Serum total glycosylation profiling for non-invasive diagnosis of liver cirrhosis in people with chronic hepatitis B

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Key Messages

1. A high-throughput assay for quantitative profiling of N-glycans attached to serum glycoproteins has been established.
2. A panel of serum N-glycans were identified as potential biomarkers for diagnosing liver cirrhosis and liver fibrosis.
3. Four glycan peaks of 1341.5, 1829.7, 1933.3, and 2130.3 m/z were all able to detect liver fibrosis and cirrhosis with 85% accuracy.

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Introduction

The persistent hepatic inflammation caused by chronic hepatitis B (CHB) infection leads to progressive liver fibrosis, and eventually, liver cirrhosis. Both liver fibrosis and cirrhosis are reversible if treated early. Knowledge of the stage of liver fibrosis is essential for prognostication and decisions about anti-viral treatment.[1,2] Liver biopsy is the gold standard for assessing liver fibrosis based on histological scoring systems.[3] A liver biopsy assessment is recommended whenever anti-viral treatment is considered,[4] but this is an uncomfortable and sometimes risky procedure, so is not suitable for the routine follow-up of CHB patients. Therefore, serum markers that can reliably detect liver cirrhosis are needed, but those currently available are not sufficiently sensitive for effective detection of liver cirrhosis.

In chronic hepatitis C (CHC) infection, serum markers have been used to predict liver fibrosis. It has been suggested that algorithms based on biochemical and haematological markers can correlate with liver fibrosis.[5,6] A commercially available test (FibroTest, BioPredictive SAS, Paris, France) based on a panel of serum protein markers related to liver fibrosis has been developed.[7] Serum-based assays can be used to assess and monitor liver fibrosis in CHC, with area under the 'receiver operator characteristics' curve being 80 to 90%.

In CHB, similar models based on serum biochemical markers have only achieved moderate correlation with liver fibrosis, and show about 50% sensitivity for detecting significant fibrosis.[8] These less encouraging results may be related to CHB's more complicated natural history, as it is characterised by intermittent exacerbations with different disease phases related to the HBeAg status, whereas CHC is generally an indolent, progressive disease.[9]

Considerable evidence indicates that the N-linked carbohydrate side-chains (ie N-glycans) of serum glycoproteins are altered in patients with liver cirrhosis. There are increased degrees of fucosylation of serum proteins (including haptoglobin, alpha1-acid glycoprotein, and cholinesterase) in liver cirrhosis.[10] Hyposialylated variants of haptoglobin, alpha1-antitrypsin and transferrin have been detected in patients with alcoholic cirrhosis.[11]

Glycomics—the study of the global glycan profile—is a relatively new post-genome research area. When N-glycans are released from serum glycoproteins, specific types of N-glycans have been associated with cirrhosis. The unique patterns of these N-glycans have enabled the identification of cirrhosis in patients with chronic liver disease with about 80% accuracy.[12] Liver cirrhosis is the severe, end-stage of liver fibrosis, so it is possible that the aberrant N-glycans appear earlier as liver fibrosis develops, but at lower levels. Thus, the quantitative profiling of N-glycans isolated from all serum glycoprotein may enable early diagnosis and staging of liver fibrosis.

Aims and objectives

1. Establish a high-throughput assay for quantitative profiling of N-glycans attached to serum glycoproteins.

2. Identify serum N-glycans or serum N-glycan patterns as potential biomarkers for the diagnosis of liver cirrhosis.

Methods

Quantitative profiling of serum N-glycans

N-glycans on whole serum glycoproteins were released using enzymatic digestion, cleaned by hydrophilic chromatography, and profiled with a Ciphergen ProteinChip Reader (Ciphergen Biosystems Inc, Fremont [CA], US) in linear MALDI-TOF MS mode with the use of a gold chip array and 'super-DHB' matrix (2,5-dihydroxy benzoic acid and 2-hydroxy-5-methoxybenzoic acid) supplemented with NaCl. The glycan peaks with signal-to-noise ratios of <3 among the mass spectra were quantified using the Biomarker Wizard software (Ciphergen Biosystems Inc). The peak intensities were normalised with the total ion current then with the total peak intensity. Serum samples from 40 CHB patients with, and from 40 CHB patients without, cirrhosis were subjected to the serum N-glycan profiling.

Bioinformatic analysis

To identify the glycans specifically associated with disease, two criteria were used: (1) the normalised peak intensities needed to be significantly higher/lower in patients with typical fibrosis/cirrhosis than in individuals with minimal fibrosis; and (2) the normalised peak intensities needed to correlate with the degree of fibrosis. A significance analysis of microarray algorithm[13] was used to identify the glycans that were significantly higher/lower in patients with fibrosis/cirrhosis by comparing the glycomic profiles of the patients with minimal fibrosis with those for patients with typical fibrosis/cirrhosis at a median false discovery rate of 2.5%. A forward stepwise linear regression analysis was performed to select the variables with independent prediction values for constructing a diagnostic model to calculate the Fibro-Glyco index.

Results

High-throughput assay for quantitative profiling of N-glycans attached to serum glycoproteins

A high-throughput assay was established, and its linearity and reproducibility were evaluated. N-glycans on whole serum glycoproteins were released by enzymatic digestion, cleaned by hydrophilic chromatography, and profiled with a Ciphergen ProteinChip Reader in linear MALDI-TOF MS mode with the use of a gold chip array and 'super-DHB' matrix supplemented with NaCl. By examining a mixture of four standard glycans, the intra-assay and inter-assay coefficient of variations for our assay were found to be <8% and <17%, respectively. The normalised intensities of the peaks were directly proportional to the quantity of the standard glycans with correlation coefficients of >0.96.

Identification of serum N-glycan features as biomarkers for diagnosis of liver cirrhosis

We analysed the serum N-glycan from 46 patients (29 CHB patients). For the serum samples, 63 common features were identified. A bioinformatic analysis showed that the normalised intensities of 21 different glycans correlated with fibrosis stages. Individually, a glycan of m/z 1829 ($P<0.0005$) had a sensitivity of 82% and a specificity of 84% for detecting liver fibrosis, whereas a glycan of m/z 1444 ($P<0.0005$) had a sensitivity of 76.5% and a specificity of 69% for detecting liver cirrhosis. The structures of 9 out of the 21 different glycans were predicted by searching their m/z values against a glycan mass database. Glycan species containing a proximal fucose and a bisecting N-acetyl glucosamine at the branching mannose were increased in patients with liver fibrosis and cirrhosis.

Linear regression model for detecting liver fibrosis and liver cirrhosis

Four peaks at m/z 1341.5, m/z 1829.7, m/z 1933.3 and m/z 2130.3 (all $P<0.005$) were equally effective for detecting liver fibrosis. These peaks were included (without any serological markers) in the diagnostic model. Leave-one-out cross-validation showed that the diagnostic model could identify significant fibrosis (Ishak score of ≥3) and cirrhosis (Ishak score of ≥5) with 85% accuracy.

Discussion

As only 46 patients were successfully examined in this study, we are undertaking a similar study with a much larger sample size to confirm the clinical value of the present diagnostic model. Further studies are also needed to determine whether the same serum N-glycan-based model can be used to detect liver fibrosis/cirrhosis with other underlying causes, such as CHC infection and chronic alcohol abuse.

As the m/z values only enable prediction of the structures of the diagnostic glycans, further experiments are needed to confirm the predicted structure, such as tandem mass spectrometry analysis and glycosidase sequencing.

Conclusions

A high-throughput assay was developed for the quantitative profiling of N-glycans from whole serum proteins using a system originally designed for serum proteomic profiling. This novel assay identified a panel of N-glycans as potential biomarkers for the diagnosis of liver fibrosis and liver cirrhosis. After validating the clinical values of the potential diagnostic N-glycans with a larger set of patient samples, N-glycans profiling may be used as first-line detection of liver fibrosis and liver cirrhosis, followed by biopsy for confirmation. This could alleviate the necessity of performing invasive liver biopsies on those who are unlikely to have liver fibrosis and/or liver cirrhosis.

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