A Systems Biology Approach to the Pathogenesis of Obesity-related Nonalcoholic Fatty Liver Disease Using Reverse Phase Protein Microarrays for Multiplexed Cell Signaling Analysis

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Nonalcoholic fatty liver disease (NAFLD) is a common cause of chronic liver disease. Omental adipose tissue, a biologically active organ secreting adipokines and cytokines, may play a role in the development of NAFLD. We tested this hypothesis with reverse-phase protein microarrays (RPA) for multiplexed cell signaling analysis of adipose tissue from patients with NAFLD. Omental adipose tissue was obtained from 99 obese patients. Liver biopsies obtained at the time of surgery were all read by the same hepatopathologist. Adipose tissue was exposed to rapid pressure cycles to extract protein lysates. RPA was used to investigate intracellular signaling. Analysis of 54 different kinase substrates and cell signaling endpoints showed that an insulin signaling pathway is deranged in different locations in NAFLD patients. Furthermore, components of insulin receptor–mediated signaling differentiate most of the conditions on the NAFLD spectrum. For example, PKA (protein kinase A) and AKT/mTOR (protein kinase B/mammalian target of rapamycin) pathway derangement accurately discriminates patients with NASH from those with the non-progressive forms of NAFLD. PKC (protein kinase C) delta, AKT, and SHC phosphorylation changes occur in patients with simple steatosis. Amounts of the FKHR (forkhead factor Foxo1) phosphorylated at S256 residue were significantly correlated with AST/ALT ratio in all morbidly obese patients. Furthermore, amounts of cleaved caspase 9 and pp90RSK S380 were positively correlated in patients with NASH. Specific insulin pathway signaling events are altered in the adipose tissue of patients with NASH compared with patients with non-progressive forms of NAFLD. Conclusion: These findings provide evidence for the role of omental fat in the pathogenesis, and potentially, the progression of NAFLD. (Hepatology 2007;46:166-172.)

Nonalcoholic fatty liver disease (NAFLD) is an important cause of chronic liver disease worldwide. NAFLD is strongly associated with metabolic syndrome and insulin resistance, and its prevalence is on the rise. NAFLD represents a spectrum ranging from simple steatosis to nonalcoholic steatohepatitis (NASH). Accumulating evidence suggests that NASH is potentially progressive, whereas simple steatosis (SS), indicated by liver biopsies, follows a more benign course with little or no progression. Therefore, it is important to identify the biological pathways that underlie the pathogenesis of NASH and distinguish it from SS.

Proteomics profiling is the most attractive option among the available high-throughput technologies because almost all drugs target proteins. The profile of intracellular signaling events revealed by proteomics technology could help define the pathogenesis of NAFLD. Additionally, proteomics assays provide effective recapitulation of the post-translational and fluctuating phosphorylation-driven signaling events that occur at the proteome level. Phosphorylation events in the kinase-driven signal networks are particularly important for identifying disease pathogenesis and therapeutic targets. In the past, it has been difficult to measure and quantify protein phosphorylation in cells and tissue spec-
imens because of the transient nature of phosphorylation and the need for enzymatically based systems. Recently, however, antibodies have been developed that specifically recognize the phosphorylated isof orm of cell signaling proteins, providing a means of directly assessing the state of activation of any signaling protein. Applying these antibodies to new types of protein microarray platforms provides an opportunity to profile the ongoing cellular signaling network within small numbers of human cells obtained by biopsy. A new type of protein array, the reverse-phase protein microarray (RPA) isolates pure cell populations from hundreds of biopsy specimens followed by spotting a protein lysate onto nitrocellulose-coated slides. Each array is incubated with one detection protein, such as an antibody, and a single analyte endpoint is measured and directly compared across multiple samples.

We employed RPA technology to profile signaling events in human adipose tissue from patients with NAFLD and a matched group of obese controls. The study was designed to test the hypothesis that white adipose tissue (WAT) is a bioactive organ that influences the pathophysiology of NAFLD. Furthermore, we correlate changes in the specific signaling pathways active in adipose tissue from patients with different subtypes of NAFLD as a means of uncovering new biomarkers and therapeutic targets for disease mitigation.

Patients and Methods

Patient Population and Pathology Assessments. Extensive clinical data and fasting serum samples (snap-frozen at the time of surgery) were obtained from patients undergoing bariatric surgery. Omental WAT specimens were collected during the surgery and immediately snap frozen for proteomic analysis. Finally, liver biopsies were also obtained at the time of surgery and reviewed by a single pathologist for NAFLD classification (Z.G.). Each liver biopsy was assessed for steatosis, inflammation, ballooning degeneration, fibrosis, and other features. The degree of steatosis was assessed in hematoxylin-eosin–stained sections and graded as an estimate of the percentage of tissue occupied by fat vacuoles as follows: 0 = none; 1 = <5%; 2 = 6% to 33%; 3 = 34% to 66%; 4 = 66%. Other histological features evaluated in hematoxylin-eosin sections included portal inflammation, lymphoplasmacytic lobular inflammation, polymorphonuclear lobular inflammation, Kupffer cell hypertrophy, apoptotic bodies, focal parenchymal necrosis, glycogen nuclei, hepatocellular ballooning, and Mallory bodies. These histological features were graded as follows: 0 = none; 1 = mild or few; 2 = moderate; or 3 = marked or many. Fibrosis was assessed with the Masson trichrome stain. Portal fibrosis and interlobular pericellular fibrosis were graded as follows: 0, none; 1, mild; 2, moderate; or 3, marked. When present, bridging fibrosis was noted as few or many bridges, and cirrhosis was identified when parenchymal nodules surrounded by fibrous tissue were noted. Cirrhosis was further categorized as incomplete or established, depending on the degree of loss of acinar architecture.

After assessing for each pathological feature, liver biopsies were assigned to one of four diagnostic categories: (1) no fatty liver disease present, (2) simple steatosis, (3) steatosis with nonspecific inflammation, or (4) NASH. NAFLD was defined as a biopsy showing changes consistent with diagnostic categories 2 to 4. Patients were defined as having simple steatosis if they had hepatocellular fat accumulation as their sole pathological condition. Patients with steatosis and nonspecific inflammation had, in addition to fat, spotty hepatocellular dropout with focal inflammation or Kupffer cell hypertrophy. Nonalcoholic steatohepatitis was identified when, in addition to fat, lobular and hepatocellular inflammation and ballooning degeneration were identified on the hematoxylin-eosin stain. The presence of at least one unequivocal Mallory body and some degree of zone 3 pericellular fibrosis or bridging fibrosis on the trichrome stain was also consistent with the diagnosis of NASH. This study was reviewed and approved by the institutional review board of Inova Fairfax Hospital.

Adipose Tissue Processing and Protein Extraction. Two hundred milligrams of each adipose tissue sample was transferred to a specialized container (PULSE Tube) along with 1.2 ml lysis buffer containing a 1:1 mixture of 2× Tris-Glycine SDS Sample Buffer (Invitrogen Life Technologies, Carlsbad, CA) and Tissue Protein Extraction Reagent (Pierce, Rockford, IL) plus 2.5% β-mercaptoethanol, and subjected to five rapid pressure cycles in the Barocycler NEP3299 (Pressure BioSciences, West Bridgewater, MA). Each cycle consisted of 20 seconds at 35,000 psi followed by 20 seconds at ambient pressure.

Reverse-Phase Protein Microarrays. The protein lysates were loaded into 384-well plates and each serially diluted in Lysis Buffer to a 5-point dilution curve (near, 1/2, 1/4, 1/8, and 1/16). Each dilution series was printed in duplicate onto nitrocellulose-coated glass slides (Whatman, Inc., Sanford, ME) with a 2470 Arrayer (Aushon BioSystems, Burlington, MA), outfitted with 350-μm pins, for a final deposited volume of approximately 33 nl per spot. Total protein in each spot ranged from 250 ng to 4 μg. Slides were desiccated and stored at −20°C. Total protein was quantified in selected arrays that were stained with Sypro Ruby Protein Blot Stain (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions and visualized on an Affymetrix 428 Array Scanner (Santa Clara, CA). Before antibody staining, the lysate
arrays were treated with mild Reblot antibody stripping solution (Chemicon, Temecula, CA) for 15 minutes at room temperature, washed 2 × 5 minutes in phosphate-buffered saline, and then incubated for at least 5 hours in blocking solution [1g 1-block (Tropix, Bedford, MA), 0.1% Tween-20 in 500 ml phosphate-buffered saline] at room temperature with constant rocking.

**Protein Microarray Staining.** Blocked arrays were stained with antibodies on an automated slide stainer (Dako Cytomation, Carpinteria, CA) using the Catalyzed Signal Amplification System kit according to the manufacturer’s recommendation (CSA; Dako Cytomation). Briefly, endogenous kitin was blocked for 10 minutes with the biotin blocking kit (Dako Cytomation), followed by application of protein block for 5 minutes; primary antibodies were diluted in antibody diluent and incubated on slides for 30 minutes, and biotinylated secondary antibodies were incubated for 15 minutes. Signal amplification involved incubation with a streptavidin-biotin-peroxidase complex provided in the CSA kit for 15 minutes, and amplification reagent, (biotinyl-tyramide/hydrogen peroxide, streptavidin-peroxidase) for 15 minutes each. Development was completed by using diaminobenzidine/hydrogen peroxide as the chromogen/substrate. Slides were allowed to air dry after development.

We specifically chose 54 primary antibodies (Table 2) to analyze the following broad signaling pathways thought to be involved in adipokine signaling and tissue changes: (1) cell survival/insulin-related signaling; (2) cell proliferation; (3) inflammation; (4) apoptosis; (5) cytokine and chemokine-related signaling. Secondary antibodies and dilutions included: biotinylated goat anti-rabbit IgG (H+L) 1:5,000 (Vector Laboratories, Burlingame, CA); and biotinylated rabbit anti-mouse IgG 1:10 (Dako Cytomation).

**Image Analysis.** Stained slides were scanned individually on a UMAX PowerLook III scanner (UMAX, Dallas, TX) at 600 dpi and saved as TIF files in Photoshop 6.0 (Adobe, San Jose, CA). The TIF images for antibody-stained slides and Sypro-stained slide images were analyzed with MicroVigene image analysis software, version 2.200 (Vigenetech, North Billerica, MA), and Microsoft Excel 2000 software. Images were imported into Microvigen, which performed spot finding, local background subtraction, replicate averaging, and total protein normalization, producing a single value for each sample at each endpoint. These numbers were then subjected to unsupervised hierarchical clustering analysis using JMP 5.0 (SAS Institute, Cary, NC).

**Statistical Procedures.** Group comparisons were performed by non-parametric Mann-Whitney hypothesis tests. Associations between measurement pairs were tested with the use of Pearson correlation coefficients. The significance of these associations was assessed by computing the confidence interval of each correlation coefficient via Fisher’s z-transform. Categorical data were subjected to univariate and multivariate ordered probit regression analysis. Unless otherwise noted, P values ≤ 0.05 were considered significant.

**Results**

**Demographic and Clinical Data.** The patient study set included 99 patients, 70 of whom had NAFLD and 29 were designated as Obese Controls because their liver biopsies did not show any significant pathological changes. Clinical and laboratory data are summarized in Table 1.

**Analysis of Human Adipose Tissue Phosphoproteome.** Broad patient-specific heterogeneity of the active signaling events is revealed by unsupervised Bayesian clustering analysis of cellular signaling using the phospho-specific endpoints listed in Table 2 (Fig. 1). Although the underlying NAFLD phenotypes (e.g., NASH, SS, NSI) did not self-organize into specific clusters, cell signaling pathways fell into expected groupings corresponding to the time course of the changes in the activity the kinases and the levels of their phosphorylated substrates. Unsupervised Bayesian approaches allow separate assessment of the kinase phosphorylation levels and its cognate substrate (e.g., mitogen-activated protein kinase [MEK] and extracellular regulated kinase [ERK]). Even without an a priori training, many of the components of the MEK-ERK kinase pathway family were grouped in the same cluster, indicating that we were able to recapitulate active networks in lysed adipose cells. The inability of an unsupervised approach to segregate NAFLD phenotypes is not surprising, and probably results from the broad signaling differences between different NAFLD subtypes and the fact that many physiological links between adipose and liver tissues are relatively weak compared with changes within the liver itself.

To evaluate the potential for specific signaling pathways to provide for correlative outcomes, we took a more focused approach—studying and comparing the progressive form of NAFLD (NASH) with its relatively benign forms (SS and NSI) and evaluating each phosphorylation event as an independent variable.

**Molecular Network Comparison of NASH Versus Non-NASH.** To evaluate differences in molecular networks between patients with NASH and patients with the non-progressive form of NAFLD (Non-NASH), and to understand the influence of nonspecific inflammation on any subsequent analysis, we studied each phospho-specific and signaling endpoint for significance of discrimination between NASH and SS, or between NASH and NSI. Histo-
graphical analysis revealed that phosphorylation of a member of the AKT/mTOR pathway, the forkhead transcription factor (FKHR), is elevated \( P < 0.001 \) in the adipose tissue of patients with SS compared with those with NASH (Fig. 2A). Note that when all 99 morbidly obese patients were analyzed, amounts of the FKHR phosphorylated at S256 residue significantly correlated with AST/ALT ratio \( (R = 0.2706, P < 0.015) \). When the analysis is expanded to include patients with SS and NSI, decision tree partition analysis, which looks for linked principal distinguishing components, also identified the FKHR protein as an important classifier. Additionally, decision tree partitioning analysis pinpoints other members of the insulin and the AKT/mTOR pathways as being differentially phosphorylated in patients with non-progressive form of NAFLD and those with NASH (Fig. 2B).

It is significant and striking that although each of these endpoints was independently analyzed as an individual component of the pathway, whole pathways were highlighted, indicating broad, pathway-wide differences between NASH and non-progressive forms of NAFLD (Fig. 3). The results indicate elevated phosphorylation of the insulin pathway in adipose tissue of patients with non-progressive forms of NAFLD (Non-NASH) compared with NASH, except for focal adhesion kinase, for which phosphorylation appears to be relatively higher in NASH (Fig. 2B). A comparison of SS to NSI revealed additional components of the AKT/mTOR pathway, GSK3 and E1F4G, and insulin signaling pathway, SHC and PKC-delta (data not shown).

This signal pathway profiling of omental adipose specimens from patients with NAFLD appears to differentiate patients with NASH from those with the non-progressive forms of NAFLD (SS and NSI). Analysis of many disparate signaling pathways specifically highlighted the insulin signaling network as significantly affected in fatty liver disease pathogenesis.

**Molecular Network Comparison of Patients with NAFLD Versus Obese Controls.** We next analyzed phospho-signaling portraits in patients with NAFLD compared with obese controls without NAFLD to further understand pathway changes in adipose tissue, the relationship of these changes with liver pathology, and the specificity of the insulin pathway changes we observed in progressive NAFLD versus non-progressive NAFLD. Principal component analysis was also an effective tool for identifying key components of discrimination, with most obese controls segregated from subjects with NAFLD. However, although some components of the insulin pathway were part of the overall discrimination, a substantial component of the signaling differences corresponded to other pathways such as eNOS (obese control vs SS, Fig. 4A) and cAbl (obese control vs NASH, Fig. 4B). Amounts of cleaved caspase 9 and pp90RSK S380 were positively correlated \( (R = 0.5444, P < 3.44e^{-08}) \) and were increased in patients with NASH and in those with NSI compared with the obese controls or those with SS \( (P < 0.05) \). The results indicate that the derangements in adipose tissue in patients with NASH versus non-progressive form of NAFLD are fairly specific, and that other signaling
changes occur in the adipose tissue of NAFLD patients compared with the obese controls.

Discussion

This study uses a novel approach to elucidate the pathogenesis of NAFLD. Earlier studies of NAFLD have focused on histological or molecular analyses of the liver tissue. We used a systematic assessment of the influences of WAT on liver pathology to reveal new insights into the biological mechanisms of NAFLD and new therapeutic targets for treatment. We chose to focus this initial study on the global analysis of phosphorylation-driven cellular signaling within the WAT itself. We hypothesized that disease-related changes in intracellular signaling in WAT could become manifest through alterations in the autocrine and paracrine feedback loops between WAT and the liver.

These data strongly connect WAT to the pathogenesis of the NAFLD and NASH. In fact, patients with biopsy-proven NASH displayed significant alterations in the pathways related to insulin resistance (IR) compared with obese controls and patients with SS or NSI. Given the known role of WAT in the development of IR and the importance of IR in the initial steps of fat deposition within the hepatic parenchyma (the so-called “first hit”), these findings are not surprising. Interestingly, phosphorylation of the components of the insulin pathway was more pronounced in the adipose tissue of SS patients than in patients with NASH. A decrease in insulin signaling via the insulin receptor substrate-1/phosphatidylinositol 3-kinase pathway should result in further reduction of glucose uptake and utilization in the livers of patients with NASH. Also, the levels of inactive, unphosphorylated insulin receptor substrate 1 were greater in the adipose tissue of patients with NASH than those with SS, perhaps because of compensatory regulation of total receptor up-regulation in patients with a potentially progressive form of liver disease. Our initial observations suggest that the action of insulin in the adipose tissue of patients with
non-progressive NAFLD is mediated mostly through CREB/focal adhesion kinase control of glucose metabolism, the PKC lipolysis pathway and the AKT/mTOR pro-survival/apoptosis axis.

In addition to supporting the role of IR in the pathogenesis of NASH, these findings provide important potential targets for future therapeutic interventions. We also demonstrate a novel approach to the discovery of new tissue biomarkers that accurately distinguish NASH from the other subtypes of NAFLD. This initial success in measuring changes in the activation of protein signaling pathways in WAT that distinguish NASH from SS now requires prospective validation in independent clinical study sets. If these phosphoprotein endpoints are validated, they may serve as new therapeutic targets; controlling NASH by modulating WAT signaling and the autocrine and paracrine pathways that link WAT to the liver. In addition, prospective validation will provide markers with prognostic value that could help stratify patients for tailored therapy, and help monitor the efficacy of new therapeutic regimens.

This study provided a comprehensive analysis of cell signaling in human adipocytes obtained directly from clinical specimens. We used RPA along with a new pressure cycling technology for fat tissue solubilization to generate phosphoproteomic “portraits” of ongoing signaling in human fat specimens to study a cohort of patients with biopsy-proven NAFLD. Our data suggest that RPA technology is effective for analyzing WAT as a clinical input specimen for any relevant study.

The choice of WAT for this analysis made intuitive sense because it is a biologically active organ that secretes several important cytokines [e.g., IL-6, tumor necrosis factor alpha (TNF-α)] and adipokines (e.g., adiponectin, resistin, leptin) affecting the liver through portal or systemic circulation. In fact, growing evidence indicates that NASH is a consequence of disturbances in the signaling pathways originating from cytokines and adipokines, which can directly or indirectly (through IR) influence its pathogenesis or progression. Unfortunately, molecular pathways linking adipokines with their nuclear and cytoplasmic targets are largely unknown, so the profiling of these networks awaits the characterizations of corresponding pathways. This study is a step in that direction, because many phosphorylation events differentiating progressive and non-progressive forms of NAFLD also affect adipokine production and signaling. For example, earlier work shows that the propagation of the insulin signal reduces the expression of adiponectin receptors AdipoR1/R2 via the phosphoinositide 3-kinase/Foxo1-dependent pathway. Increased phosphorylation of the transcription factor FKHR/FOXO1 leads to its exclusion form the nucleus. FOXO1 suppression in the adipocytes of steatotic patients may lead to the inhibition of the adiponectin receptors, blunting the autocrine action of adiponectin. Adiponectin operates as a key regulator of adipocyte secretory function and prevents the release of several insulin resistance-inducing factors. Therefore, FKHR-dependent decimation of adiponectin may contribute to the induction and evolution of insulin resistance in the liver and skeletal muscle and stimulate accumulation of the lipids in the liver.

In summary, this in-depth study of WAT in NAFLD patients uses a systems biology approach to examine the molecular differences between progressive versus non-progressive forms of NAFLD. WAT phosphoproteomic profiling points to an alteration in insulin signaling in the...
adipose tissue of patients with NASH versus SS, and supports the hypothesis that WAT is an endocrine organ that actively participates in the pathogenesis of NAFLD. These findings support the hypothesis that adipose tissue is an active participant in liver diseases that arise from metabolic alterations characteristic of obesity. The next steps include prospective validation of these findings in larger clinical study sets of WAT, and comparisons with signaling in liver tissue itself. If these results are validated in ongoing independent studies, specific measurements of insulin signaling will help elucidate the pathogenesis of NASH, provide potential therapeutic targets for patient tailored therapy, and develop biomarkers for the progressive form of this important liver disease.

References