA_{1c} elevation was significantly associated with liver fibrosis progression, independent of weight gain, which may be a valuable therapeutic target to prevent the pathological progression of NASH.

ARTICLE HIGHLIGHTS

- It remains uncertain how diabetes and obesity contribute to histological courses of nonalcoholic fatty liver disease (NAFLD).
- Clinical features and gene expression signatures that predict or are associated with future liver fibrosis development were assessed in a serial liver biopsy study of subjects with NAFLD.
- An increase in HbA_{1c}, but not BMI, was associated with liver fibrosis progression in the generalized linear mixed model.
- Considering hepatic gene set enrichment analyses, diabetes may enhance liver fibrosis via injuring central liver sinusoidal endothelial cells that mediate inflammation and stellate cell activation during NAFLD development.

Gene expression profiles suggest that diabetes-induced hypoxia and oxidative stress injure LSECs in zone 3 hepatocytes, which may mediate inflammation and stellate cell activation, leading to liver fibrosis.

Nonalcoholic fatty liver disease (NAFLD) ranges from simple nonalcoholic fatty liver to nonalcoholic steatohepatitis (NASH) with inflammation and fibrosis. Among the

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histological features of NASH, liver fibrosis is associated with long-term outcomes, such as liver cirrhosis, liver failure, hepatocellular carcinoma, and all-cause mortality (1,2). However, despite intensive pharmaceutical efforts in drug development and clinical trials, no promising therapy against liver fibrosis has been established (3). It is well recognized that obesity is a leading cause of NAFLD. Still, it remains unclear what clinical features besides obesity are responsible for the pathological progression of liver fibrosis, especially in Japanese patients, who generally are not severely obese, in contrast to Western patients.

In the natural course of posttransfusion chronic hepatitis C, type 2 diabetes accelerates liver fibrosis and subsequent development of hepatocellular carcinoma and mortality (4), suggesting a causal role of hyperglycemia in liver fibrosis. Consistently with this observation, our previous comprehensive analysis of the hepatic gene expression profile revealed that in the livers of subjects with diabetes, expression of the genes in the transforming growth factor-β1family, together with angiogenic factors such as platelet-derived growth factor, are cooperatively upregulated (5). These cytokines activate stellate cells to myofibroblasts that produce collagen. Indeed, genes for collagens are upregulated in the liver of patients with type 2 diabetes, even those without NASH (6). These findings suggest that hyperglycemia independently accelerates liver fibrosis in various liver diseases.

In a small-scale serial liver biopsy analysis of 39 Japanese patients with NAFLD, we previously found that reduction in HbA_{1c} and insulin treatment independently contribute to alleviating liver fibrosis (7). However, the small number of subjects and short observation periods prevented us from confirming the significance of the findings in the analyses of subgroups with and without diabetes. In addition, gene expression signatures underlying pathological progression of human NAFLD/NASH remain underinvestigated.

To identify the clinical parameters that predict and determine the development of liver fibrosis, we extended the previous study by increasing the number of subjects with serial liver biopsies and observation periods and analyzed the trajectories of liver fibrosis by the presence or absence of diabetes using trend tests and a generalized linear mixed model. Furthermore, we examined serial global hepatic gene expression profiles associated with hepatic fibrosis and glycemic control to address the molecular basis underlying hepatic fibrosis progression.

RESEARCH DESIGN AND METHODS

In this prospective cohort study, subjects who had undergone two or more serial liver biopsies for NAFLD since 1998, with a maximum of 15 years between biopsies, were observed. Subjects' eligibility and exclusion criteria are described in the Supplementary Appendix.

As shown in Supplementary Fig. 1, we initially recruited 350 subjects with histologically confirmed NAFLD who had undergone liver biopsy from 1998 to 2014 at Kanazawa University Hospital. Of these subjects, 121 had undergone serial liver biopsies. Histology of the serial liver biopsy samples from these 121 subjects was reanalyzed by a pathologist who was not allowed access to the subjects' clinical information. Three subjects were excluded after histological reexamination (one with hepatic sarcoidosis and two without significant liver steatosis). The accepted subjects were followed until December 2017. Finally, 118 subjects diagnosed histologically with NAFLD were enrolled (Supplementary Fig. 1).

The study was conducted with the approval of the ethics committee of Kanazawa University Hospital in accordance with the Declaration of Helsinki. All subjects provided written informed consent.

Data Collection

Clinical information was obtained for each subject, including age, sex, body measurements, alcohol consumption, medical history, medication use, and laboratory tests. Details are provided in the Supplementary Material.

Histological Examination of the Liver

Biopsies were obtained after a thorough clinical evaluation and the receipt of signed informed consent from each subject and were performed during hospitalization. Liver biopsy slides were stained with hematoxylin-eosin, Azan Mallory, and silver reticulin impregnation. A single pathologist (K.H.) blind to both clinical information and the order in which the biopsies had been obtained analyzed all biopsies. The NAFLD activity score was calculated as the unweighted sum of the scores for steatosis (0–3), lobular inflammation (0–3), and ballooning (0–2) as reported by Kleiner et al. (8). NASH was histologically diagnosed based on the presence of ballooned hepatocytes with lobular hepatitis along with >5% steatosis. Fibrosis was scored as 0 (normal connective tissue), 1 (perisinusoidal fibrosis in zone 3), 2 (perisinusoidal fibrosis in zone 3 with portal fibrosis), 3 (bridging fibrosis), or 4 (cirrhosis) according to the standard criteria developed by Brunt et al. (9).

Hepatic Gene Expression

We examined serial global hepatic gene expression profiles associated with hepatic fibrosis and glycemic control to address the molecular basis underlying hepatic fibrosis progression. Hepatic gene expression profiles were examined in 33 patients using serial liver biopsy samples, as previously reported (10). Details are provided in the Supplementary Appendix.

Among 33 subjects, the histological stage of liver fibrosis progressed in 6, improved in 7, and did not change in others. Regarding HbA_{1c} levels, values increased in 9 subjects and decreased in 16 (Supplementary Tables 3*B* and *C*).

Statistical Analysis

First, we used trend tests to identify clinical parameters that predict liver fibrosis development. Next, we used a generalized linear mixed model for multilevel analyses of continuous

liver biopsy to determine the pathological conditions and parameters contributing to liver fibrosis development (details provided in the Supplementary Appendix). SPSS version 27.0 software (IBM Corporation, Armonk, NY) was used for all statistical analyses. $P < 0.05$ was considered to indicate statistical significance.

Data and Resource Availability

All data are included in the article and supplementary materials. The data sets and resources generated and analyzed during the study are available from the corresponding author upon reasonable request.

RESULTS

Baseline Data

This study included 118 subjects (67 males and 51 females) diagnosed histologically as having NAFLD (Supplementary Fig. 1). These subjects had a mean duration of follow-up of 3.8 ± 3.45 years (maximum 15 years). A total of 342 liver biopsy samples were systematically rescored according to the classifications. The baseline clinical, laboratory, and histological characteristics of the 118 subjects are described in Supplementary Table 1. The mean (SD) age, BMI, and HbA_{1c} scores of these subjects with NAFLD were 50.7 (14.1) years, 28.8 (5.6) kg/m^2, and 7.6% (2.0%), respectively. At baseline, 86 (72.9%) of the 118 subjects had type 2 diabetes. At the initial liver biopsy, 26 subjects had nonalcoholic fatty liver, and 92 had NASH.

Baseline Clinical Factors Affecting Final Liver Histology

First, a trend test was performed to identify baseline clinical factors associated with liver histological fibrosis scores at the end of the follow-up (Table 1). Hepatic fibrosis at follow-up was positively correlated with age ($P = 0.001$), AST ($P = 0.001$), AST/ALT ratio ($P < 0.001$), fibrosis-4 (FIB-4) index ($P < 0.001$), treatment with ursodeoxycholic acid ($P = 0.002$), and having a history of hepatocellular carcinoma ($P = 0.003$) at baseline. It was negatively correlated with total cholesterol ($P = 0.011$) and platelet count ($P < 0.001$). On the other hand, HbA_{1c} and BMI at baseline were not associated with hepatic fibrosis at follow-up (Table 1).

Changes in Clinical Parameters Associated With the Progression of Liver Pathology

Next, we performed multivariable analyses to evaluate associations between changes in clinical parameters and the progression of liver pathology over time using generalized linear mixed models, adjusting for covariates. In the generalized linear mixed model analysis, the number of liver biopsies and age did not independently affect liver fibrosis progression (Table 2). AST ($P = 0.022$), γ-glutamyl transferase (GGT) ($P = 0.038$), HbA_{1c} ($P = 0.038$), and treatment with ursodeoxycholic acid ($P = 0.043$) were positively correlated and platelet count ($P < 0.001$) negatively correlated with the progression of liver fibrosis in all subjects (Table 2). To discriminate the interaction of these factors with diabetes, we performed multivariable analyses separately in subjects with and without diabetes. In the group with diabetes, HbA_{1c} ($P = 0.038$) and GGT ($P = 0.025$) were positively correlated and platelet count ($P = 0.001$) negatively correlated with the progression of hepatic fibrosis. On the other hand, in the group without diabetes, AST ($P = 0.011$) was positively correlated and ALT ($P = 0.044$) negatively correlated with the progression of hepatic fibrosis (Table 2). Administration or discontinuation of ursodeoxycholic acids, but not insulin, metformin, and sulfonylureas, was significantly associated with changes in liver fibrosis scores.

We calculated the coefficients of interaction by type 2 diabetes and HbA_{1c} using a generalized linear mixed model with adjustments for sex, age, and BMI. As shown in Supplementary Table 2, diabetes tended to interact with HbA_{1c} and fibrosis progression ($P = 0.054$).

Hepatic Gene Expression Profiles Associated With Hepatic Fibrosis Progression

To address the molecular basis underlying liver fibrosis progression, we examined serial hepatic gene expression profiles associated with liver fibrosis and glycemic control using RNA sequencing (RNA-seq) in 33 subjects. Baseline clinical characteristics in these subjects are described in Supplementary Table 3*A*. Among 33 subjects, the histological stage of liver fibrosis progressed in 6, improved in 7, and did not change in others. Regarding HbA_{1c} levels, values increased in 9 subjects and decreased in 16 (Supplementary Tables 3*B* and *C*).

Pathway analyses identified genes coordinately altered in the liver of subjects with hepatic fibrosis progression (Supplementary Table 5) or HbA_{1c} elevation (Supplementary Table 6) during the course. To further address which components of resident cells participate in alleviating liver fibrosis and glycemic control, we performed gene set enrichment analyses using gene sets associated with resident cells in the human liver defined by single-cell RNA-seq analyses (11). Tables 3 and 4 show the coordinately altered pathways associated with the progression or regression of fibrosis and the elevation or reduction in HbA_{1c} levels, respectively.

DISCUSSION

This study is the first to use a trend test and a generalized linear mixed model to reveal the factors contributing to the long-term real-world histological course of the liver in subjects with NAFLD. Trend analyses revealed that the baseline FIB-4 Index and its components (age, platelet count, AST, and ALT) predicted the future progression of liver fibrosis. The FIB-4 Index reflects the real-time status of hepatic fibrosis in subjects with NAFLD in the current study (data not shown), as previously reported (12,13). In a study evaluating the prevalence of NAFLD in type 2 diabetes, the estimated fibrosis score by FIB-4 Index was consistent with the actual score obtained by liver biopsy (14). In addition, of the 4,899 patients with type 1

Table 1—Trend analysis between final fibrosis score and baseline variables

	Final fibrosis score					
	0	1	2	3	4	*P*
Age (years)	46.8 (10.6)	46.5 (14.9)	54.4 (15.1)	56.8 (12.7)	57.0 (6.7)	0.001**
Sex (male:female)	15:3	23:25	14:9	9:12	6:2	0.39
Type 2 diabetes	12 (14.0)	36 (41.9)	16 (18.6)	18 (20.9)	4 (4.7)	0.963
Hypertension	6 (11.3)	18 (34.0)	13 (24.5)	13 (24.5)	3 (5.7)	0.103
Dyslipidemia	16 (18.6)	37 (43.0)	15 (17.4)	14 (16.3)	4 (4.7)	0.022*
BMI (kg/m^2)	26.0 (2.8)	29.6 (6.7)	29.7 (5.6)	29.2 (4.5)	27.0 (4.6)	0.110
AST (IU/L)	33.6 (15.5)	40.7 (26.7)	42.8 (22.9)	53.5 (27.6)	101.9 (109.7)	0.001**
ALT (IU/L)	64.6 (39.1)	65.7 (48.8)	63.9 (39.0)	72.4 (47.4)	91.6 (85.6)	0.443
AST/ALT ratio	0.61 (0.26)	0.68 (0.21)	0.75 (0.25)	0.85 (0.26)	1.15 (0.49)	<0.001**
GGT (IU/L)	72.2 (54.7)	71.1 (51.1)	58.2 (34.6)	95.3 (65.5)	96.4 (38.4)	0.056
HbA_{1c} (%)	7.4 (1.6)	8.0 (2.4)	7.7 (2.1)	6.8 (1.3)	7.0 (1.7)	0.232
Fasting plasma glucose (mg/dL)	127.1 (35.3)	134.9 (38.8)	131.9 (67.1)	113.9 (27.8)	133.5 (46.6)	0.229
Platelets (× 10^9/L)	246.1 (78.0)	239.3 (50.6)	225.5 (52.5)	194.7 (73.2)	134.6 (48.0)	<0.001**
FIB-4 Index	0.91 (0.44)	1.06 (0.66)	1.48 (0.99)	2.21 (1.35)	4.75 (2.79)	<0.001**
Total cholesterol (mg/dL)	200.7 (46.8)	200.9 (46.1)	187.0 (28.2)	189.7 (51.1)	165.3 (43.9)	0.011*
Triglycerides (mg/dL)	153.4 (118.4)	158 (89.1)	147.4 (84.6)	137.0 (96.9)	128.4 (78.7)	0.276
HDL cholesterol (mg/dL)	46.4 (13.6)	47.5 (10.7)	44.8 (10.3)	47.6 (15.5)	41.1 (11.1)	0.383
Treatment						
Insulin	4 (28.6)	4 (28.6)	4 (28.6)	2 (14.3)	0 (0)	0.273
Metformin	2 (11.8)	10 (58.8)	1 (5.9)	4 (23.5)	0 (0)	0.461
Sulfonylureas	4 (22.2)	7 (38.9)	4 (22.2)	3 (16.7)	0 (0)	0.281
Ursodeoxycholic acid	0 (0)	0 (0)	0 (0)	1 (33.3)	2 (66.7)	0.002**
History of pancreatic surgery	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0.036*
History of hepatocellular carcinoma	0 (0)	2 (25.0)	0 (0)	4 (50.0)	2 (25.0)	0.003**

Data are mean (SD) or *n* (%) unless otherwise indicated. Jonckheere-Terpstra trend test was used for continuous variables and Cochran-Armitage trend test for categorical variables. *$P < 0.05$, **$P < 0.01$.

diabetes, advanced liver fibrosis was present in 6.7% based on a FIB-4 Index of ≥2.67 (15).

Following the trend analysis, we created a generalized linear mixed model to identify the therapeutic target against liver pathology progression in NAFLD. In subjects with NAFLD and diabetes, an increase in HbA_{1c} was significant, and an increase in BMI tended to be associated with the progression of liver fibrosis. Unlike in the subjects with NAFLD and diabetes, neither changes in HbA_{1c} nor BMI were associated with fibrosis progression in subjects without diabetes.

In the current study, baseline levels of and changes in BMI, often associated with insulin resistance, were not significantly associated with liver fibrosis development (Tables 1 and 2). In our recent intervention study, reduction in HbA_{1c}, but not BMI, was significantly associated with a reduction in liver fibrosis in patients with type 2 diabetes and NAFLD treated with the sodium–glucose cotransporter 2 inhibitor tofogliflozin (10). On the basis of these findings, we speculate that insulin resistance exerts limited effects on liver fibrosis progression, at least in relatively slender Japanese patients with NAFLD.

The following two studies support our conclusion. In a serial liver biopsy study in 103 subjects with NAFLD, multivariable analysis showed that diabetes was the cause of the progression of liver fibrosis (16). In addition, it was reported that diabetes incidence increased as liver fibrosis progressed in a serial liver biopsy study of 59 subjects with NAFLD (17). However, these studies did not use a generalized mixed model or identify biomarkers that predict and are associated with fibrosis progression in subjects with type 2 diabetes.

In the current study, surrogate liver enzymes were also distinct between subjects with NAFLD and with and without diabetes. GGT, like HbA_{1c}, was significantly associated with the progression of liver pathology in the group with NAFLD and diabetes. In contrast, changes in ALT and AST were negatively and positively associated with fibrosis progression, respectively, in the group with NAFLD but without diabetes. These findings are consistent with a

Table 2—Generalized linear mixed model for fibrosis score in adult patients with NAFLD

	Total			Type 2 diabetes			No diabetes		
	Standardized coefficient	95% CI	*P*	Standardized coefficient	95% CI	*P*	Standardized coefficient	95% CI	*P*
Number of liver biopsies	−0.11	−0.323 to 0.107	0.322	−0.05	−0.290 to 0.201	0.720	−0.33	−0.986 to 0.321	0.307
Sex	0.11	−0.059 to 0.273	0.205	0.10	−0.104 to 0.303	0.335	0.11	−0.241 to 0.465	0.524
Age	0.09	−0.102 to 0.286	0.351	0.16	−0.072 to 0.384	0.179	−0.01	−0.431 to 0.417	0.974
Observation period	−0.03	−0.247 to 0.189	0.792	−0.09	−0.348 to 0.161	0.467	0.07	−0.566 to 0.714	0.815
BMI	0.16	0.000 to 0.319	0.050	0.17	−0.026 to 0.358	0.089	0.22	−0.100 to 0.546	0.170
AST	0.29	0.042 to 0.538	0.022	0.21	−0.088 to 0.499	0.168	0.87	0.215 to 1.532	0.011
ALT	−0.21	−0.458 to 0.04	0.099	−0.16	−0.443 to 0.129	0.279	−0.73	−1.443 to −0.021	0.044
GGT	0.17	0.009 to 0.332	0.038	0.21	0.026 to 0.383	0.025	0.38	−0.051 to 0.812	0.082
Fasting plasma glucose	−0.06	−0.209 to 0.086	0.415	−0.05	−0.210 to 0.115	0.562	0.07	−0.209 to 0.353	0.604
HbA_{1c}	0.16	0.009 to 0.308	0.038	0.17	0.009 to 0.326	0.038	0.04	−0.240 to 0.313	0.790
Platelets	−0.34	−0.500 to −0.173	<0.001	−0.32	−0.497 to −0.138	0.001	−0.28	−0.764 to 0.197	0.238
Total cholesterol	−0.11	−0.262 to 0.037	0.138	−0.04	−0.212 to 0.125	0.613	−0.35	−0.758 to 0.061	0.092
Triglycerides	−0.07	−0.212 to 0.078	0.362	−0.08	−0.238 to 0.086	0.356	0.03	−0.346 to 0.397	0.891
HDL cholesterol	−0.13	−0.279 to 0.022	0.094	−0.15	−0.326 to 0.026	0.093	−0.12	−0.489 to 0.244	0.499
Treatment									
Insulin	0.01	−0.106 to 0.134	0.823	0.04	−0.106 to 0.178	0.617			
Metformin	0.06	−0.066 to 0.193	0.338	0.09	−0.056 to 0.239	0.224	0.21	−0.022 to 0.436	0.075
Sulfonylureas	−0.05	−0.192 to 0.089	0.472	−0.01	−0.174 to 0.153	0.902			
Ursodeoxycholic acid	0.17	0.005 to 0.342	0.043	0.16	−0.020 to 0.331	0.082	0.50	−0.106 to 1.110	0.102
History of pancreatic surgery	−0.05	−0.222 to 0.119	0.551	−0.08	−0.291 to 0.131	0.454			
History of hepatocellular carcinoma	0.00	−0.183 to 0.184	0.994	0.01	−0.193 to 0.217	0.911	−0.53	−1.219 to 0.170	0.134

Table 3—Gene set enrichment analysis using resident cells gene sets in human liver defined by single-cell RNA-seq analyses in subjects with NAFLD with fibrosis regression or progression

	Gene set	Representative genes	Networks ($P < 0.05$)	Number of genes	LS permutation *P*	KS permutation *P*	Up- or downregulated
Fibrosis progression ($n = 6$)							
1	Zone 3 hepatocytes	*ADGRG6*, *NKG7*, *ATP13A3*, *TPM1*, *SLC7A2*, *RCL1, IL1RAP*, *PC*, *F5*, *C6*, *PDXDC1*, *ERGIC1*	Blood coagulation Inflammation_protein C signaling Development_skeletal muscle development Muscle contraction Cell adhesion_platelet-endothelium-leukocyte interactions Cytoskeleton_actin filaments Inflammation_innate inflammatory response	1,263	<0.00001	<0.00001	Down
2	Zone 1 hepatocytes	*HMGCS1*, *SLC7A2*, *C6*	Inflammation_complement system Proteolysis_ubiquitin-proteasomal proteolysis	161	<0.00001	<0.00001	Down
3	Stellate cells	*EFEMP2*, *WFDC1*, *LMOD1*, *TPM2*, *TPM1*, *GEM*, *MYL9*	Development_skeletal muscle development Muscle contraction Cytoskeleton_actin filaments Cell cycle_G2/M	65	0.00144	0.1542	Up
4	NK-like cells	*ACAP1*		81	0.0042	0.00005	Down
5	Noninflammatory macrophages	*ACAP1*		81	0.00431	0.00005	Down
6	Central LSECs	*NRP1*, *APP*, *SCARF1*, *BTNL9*	Cell adhesion_amyloid proteins Reproduction_feeding and neurohormone signaling Protein folding_ER and cytoplasm	260	0.00488	0.00233	Down
7	γδ-T cell 1	*NKG7*		46	0.01107	<0.00001	Down
8	αβ-T cell	*NKG7*		46	0.01138	0.00002	Down
9	Zone 2 hepatocytes			138	0.01813	<0.00001	Down
10	Cholangiocytes			59	0.03225	0.00119	Up
Fibrosis regression ($n = 7$)							
1	Inflammatory macrophages	*RGS18*, *TYROBP, CD68*, *MS4A7*, *ARPC1B*, *LST1*, *C3AR1*	Inflammation_TREM1 signaling Inflammation_NK-cell cytotoxicity Apoptosis_anti-apoptosis mediated by external signals via PI3K/AKT	223	<0.00001	0.00036	Down
2	Zone 2 hepatocytes	*TTC36*		138	0.00223	<0.00001	Up

Continued on p. 1303

Table 3—Continued

	Gene set	Representative genes	Networks ($P < 0.05$)	Number of genes	LS permutation P	KS permutation P	Up- or downregulated
3	Zone 3 hepatocytes	*TTC36*, *TYROBP*, *SORBS2*, *ARPC1B*	Inflammation_TREM1 signaling Inflammation_NK-cell cytotoxicity Apoptosis_anti-apoptosis mediated by external signals via PI3K/AKT	729	0.05933	0.00024	Up

Resident cell gene sets in liver were taken from MacParland et al. (11). Gene sets were taken from 20 clusters of genes consisting of hepatocytes (zones 1–6), cholangiocytes, central LSECs, periportal LSECs, portal endothelial cells, stellate cells, inflammatory macrophages, noninflammatory macrophages, αβ-T cells, γδ-T cells 1, γδ-T cells 2, NK cells, mature B cells, plasma cells, and erythroid cells. *ACAP1*, ArfGAP with coiled-coil, ankyrin repeat and PH domains 1; *ADGRG6*, adhesion G protein-coupled receptor G6; *APP*, amyloid β precursor protein; *ARPC1B*, actin-related protein 2/3 complex subunit 1B; *ATP13A3*, ATPase 13A3; *BTNL9*, butyrophilin-like 9; *C3AR1*, complement C3a receptor 1; C6, complement C6; *CD68*, CD68 molecule; *EFEMP2*, EGF-containing fibulin extracellular matrix protein 2; ER, endoplasmic reticulum; *ERGIC1*, endoplasmic reticulum-Golgi intermediate compartment 1; *F5*, coagulation factor V; *GEM*, GTP binding protein overexpressed in skeletal muscle; *HMGCS1*, 3-hydroxy-3-methylglutaryl-CoA synthase 1; *IL1RAP*, interleukin 1 receptor accessory protein; *LMOD1*, leiomodin 1; *LST1*, leukocyte-specific transcript 1; *MS4A7*, membrane spanning 4-domains A7; *MYL9*, myosin light chain 9; NK, natural killer; *NKG7*, natural killer cell granule protein 7; *NRP1*, neuropilin 1; *PC*, pyruvate carboxylase; *PDXDC1*, pyridoxal-dependent decarboxylase domain containing 1; PI3K, phosphoinositide 3-kinase; *RCL1*, RNA terminal phosphate cyclase-like 1; *RGS18*, regulator of G protein signaling 18; *SCARF1*, scavenger receptor class F member 1; *SLC7A2*, solute carrier family 7 member 2; *SORBS2*, sorbin and SH3 domain containing 2; *TPM1*, tropomyosin 1; *TPM2*, tropomyosin 2; TREM1, triggering receptor expressed on myeloid cells 1; *TTC36*, tetratricopeptide repeat domain 36; *TYROBP*, transmembrane immune signaling adaptor; *WFDC1*, WAP four-disulfide core domain 1. KS, Kolmogorov-Smirnov test; LS, least squares test.

previous report that elevated GGT is independently associated with undiagnosed advanced hepatic fibrosis in patients with type 2 diabetes (18) and suggest that a unique pathophysiology may underlie the progression of liver fibrosis between NAFLD with and without diabetes.

On the basis of these findings, we propose a disease entity, "diabetic steatohepatitis," in which hyperglycemia exacerbates liver fibrosis independent of weight gain. GGT, rather than ALT and AST, may be the best surrogate liver enzyme for diabetic steatohepatitis. Itoh et al. (19) in Japan first reported cirrhotic NASH in five postmenopausal women with obesity and type 2 diabetes. They named its pathology "diabetic cirrhosis" in 1979, prior to the proposal of the NASH pathology by Ludwig et al. (20) in 1980.

To address which components of resident cells participate in alleviating liver fibrosis and glycemic control, we performed gene set enrichment analyses using gene sets associated with resident cells in the human liver defined by single-cell RNA-seq analyses (11). Broad ranges of hepatocytes seemed to be functionally impaired because genes involved in zone 1, zone 2, and zone 3 hepatocytes, including genes encoding coagulation factor F5 and complement C6, were coordinately downregulated in association with liver fibrosis progression (Table 3), suggesting extensive hepatocyte damage during fibrosis progression.

Liver sinusoidal endothelial cells (LSECs) act as a platform for the adhesion of liver-resident noninflammatory macrophages, Kupffer cells, and stellate cells (21). In combination with Kupffer cells, LSECs themselves have a vital scavenger function. Conversely, Kupffer cells protect LSECs from Fas-dependent apoptosis in sepsis (22). In the current study, genes involved in LSECs in zone 3 (central LSEC) and noninflammatory macrophages were coordinately downregulated with the progression of liver fibrosis (Table 3). Inadequately controlled diabetes may injure LSECs in zone 3 and Kupffer cells, which mediate crosstalk between inflammatory cells and stellate cells in the space of Disse, leading to liver fibrosis.

Gene sets of stellate cells and cholangiocytes were coordinately upregulated with the progression of liver fibrosis. Cholangiocytes release autocrine or paracrine factors that regulate proliferative responses and activate hepatic stellate cells into activated myofibroblasts (23), suggesting cholangiocyte proliferation as a potential therapeutic target against liver fibrosis in NASH.

Genes involved in the inflammatory macrophages were coordinately downregulated in association with fibrosis regression. However, unexpectedly, genes involved in immune cells, such as natural killer–like cells, γδ-T cells, and αβ-T cells, were coordinately downregulated in association with fibrosis progression. We interpret these profiles as that some clusters of inflammatory cells may be downregulated in association with so-called burnout NASH during fibrosis progression.

In association with the HbA_{1c} elevation, genes involved in response to hypoxia and oxidative stress in zone 2 and

Table 4—Gene set enrichment analysis using resident cells gene sets in human liver defined by single-cell RNA-seq analyses in subjects with NAFLD with HbA_{1c} reduction or elevation

	Gene set	Representative genes	Networks	Number of genes	LS permutation *P* value	KS permutation *P* value	Up- or downregulated
HbA_{1c} elevation (*n* = 9)							
1	Erythroid cells	*NFIX*, *GABARAPL2*, *BSG*, *MPP1*, *PTGES3*, *SKP1*, *EMC3*, *RBM38*, *TUBA1B*, *SNX3*, *REXO2, SIAH2, FBXO7*, *ACP1*, *TRIM58*, *OAZ1*	Cell cycle_meiosis Cytoskeleton_cytoplasmic microtubules Proteolysis_proteolysis in cell cycle and apoptosis Apoptosis_apoptotic nucleus	101	0.00005	0.00058	Up
2	Zone 2 hepatocytes	*MCEE*, *SLC25A3*, *DECR1*, *PTGR1*, *UQCRFS1*, *GAPDH*, *SUCLG1*, *ATP6V1G1*, *GSTO1*, *ESD*, *COX5A*, *NDUFA12*	Response to hypoxia and oxidative stress Signal transduction_nitric oxide signaling	138	0.00017	0.00096	Up
3	Zone 3 hepatocytes	*SDHC*, *ANXA10*, *TUBB4B*, *PSMD7*, *PSMB5*, *MCEE*, *AK3*, *TPI1*, *YIF1A*, *TIMM17A*, *MRPL37*, *REEP5*, *DECR1*, *PTGR1*, *FDX1*, *UQCRFS1*, *FNDC4*, *GAPDH*, *SUCLG1*, *GOT1*, *EIF5A*, *ARPC2*, *LGALS1*, *NPC2*, *MRPL46*, *GSTO1*, *AP2S1*, *LAPTM4A*, *GOT2*, *LECT2*, *CLDN1*, *SKP1*, *TMEM59*, *EMC3*, *UQCRC1*, *AGMO*, *PRDX3*, *PSMA5*, *ETFA*, *PHPT1*, *ENO1*, *AHSG*, *ESD*, *RER1*, *COX5A*, *MDH2*, *RCAN1*, *KRT8*, *CSTB*, *OAZ1*, *GLRX*, *GHITM*, *AADAC*, *RAB2A*, *KNG1*, *LEAP2*	Response to hypoxia and oxidative stress Proteolysis_ubiquitin-proteasomal proteolysis Translation_translation in mitochondria Cytoskeleton_regulation of cytoskeleton rearrangement Immune response_antigen presentation Cell adhesion_integrin-mediated cell matrix adhesion	729	0.0007	0.0033	Up
HbA_{1c} reduction (*n* = 16)							
1	Stellate cells	*EMILIN1*, *PDGFRA*, *COL3A1*, *CCBE1*, *GGT5*	Cell adhesion_platelet-endothelium-leukocyte interactions Development_EMT_regulation of epithelial-to-mesenchymal transition Reproduction_spermatogenesis, motility and copulation Cytoskeleton_macropinocytosis and its regulation Apoptosis_anti-apoptosis mediated by external signals via NF-κB	65	0.00253	0.08226	Down

Continued on p. 1305

Table 4—Continued

Gene set	Representative genes	Networks	Number of genes	LS permutation *P* value	KS permutation *P* value	Up- or downregulated
		Apoptosis_anti-apoptosis mediated by external signals via MAPK and JAK/STAT				
		Cell cycle_G2-M				
		Cell adhesion_cell-matrix interactions				
		Apoptosis_anti-apoptosis mediated by external signals via PI3K/AKT				
		Signal transduction_NOTCH signaling				
		Reproduction_male sex differentiation				

Resident cells gene sets in liver were taken from MacParland et al. (11). Gene sets were taken from 20 clusters of genes consisting of hepatocytes (zones 1–6), cholangiocytes, central LSECs, periportal LSECs, portal endothelial cells, stellate cells, inflammatory macrophages, noninflammatory macrophages, αβ-T cells, γδ-T cells 1, γδ-T cells 2, natural killer cells, mature B cells, plasma cells, and erythroid cells. *AADAC*, arylacetamide deacetylase; *ACP1*, acid phosphatase 1; *AGMO*, alkylglycerol monooxygenase; *AHSG*, α 2-HS glycoprotein; *AK3*, adenylate kinase 3; *ANXA10*, annexin A10; *AP2S1*, adaptor-related protein complex 2 subunit σ 1; *ARPC2*, actin-related protein 2/3 complex subunit 2; *ATP6V1G1*, ATPase H^+ transporting V1 subunit G1; *BSG*, basigin; *CCBE1*, collagen and calcium binding EGF domains 1; *CLDN1*, claudin 1; *COL3A1*, collagen type III α1 chain; *COX5A*, cytochrome c oxidase subunit 5A; *CSTB*, cystatin B; *DECR1*, 2,4-dienoyl-CoA reductase 1; *EIF5A*, eukaryotic translation initiation factor 5A; *EMC3*, ER membrane protein complex subunit 3; *EMILIN1*, elastin microfibril interfacer 1; *ENO1*, enolase 1; *ESD*, esterase D; *ETFA*, electron transfer flavoprotein subunit α; *FBXO7*, F-box protein 7; *FDX1*, ferredoxin 1; *FNDC4*, fibronectin type III domain containing 4; *GABARAPL2*, GABA type A receptor associated protein like 2; *GGT5*, γ-glutamyltransferase 5; *GHITM*, growth hormone–inducible transmembrane protein; *GLRX*, glutaredoxin; *GOT1*, glutamic-oxaloacetic transaminase 1; *GOT2*, glutamic-oxaloacetic transaminase 2; *GSTO1*, glutathione *S*-transferase ω-1; *KNG1*, kininogen 1; *KRT8*, keratin 8; KS, Kolmogorov-Smirnov; *LAPTM4A*, lysosomal protein transmembrane 4 α; *LEAP2*, liver enriched antimicrobial peptide 2; *LECT2*, leukocyte cell–derived chemotaxin 2; *LGALS1*, galectin 1; LS, least squares; MAPK, mitogen-activated protein kinase; *MCEE*, methylmalonyl-CoA epimerase; *MDH2*, malate dehydrogenase 2; *MPP1*, membrane palmitoylated protein 1; *MRPL37*, mitochondrial ribosomal protein L37; *MRPL46*, mitochondrial ribosomal protein L46; *NDUFA12*, NADH:ubiquinone oxidoreductase subunit A12; *NFIX*, nuclear factor I X; NF-κB, nuclear factor-κB; *NPC2*, NPC intracellular cholesterol transporter 2; *OAZ1*, ornithine decarboxylase antizyme 1; *PDGFRA*, platelet-derived growth factor receptor α; *PHPT1*, phosphohistidine phosphatase 1; PI3K, phosphoinositide 3-kinase; *PRDX3*, peroxiredoxin 3; *PSMA5*, proteasome 20S subunit α5; *PSMB5*, proteasome 20S subunit α6; *PSMD7*, proteasome 26S subunit, non-ATPase 7; *PTGES3*, prostaglandin E synthase 3; *PTGR1*, prostaglandin reductase 1; *RAB2A*, RAB2A, member RAS oncogene family; *RBM38*, RNA binding motif protein 38; *RCAN1*, regulator of calcineurin 1; *REEP5*, receptor accessory protein 5; *RER1*, retention in endoplasmic reticulum sorting receptor 1; *REXO2*, RNA exonuclease 2; *SDHC*, succinate dehydrogenase complex subunit C; *SIAH2*, siah E3 ubiquitin protein ligase 2; *SKP1*, S-phase kinase associated protein 1; *SLC25A3*, solute carrier family 25 member 3; *SNX3*, sorting nexin 3; *SUCLG1*, succinate-CoA ligase GDP/ADP-forming subunit α; *TIMM17A*, translocase of inner mitochondrial membrane 17A; *TMEM59*, transmembrane protein 59; *TPI1*, triosephosphate isomerase 1; *TRIM58*, tripartite motif containing 58; *TUBA1B*, tubulin α1b; *TUBB4B*, tubulin β 4B class Ivb; *UQCRC1*, ubiquinol-cytochrome c reductase core protein 1; *UQCRFS1*, ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1; *YIF1A*, Yip1 interacting factor homolog A, membrane trafficking protein.

3 hepatocytes were coordinately upregulated (Table 4). The findings potentially may reflect the impaired oxygen-binding capacity of HbA_{1c} and central LSEC injury in the relatively hypoxic zone 3 area. Acceleration of hypoxia in zone 3 would worsen hepatocyte damage and stellate cell activation that might trigger inflammation and fibrosis in zone 3. Indeed, genes involved in stellate cells were coordinately downregulated in association with HbA_{1c} reduction, suggesting that this pathway may be a common target of diabetes and liver fibrosis.

In conclusion, our generalized linear mixed model analysis indicates that an increase in HbA_{1c} is significantly associated with hepatic fibrosis progression in subjects with NAFLD and diabetes. Therefore, in subjects with diabetes, hyperglycemia, together with obesity, may be the essential therapeutic target against the progression of NAFLD to cirrhosis and hepatocellular carcinoma. Hepatic gene expression profiles suggest that diabetes-induced hypoxia and oxidative stress injure LSECs in zone 3, which may mediate inflammation and stellate cell activation, leading to liver fibrosis.

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