Uto et al. Proteome Science 2010, 8:70
http://www.proteomesci.com/content/8/1/70

PROTEOME SCIENCE

REVIEW Open Access

Clinical proteomics for liver disease: a promising approach for discovery of novel biomarkers

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Abstract

Hepatocellular carcinoma (HCC) is the fifth most common cancer and advanced hepatic fibrosis is a major risk factor for HCC. Hepatic fibrosis including liver cirrhosis and HCC are mainly induced by persistent hepatitis B or C virus infection, with approximately 500 million people infected with hepatitis B or C virus worldwide. Furthermore, the number of patients with non-alcoholic fatty liver disease (NAFLD) has recently increased and NAFLD can progress to cirrhosis and HCC. These chronic liver diseases are major causes of morbidity and mortality, and the identification of non-invasive biomarkers is important for early diagnosis. Recent advancements in quantitative and large-scale proteomic methods could be used to optimize the clinical application of biomarkers. Early diagnosis of HCC and assessment of the stage of hepatic fibrosis or NAFLD can also contribute to more effective therapeutic interventions and an improve prognosis. Furthermore, advancements of proteomic techniques contribute not only to the discovery of clinically useful biomarkers, but also in clarifying the molecular mechanisms of disease pathogenesis by using body fluids, such as serum, and tissue samples and cultured cells. In this review, we report recent advances in quantitative proteomics and several findings focused on liver diseases, including HCC, NAFLD, hepatic fibrosis and hepatitis B or C virus infections.

Introduction

Diagnostic methods for hepatocellular carcinoma (HCC) include imaging, such as abdominal ultrasonography and computed tomography (CT), and measurement of serum tumor markers. Alpha-fetoprotein (AFP), AFP lectin fraction L3 (AFP-L3), and des-gamma-carboxy prothrombin (DCP, also known as PIVKA-II) are widely used clinically as serum tumor markers of HCC. However, the sensitivity of AFP or DCP for detecting early stage HCC is only 30-60% [1-4]. Although combination measurements of AFP and DCP can improve the diagnostic performance, the diagnostic accuracy is still low for HCC lesions of ≤2 cm. Therefore, the development of a new diagnostic method for early stage HCC is needed to improve outcomes [5-7].

The main cause of liver cirrhosis and HCC is persistent hepatitis B or C virus infection. The degree of hepatic fibrosis is associated with the occurrence of HCC, and serum hyaluronic acid and type IV collagen levels are used for diagnosis of hepatic fibrosis including cirrhosis, but these markers do not always reflect the stage of hepatic fibrosis assessed by liver biopsy [8,9]. In addition, the incidence of nonalcoholic fatty liver diseases (NAFLD) has increased worldwide, but no specific biomarker is available and invasive liver biopsy is still required for definite diagnosis of NAFLD, especially for nonalcoholic steatohepatitis (NASH), which can progress to cirrhosis and HCC [10,11]. Therefore, there is a need to identify blood (serum or plasma) markers that are specific for early diagnosis of HCC, prediction of carcinogenesis from liver cirrhosis, progression of liver cirrhosis, and diagnosis of NASH. These analyses may also aid in the elucidation of the mechanism(s) underlying the pathogenesis of hepatitis and hepatocarcinogenesis.

Proteomics is the term used for exhaustive analysis of protein structure and function in an organ or tissue. The levels of gene expression and protein production are not necessarily proportional, and protein activity is frequently regulated by posttranslational modifications such as phosphorylation [12,13]. Proteomics is useful for elucidation of the pathology and discovery of disease markers for HCC and chronic liver diseases. Serum and plasma are readily used as clinical samples since they can be obtained using less invasive methods. If a biomarker

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associated with the pathology, disease progression or efficacy of treatment is identified in serum or plasma, it can be easily applied for early or differential diagnosis of diseases. Recent advances in methods for mass spectrometric analysis, including protein labeling and amino acid analysis, facilitate highly sensitive and exhaustive proteomic analysis of patient samples. These advances in proteomics techniques have promoted exploration of biomarkers for malignant tumors including HCC and for chronic liver diseases including liver cirrhosis, NAFLD and chronic hepatitis B or C. In this review, we provide an overview of recent findings in proteomic analysis of those liver diseases.

A - Clinical proteomics

For the efficient discovery of biomarkers, more quantitative and reproducible techniques are required. Therefore, differential analysis of protein expression is frequently used in clinical proteomics. Quantitative proteomic approaches can be separated into both labeling (Table 1) and labeling-free methods (Table 2), and the labeling method is separated into gel-based and non-gel-based methods. The most typical method of the gel-based differential approach is two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) [14,15]. On the other hand, non-gel-based methods include some stable isotope-labeling methods, such as cleavable isotope-coded affinity tags (cICAT) [16], stable isotope labeling by amino acids in cell culture (SILAC) [17,18], 2-nitrobenzenesulfenyl (NBS) labeling [19] and protein quantitation using isobaric tags for relative and absolute quantitation (iTRAQ) [20]. In addition, labeling-free methods; surface enhanced laser desorption ionidization (SELDI) methods [21] and ClinProt® systems [22] based on affinity-column or -beads chromatographic methods were beneficial to analyze the blood samples (Table 2). Moreover, molecular information can be obtained from comparison of multiple samples in a single analysis with these methods. The techniques of separation and detection on mass spectrometric analysis and molecular identification have also progressed with improvement in accuracy. The development of high-sensitivity, high-throughput, and exhaustive analytical methods has facilitated identification of trace proteins in biological samples, and clinical proteomics is now performed using new protein analysis techniques. However, these are mostly basic studies, rather than disease-based proteomics useful for bedside diagnosis and prediction of therapeutic effects. Thus, proteomics studies of the association between clinical data and results obtained from cells, tissues and clinical samples are required.

B - Proteomic analysis of hepatocellular carcinoma

1 - Serum proteomics in patients with HCC

Protein separation by 2-dimensional electrophoresis (2-DE) is a well-established and widely used method with easy handling and good reproducibility. In a 2-DE study of protein expression in sera of 5 patients with HCC (2 hepatitis B virus surface [HBs] antigen-positive cases, 2 hepatitis C virus [HCV] antibody-positive cases, and one case negative for both] and healthy subjects, 317 proteins were separated and identified, of which 6 (annexin VI isoform 1, complement component 9, ceruloplasmin, and serum amyloid A4, A2 and A1 isoform 2) were proposed as diagnostic markers for HCC [23]. In

Table 1 Quantitative proteomic techniques that have been applied to clinical proteomics using labeling method

Methods	Type of method	Labeling reagents	Interests	Comparable number of samples/assay	References
2D-DIGE	Gel-based	Cy2, Cy3, Cy5, IC3-OSu, IC5-OSu	Most frequently used gel-based method	2 samples	[14], [15]
cICAT	Non-gel based	^{12}C-ICAT (light) ^{13}C-ICAT (heavy) Labeled to cysteine thiol group	Most frequently used isotope labeling method	2 samples	[16]
SILAC	Non-gel based	^{12}C- or ^{14}N-lysine and arginine (light) ^{13}C- or ^{15}N-lysine and arginine (heavy) Incorporated into cultured cells	Pre-labeling method. Cell lysates and conditioned media can be analyzed.	2 samples	[17], [18]
NBS	Non-gel based	^{12}C-NBS (light) ^{13}C-NBS (heavy) Labeled to tryptophan indole group	Simple MS spectra can be obtained because there is less tryptophan in protein sequences.	2 samples	[19]
iTRAQ	Non-gel based	Isobaric tags (m/z 305, in total) (m/z, reporter) + (m/z, balancer): (113) + (192), (114) + (191), (115) + (190), (116) + (189), (117) + (188), (118) + (187), (119) + (186), (121) + (183) Labeled to lysine amino group	Expression ratio can be used to quantify the signal intensity of reporter peaks. Many samples can be assayed in one experiment.	2 ~ 8 samples	[20]

2D-DIGE; two-dimensional fluorescence difference gel electrophoresis, cICAT; cleavable isotope-coded affinity tags, SILAC; stable isotope labeling by amino acids in cell culture, NBS; 2-nitrobenzenesulfenyl, iTRAQ; isobaric tags for relative and absolute quantitation, Cy; cyanine.

Table 2 Quantitative proteomic techniques that have been applied to clinical proteomics using labeling-free method

Methods	Types of chips or magnetic beads	Interests	References
ProteinChip SELDI	IMAC30 (metal modified), CM10 (cation exchanging), WCX2 (weak cation exchanging) Q10 (anion exchanging), H50 (reverse-phase), H4 (reverse-phase), NP20 (normal-phase), Gold	Analyses: a few μl of serum/plasma (without removal of abundant proteins), urine, cell/tissue lysates and conditioned media Identification: MS/MS, LC-MS/MS	[21]
ClinProt®	Profiling: WCX, WAX, HIC8, IMAC-Cu Large-protein beads: HIC1, HIC3 Peptides beads: HIC18 Phospho beads: IMAC-Fe Glyco beads: LAC ConA, ConAC boronic Antibody capture beads: IAC ProtG	Performance: Many samples can be assayed in parallel.	[22]

SELDI; surface enhanced laser desorption ionidization, MS; mass spectrometry, LC; liquid chromatography.

sera of patients with hepatitis B virus (HBV)-related HCC investigated by 2-DE, 8 proteins with significant differences in expression levels compared to controls were identified (transferrin, transthyretin, α1-antitrypsin, clusterin, haptoglobin α2 chain, ceruloplasmin, heat-shock protein 27 [HSP27], and α-fetoprotein), and HSP27 was positive in 90% of the HCC cases, showing its value for HCC screening [24]. Useful diagnostic markers may be discovered in proteins directly identified by 2-DE separation of patient serum, followed by extraction of protein spots from the gel and identification by peptide-mass fingerprinting (PMF) and MS/MS analysis. However, albumin, globulin, transferrin, and antitrypsin account for about 90% of the serum protein composition, and the large amounts of these proteins interfere with separation of serum proteins by 2-DE, and make separation and analysis difficult. To analyze serum using 2-DE, removal of these abundant proteins and subsequent detection of changes in trace protein levels are necessary. Ang et al. removed albumin by pretreatment of sera from patients with HCC and chronic liver disease (CLD) using lectin and then compared the glycosylated haptoglobin expression level using 2-DE [25]. Expression of glycosylated haptoglobin was increased in the HCC group, and the level was higher in advanced HCC compared to early stage HCC, suggesting that glycosylated haptoglobin is useful for diagnosis and prediction of the HCC stage [25]. These findings also suggest that pretreated serum is better than non-treated serum for 2-DE analysis.

There are two methods of serological diagnosis using the ProteinChip SELDI system: one uses identification of individual proteins and functional analysis, and the other is based on a classification (decision tree) method established by data mining without protein identification. Identification of a protein corresponding to a target peak is difficult using the ProteinChip SELDI system, and the classification-based diagnostic method (multi-marker analysis) is frequently used, in which identification of each protein corresponding to an individual peak is not necessary. The disease and control groups are differentiated based only on the expression levels of several protein peaks. We established a classification method based on 7 peaks that were highly distinguishable between HCV-related HCC and HCV-related CLD, and showed that this method is applicable for diagnosis of both early stage and advanced HCC [26]. This approach was capable of detecting HCC earlier than detection of tumorous lesions by abdominal ultrasonography, and was more useful for early diagnosis than current tumor markers such as AFP and DCP. Similarly, Zinkin et al. developed a diagnostic method using 11 protein peaks detected by the ProteinChip SELDI system, and found a sensitivity and specificity for diagnosis of HCV-related HCC of 79% and 86%, respectively [27]. The diagnostic sensitivity and specificity do not differ significantly from those of methods using current HCC markers (AFP, AFP-L3 fraction, and DCP), but the performance for diagnosis of small HCC of ≤2 cm was better than that for methods using current markers.

He et al. selected 3 protein peaks (5890, 11615, and 11724 Da) in serum that showed significant differences in HBV-related HCC patients compared to HBV patients without HCC, and found that HBV-related HCC could be diagnosed in almost 100% of cases based on these proteins [28]. The SELDI method was combined with 2-DE to identify the protein with a peak at 11615 Da as serum amyloid A (SAA). However, the positive rate was also high in patients with HBV-related CLD in this analysis, indicating that the method is not

specific for HCC. Cui et al. reported that patients with HBV-related HCC or CLD could be distinguished from healthy subjects by multi-marker serum analysis with sensitivity and specificity of 90% or higher [29], but it is doubtful whether this method could be used for differentiation of HCC from CLD. Similarly, sensitivity of 100% and specificity of 92 or 97% have been reported for diagnosis of HBV-related HCC [30], but the control group consisted of healthy subjects without CLD and the utility for early diagnosis of HCC in patients with cirrhosis is doubtful. Göbel et al. established a method for differentiation of HCV-related HCC from liver cirrhosis without HCC using 4 protein peaks at 7486, 12843, 44293, and 53598 Da (multi-marker analysis), and found that the method was useful for diagnosis of early stage HCC [31]. Ward et al. also reported a multi-marker analysis with 94% sensitivity, 86% specificity, and 0.92 AUROC [32]. Thus, multi-marker analysis is useful for diagnosis. However, a ProteinChip SELDI system is necessary for analysis, and this system is expensive compared with commercially available kits such as those for ELISA, and not all facilities can use this system. Moreover, the peak protein intensity detected by a protein chip system may vary among analytical devices and facilities, and further technical advances are needed for clinical application of multi-marker analysis for diagnosis of early stage HCC (Table 3).

In analysis using cICAT and liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS), Kang et al. compared serum proteins between 9 cases of HCC and 9 cases of liver cirrhosis, and identified 31 proteins with differences in expression levels. Of these proteins, significantly enhanced expression of α1-acid glycoprotein (AGP) was observed in the HCC validation group (HCC; N = 52, liver cirrhosis; N = 40), showing that AGP is a candidate serum diagnostic marker for HCC [33]. Thus, serum proteomic analysis using a combination of cICAT and LC-ESI-MS/MS can be used for direct identification of potential protein markers.

AFP and DCP are frequently used in current diagnosis of HCC. These proteins are directly expressed by tumors and their blood levels are reduced by local treatment or tumor resection. Therefore, they serve as indices for diagnosis, therapeutic effect and recurrence. In contrast, the proteins identified by serum protein expression analysis described above are not necessarily produced by the tumor (for example, they may be produced by immune cells that act on tumor cells) and this may be limitation of current biomarker exploration by serum analysis. Moreover, some proteins in HCC change with progression of the pathology of the underlying diseases of chronic hepatitis and liver cirrhosis. Therefore, specificity is of importance in clinical proteomic analysis using serum from patients with HCC.

2 - Tissue proteomics in patients with HCC

HCC tissue has been widely used in proteomics because a large amount of tissue can be obtained relatively easily from surgical specimens [34-41]. In a study in which protein expression in liver tissue was investigated by 2-DE in 40 cases of HBV-related HCC and 36 control subjects (20 patients with liver cirrhosis and 16 normal liver tissues from residual grafts of liver donors), 14 proteins with ≥2-fold changes in expression level were identified in patients with HCC compared to the controls [34]. Of these proteins, vimentin expression was

Table 3 The peaks detected by ProteinChip SELDI in patients with hepatocellular carcinoma

Subjects	Protein/Peptide Peaks (m/z)	Type of Protein Chip	Identification	References
HCV-related HCC *vs.* non-HCC	3444, 3890, 4067, 4435, 4470, 7770	CM10	ND	[26]
HCC *vs.* non-HCC (cirrhosis)	3687, 3906, 26457 11853, 11873, 11887, 13391 11319, 17783, 17906, 18021	CM10, IMAC30, H50,	13391-Da; Cystatin C	[27]
HBV-related HCC *vs.* non-HCC	5890, 11615, 11724	IMAC30-Cu	ND	[28]
CHB LC HCC (*vs.* Healthy control)	22842 (up), 2957, 2049 (down), 2049 (up), 3166 (down), 23381, 28040 (up), 2018 (down)	WCX2	ND	[29]
HBV-related HCC *vs.* Healthy control	7777, 9250, 16200	WCX2	ND	[30]
HCV-related HCC *vs.* HCV-related LC	2873, 6646, 7775, 10525, 67867	CM10	6646-Da; Apolipoprotein C-I	[31]
HCV-related HCC *vs.* HCV-related LC	22960, 23530	IMAC30	ND	[32]

SELDI; surface-enhanced laser desorption and ionization mass spectrometry, HCV; hepatitis C virus, HCC; hepatocellular carcinoma, HBV; hepatitis B virus, CHB; chronic hepatitis B, LC; liver cirrhosis, ND; not done.

significantly elevated in the HCC group. The vimentin level was also elevated in serum from the HCC patients, which was useful for diagnosis of HCC lesions of ≤2 cm. Comparison of HCC and normal liver tissues using 2D-DIGE has shown reduced expression levels of proteins associated with metabolism and increased expression of stress-related proteins of the HSP family in HCC, with aldo-keto reductase 1C2, thioredoxin, and transketolase proposed as HCC markers [35,36]. Luk et al. analyzed liver tissue of 146 patients by 2-DE+MS/MS and detected 1800 protein spots. Three of these protein spots with strong expression in HCC were identified as Hsp27, Hsp70 and glucose-regulated protein (GRP) 78. Hsp27 was found to be highly correlated with AFP, and GRP78 was associated with venous tumor invasion [37]. Sun et al. performed protein expression analysis to search for HCC biomarkers using liver tissue samples from 59 patients with liver diseases (39 with HCC and 20 with liver cirrhosis) and 16 healthy subjects, and 63 plasma samples (35 patients with HCC, 16 with liver cirrhosis, and 12 healthy subjects). In both liver tissue and plasma, lamin B1 (LMNB1) expression was significantly enhanced in HCC patients compared to healthy subjects, and the LMNB1 expression level was associated with the cancer stage, leading to the conclusion that this protein is a useful biomarker for early stage HCC [38]. An increased clathrin heavy chain level and a lower formiminotransferase cyclodeaminase level in 2-DE analysis of HCC tissue have also been proposed to be useful for diagnosis of early stage HCC [39], and APC-binding protein EB1 expression in resected HCC specimens has been related to the survival and recurrence rates after resection [40].

Use of whole resected liver specimens containing HCC for proteomic analysis allows detection of proteins expressed not only by cancer cells, but also by cells infiltrating around the tumor and by interstitial cells. Selection of cancer tissue by laser capture microdissection (LCM) followed by 2-dimensional liquid chromatography tandem mass spectrometry (2D-LC/MS-MS) can be used to identify proteins that differ quantitatively between disease and control tissue [41]. This approach is useful for analysis of surgical samples and may help to improve the understanding of the mechanism of carcinogenesis in HCC.

C - Proteomic analysis of nonalcoholic fatty liver disease (NAFLD), hepatic fibrosis and liver cirrhosis

1 - Serum proteomics of NAFLD, hepatic fibrosis and liver cirrhosis

The number of cases of NAFLD including NASH has shown a recent increase, and NASH is a risk factor for HCC. NASH has a pathology similar to that of alcoholic liver diseases and is accompanied by inflammation and fibrosis that progresses to liver cirrhosis and HCC. Excess nutrition, obesity, insulin resistance, and oxidative stress are thought to be involved in the development and progression of NASH, but the molecular mechanisms remain uncertain. About 30% of subjects in a health check-up in Japan showed abnormalities in serum markers of liver function such as ALT, AST and γ-GTP, and most cases were assumed to be NAFLD. Identification of patients with NASH among those with NAFLD is very important for prevention of liver cirrhosis and HCC through intensive treatment. However, invasive liver biopsy is currently required for diagnosis of NASH, since no specific serum marker is available for use in a noninvasive test [10]. In serum proteomics in patients with NAFLD, four apolipoproteins and CD5 antigen-like protein (CD5L) were identified by 2-DE analysis. Of these proteins, CD5L reflects the severity of hepatic fibrosis in NAFLD and its serum level increases in cases with severe fibrosis, suggesting that it may serve as a diagnostic marker of NASH [42]. An analysis of sera of patients with NAFLD using the ProteinChip SELDI system identified 4 protein peaks with significant changes in patients with NASH compared to obese patients without NAFLD [11]. These peaks may be useful for diagnosis of NASH, but the corresponding proteins have yet to be identified. Interestingly, mRNA expression in liver tissue was analyzed using a microarray in the same patients in this study, and this analysis may advance the understanding of the molecular mechanism of development and progression of NAFLD. Currently, the association between serum protein expression and gene expression in liver tissue is unclear.

Bell et al. analyzed sera of 85 patients with NAFLD, including cases of NASH and simple fatty liver by LC/MS-MS and identified 1738 proteins, of which 9 reflected differences in fibrosis among the NASH patients and 21 were proposed as useful biomarkers to distinguish NASH (F3/F4) with advanced fibrosis from simple fatty liver [43]. Unfortunately, no single protein for discrimination between simple fatty liver and NASH was obtained in this analysis. A panel diagnostic method using fibrinogen β chain, retinol binding protein 4, serum amyloid P component, lumican, transgelin 2, CD5L, complement component C7, insulin-like growth factor acid labile subunit, and transgelin 2 has been developed that discriminates among healthy subjects, patients with simple fatty liver, and patients with NASH with high power. In this report, serum was separated by nano-HPLC in proteomic analysis, and proteins were identified and quantified by electrospray ionization (ESI) [43]. Such combination method has high-resolution and relatively favorable quantitative performance with a very small amount of sample, therefore is capable of

identifying many proteins, which has led to expansion of its use. However, whether the identified proteins are truly specific to NAFLD and useful for diagnosis of NASH remains to be investigated.

Proteomic analysis in patients with liver cirrhosis has also been performed to search for hepatic fibrosis markers. Poon et al. developed a scoring system for diagnosis of hepatic fibrosis using 10 factors: 7 protein peaks detected using the ProteinChip SELDI system, and the ALT, total protein, and bilirubin levels in serum. Using this approach, liver cirrhosis was diagnosed with 94% sensitivity and 91% specificity [44]. Morra et al. also showed that a differentiation method based on 8 protein peaks was more useful for evaluation of hepatic fibrosis than the existing Fibro Test (an method that uses α2-macroglobulin, haptoglobin, apolipoprotein A1, total bilirubin, and γ-GTP) [45]. Göbel et al. used 5 serum protein peaks in development of a multi-marker method to differentiate between cirrhotic and non-cirrhotic livers (F1 and F2), and showed that this method could be used to diagnose liver cirrhosis with 80% sensitivity and 67% specificity [31].

In 2-DE analysis of sera from patients with HCV-related CLD, the levels of inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) fragments, α1-antichymotrypsin, apolipoprotein L1, prealbumin, albumin, paraoxonase/arylesterase 1, and zinc-α2-glycoprotein were reduced and those of CD5 antigen-like protein (CD5L) and β2 glycoprotein I (β2GPI) were elevated in the liver cirrhosis group [9]. Using a similar method, the levels of Mac-2-binding protein, α2-macroglobulin and hemopexin were found to be elevated and those of α1-antitrypsin, leucine-rich α2-glycoprotein and fetuin-A were decreased in advanced liver fibrosis [46]. Identification of serum protein peaks that are altered in liver cirrhosis and verification of their utility in the diagnosis of liver cirrhosis may lead to the discovery of novel diagnostic markers. Callewaert et al. have also recently reported an interesting glycoproteomics approach in developing the methodology for a DNA sequencer-based total serum protein-linked N-glycans [47]. Their methodology allows for high-throughput fingerprinting and sequencing of N-glycans that are present on picomolar amounts of glycoproteins. Using this method, they compared the serum protein-linked N-glycan profiles from compensated cirrhotic and non-cirrhotic chronic liver disease patients, to successfully distinguish the pathogenesis of both disease populations with 79% sensitivity and 86% specificity [48].

2 - Tissue proteomics of NAFLD, hepatic fibrosis and liver cirrhosis

To predict the progression of NASH and/or hepatic fibrosis, it is important to gain a better understanding of the pathogenesis and molecular mechanism(s) responsible. To understand the pathogenesis of NAFLD, tissue proteomics is also considered to be a more effective tool. Younossi et al. constructed a model to predict NASH and advanced hepatic fibrosis based upon protein microarray-phosphoproteomics using liver biopsy samples, blood samples and visceral adipose tissue [49]. In this model, using the parameters of age, race, gender, diabetes status, AST, phosphorylated-Akt (Ser 473) and phosphorylated-insulin receptor substrate 1 (IRS1) (Ser 612), it was possible to predict NASH with AUC = 0.860 (81.3% sensitivity and 87.0% specificity) [49]. Charlton et al. compared the protein expression profiles in four groups of liver tissue samples (obese normal, simple steatosis, NASH-mild [inflammation grade 1, fibrosis stage 0-1] and NASH-progressive group [fibrosis stage 2-4]) from obese patients using the combination of iTRAQ with LC-MS/MS [50]. They identified a total of 1362 hepatic-expressed proteins, and found that a 40-kDa keratin sulfate proteoglycan was significantly overexpressed in a progressive manner in NASH (-mild and -progressive), whereas, fatty acid binding protein-1 (FABP-1) was underexpressed in both states of NASH [50].

Several groups have performed proteomic analyses using liver tissue from patients with HCV-related chronic liver disease. Diamond et al. performed a quantitative proteomic analysis of HCV-infected human liver tissue from patients at different stages of fibrosis using $^{16}O/^{18}O$ stable isotope labeling combined with the accurate mass and time tag approach, and revealed that 210 of 1641 proteins, including those associated with carbohydrate and fatty acid metabolism and the mitochondrial oxidative stress response, exhibited statistically significant differences that were associated with the fibrosis stage [51]. Mölleken et al. compared the protein expression in hepatocytes and cells from the cirrhotic septa of patients with end-stage liver disease associated with HCV infection at the time of liver transplantation using 2-DE-LC-MS/MS [52]. Several structural proteins were upregulated in cells from fibrotic septa, which were assumed to have arisen from activated hepatic stellate cells. One of these identified proteins, microfibril-associated protein-4 (MFAP-4) was subsequently measured in serum, and were shown to increase as the fibrosis stage increased. Although this marker was not able to discriminate between mild and moderate fibrosis, MFAP-4 was more useful to diagnose cirrhosis associated with HCV infection compared with alcoholic liver cirrhosis. In addition, this report could extend the concept of tissue proteomics into the discovery of serum biomarkers.

D - Proteomic analysis of hepatitis B or C virus infection

HBV and HCV can induce both acute and chronic necroinflammatory liver disease, and chronic infection with both viruses has a very high risk of developing into

HCC. Thus, biomarkers reflecting the pathogenesis of viral infection and/or chronic hepatitis are also necessary to elucidate new potential therapeutic approaches. However, reports of biomarkers that can predict viral infection or the mechanism of hepatitis have not been fully investigated.

He et al. compared sera from normal, HBV infected low- and high-necroinflammatory scoring patients using 2-DE, and identified that the expression of seven proteins, haptoglobin β and α2 chain, apolipoprotein A-I and A-IV, α1-antitrypsin, transthyretin and DNA topoisomerase IIβ correlated with the HBV necroinflammatory scores [53]. More recently, Ren et al. performed a serum proteomic analysis of HBV infection. They compared the changes in serum proteins in patients with acute-on-chronic liver failure (AoCLF) with those in normal subjects or in patients with chronic hepatitis B using 2-DE, and identified 12 of 23 differentially expressed proteins [54]. In this analysis, serum levels of α1-acid glycoprotein was one of the proteins that were significantly decreased in patients with AoCLF [54]. Chen's group performed several *in vitro* proteomic investigations of HBV-infected HepG2 hepatoma cells to evaluate the protein changes associated with virus infection. Using the combined methods of iTRAQ with 2D-LC-MS/MS, they compared the protein expression in non-infected HepG2 with HBV-infected HepG2 cells to identify several proteins that were down-regulated in HBV-infected cells, including S100A6 and annexin A2 [55,56]. On the other hand, the influence of HCV infection is often assessed *in vitro* using the HCV replicon system [57]. Jacobs et al. performed a large-scale proteome analysis of the Huh-7.5 cell line, containing a full-length HCV replicon with the multidimensional LC-MS/MS technique [58]. Then, they identified 4,200 proteins, including lipid metabolism-related proteins, expressed in Huh-7.5 cells. A total of 1,500 proteins were also detected from liver biopsies from HCV-infected patients. More recently, Singaravelu et al. utilized a unique labeling probe, a non-directed phenyl sulfonate ester probe, PS4, which was labeled to a nucleophilic residue within the active site of the enzyme molecules to profile the alteration of activity levels during HCV replication during Huh-7 HCV subgenomic replicon [59]. Nineteen active proteins including protein disulfide isomerase-associated 4, heat shock 70 kDa protein 5 were then identified by 2-DE-LC-MS/MS. Thus, proteomic analysis using HCV replicon is thought to be useful for understanding the mechanism of HCV infection and replication.

E - Prospects for proteomics in liver diseases

Analysis of phosphorylated or glycosylated peptides and proteins is increasingly important in biomarker studies [60]. In addition to identification and localization of modified sites, analysis of their variation may provide important clues to complex biological functions and for exploration of disease biomarkers and new drug development. The outline of proteomic analysis for phosphor- and glyco-ptrotein was shown in Figure 1. Plectin-1 (phospho-Ser-4253) and alpha-HS-glycoprotein (phospho-Ser 138 and 312) have been identified as biomarkers of HCC in an analysis targeting phosphorylated proteins [61].

Differences in carbohydrate chains bound to the same protein in cancer and normal cells are well known, and proteomics focusing on differences in glycosylation of proteins has been performed [62-65]. Different glycosylation patterns of proteins in HCC tissue and plasma have been reported in a study in which HCC samples were pretreated with lectin-bound agarose and the resulting glycoprotein was analyzed by 2D-DIGE and MALDI-TOF/MS. Analysis of plasma by nano-LC/MS-MS showed increased expression of human liver carboxylesterase 1 (hCE1) in HCC patients [66]. Block et al. showed that the hyperfucosylated Golgi Protein 73 (GP73) was elevated in the serum of patients with HCC based upon targeted glycoproteomics using the combined method of HPLC with 2-DE [67]. Lee et al. labeled proteins in the plasma of HCC patients and healthy controls using iTRAQ and identified 14 high-level N-linked sugar chains in the HCC group. Two of the associated proteins were identified as vitronectin (Asn-169, 242) and antithrombin III (Asn-225), and the changes in the sugar chains were proposed as potential markers of HCC [68].

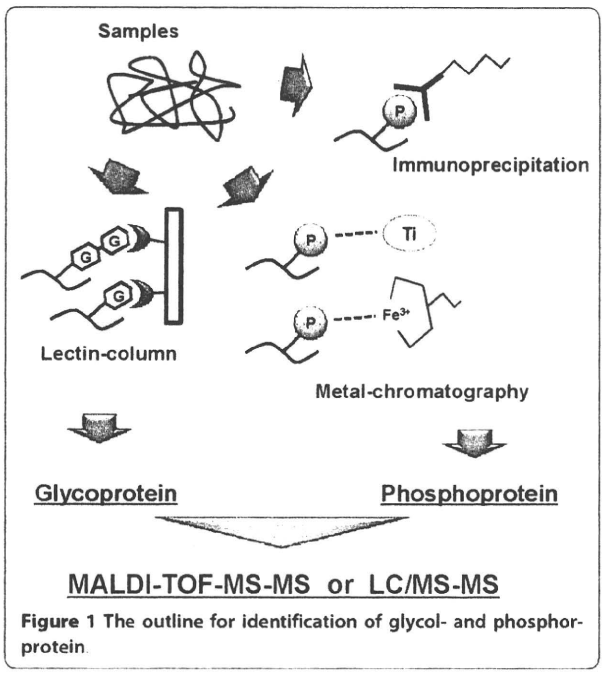

Figure 1 The outline for identification of glycol- and phosphor-protein

In another study, the level of fucosylated α1 acid glycoprotein (AGP) was found to be higher in patients with HBV-related HCC compared to controls, although there was no significant difference in the total AGP level in serum between the patients and controls [64]. Peptidomics targeting low-molecular-weight peptides [69] and fragmentomics targeting protein fragments [70,71] may also be useful in the search for liver disease markers.

Paradis et al. used the ProteinChip SELDI system to analyze sera from 96 patients with chronic hepatitis C who were treated with interferon and ribavirin and found that changes in the number of protein peaks during the treatment course was significantly greater in patients who responded to the treatment compared to non-responders [72]. The therapeutic effect could be predicted with an AUROC of 0.75 using a differentiation method based on a combination of the peak levels of 2 proteins, fibrosis stage, and viral genotype. Therefore, proteomic analysis may also allow prediction of therapeutic effects and identification of proteins related to these effects, in addition to diagnosis of liver diseases.

Conclusion

In recent decades, proteomic technologies based on mass spectrometry have been developed, and the reliability of these technologies continues to improve. Such advancements in proteomic techniques could contribute to the discovery of clinically useful biomarkers and the elucidation of the molecular mechanisms involved in disease pathogenesis. However, such advanced techniques are not necessarily utilized broadly and effectively because of the costs associated with the introduction of these technologies and the conscious differences that exist between developers and users of the application of identified biomarkers in clinical practice. Thus, as developers it is essential to make it clear as to how to use identified biomarker candidates appropriately.

In this review, we provided a survey of recent advances of proteomic investigations and several findings focused in liver diseases, including NAFLD, viral hepatitis, hepatic fibrosis, liver cirrhosis, and HCC. A low correlation between mRNA and protein expression levels has been found using exhaustive protein expression analysis [73]. Compared to detection of gene expression using DNA microarray analysis, techniques such as time-of-flight MS used in proteomics have relatively weak reproducibility and operability, and have not been developed sufficiently to allow wide use at all facilities. However, analysis of changes in protein expression is essential to investigate pathological conditions and reactions in vivo because processes at the organ, tissue and cellular levels are mostly regulated by proteins. About 20 proteins, including albumin and immunoglobulin, account for 99% of total serum protein, and proteins that may serve as biomarkers are present in trace amounts that account for the remaining 1%. Therefore, a more sensitive detection system to search for biomarkers is required, and this may allow discovery of clinically useful markers for all liver diseases. In addition, clarifying the profile of glycol- and phosphor-proteins may also be very important in understanding the pathogenesis of HCC and other liver diseases. The detection of such post-translational modification of proteins may reflect the pathogenesis of disease states more sensitively and specifically than methods that only examine the fluctuation of protein expression, as the profile of glycol- and phosphor-proteins in cancer cell-surface and -secreted protein are distinct from those in normal cells.

Proteins are assumed to be key molecules that define the characteristics and dynamics of cells and control biological reactions. Therefore, investigation of changes in protein expression levels is very important in understanding disease pathology. Further advances in proteomics techniques and establishment of simple and quantitative performance comparable to that of DNA microarrays are likely to promote proteomic studies and lead to further breakthroughs in clinical proteomics.

Acknowledgements

This work was supported in part by grants from the Ministry of Education, Science and Culture of Japan, the Ministry of Health, Labour and Welfare of Japan and Takeda Science Foundation.

Authors' contributions

HU carried out the interpretation of the data and preparation of the manuscript. SK and YT had contributed to the manuscript preparation. HT has contributed to the overall conception and critical review of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 6 August 2010 Accepted: 31 December 2010
Published: 31 December 2010

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doi:10.1186/1477-5956-8-70
Cite this article as: Uto *et al.*: Clinical proteomics for liver disease: a promising approach for discovery of novel biomarkers. *Proteome Science* 2010 **8**:70.

Hepatology Research

Hepatology Research 2010; **40**: 438–445 doi: 10.1111/j.1872-034X.2009.00615.x

Short Communication

Identification of a novel biomarker for oxidative stress induced by hydrogen peroxide in primary human hepatocytes using the 2-nitrobenzenesulfenyl chloride isotope labeling method

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Aim: Oxidative stress is involved in the progression of non-alcoholic steatohepatitis (NASH). However, there are few biomarkers that are easily measured and accurately reflect the disease states. The aim of this study was to identify novel oxidative stress markers using the 2-nitrobenzenesulfenyl (NBS) stable isotope labeling method and to examine the clinical utility of these diagnostic markers for NASH.

Methods: Proteins extracted from phosphate buffered saline- and hydrogen peroxide-loaded human primary hepatocyte were labeled with the [^{12}C]- and [^{13}C]-NBS reagents, respectively. Pairs of peaks with 6-Da differences in which the [^{13}C]-NBS labeling was more intense than the [^{12}C]-NBS labeling were detected by MALDI-TOF/MS and identified by MS/MS ion searching.

Results: Four pairs of peaks, m/z 1705–1711, m/z 1783–1789, m/z 1902–1908 and m/z 2790–2796, were identified as cytochrome *c* oxidase VIb (COX6B), liver carboxylesterase 1 (CES1), carbamoyl-phosphate synthase 1 (CPS1) and superoxide dismutase (MnSOD), respectively. Furthermore, serum MnSOD protein levels were significantly higher in NASH patients than in simple steatosis (SS) patients. The serum MnSOD levels tended to increase in parallel with the stage of fibrosis.

Conclusion: The NBS labeling technique was useful to identify biomarkers. Serum MnSOD may be a useful biomarker that can distinguish between SS and NASH.

Key words: 2-nitrobenzenesulfenyl, oxidative stress, MnSOD, non-alcoholic steatohepatitis

INTRODUCTION

IN SEVERAL LIVER diseases, including non-alcoholic steatohepatitis (NASH) and chronic hepatitis C (CHC), oxidative stress is a major pathogenetic event. Lipid peroxidation, free radical generation, CYP2E1 induction and mitochondrial dysfunction are known to induce oxidative stress and contribute to the progression of NASH and CHC.[1–3] Therefore, oxidative stress markers should be biomarkers that reflect the pattern and strength of oxidative stress and disease progression. Several oxidative stress markers for liver diseases including 8-hydroxy-2′-deoxyguanosine (8-OHdG), superoxide dismutase (SOD) and thioredoxin are well known. However, the clinical significance of these markers has not been fully evaluated.[4–6] Thus, oxidative stress markers that accurately reflect disease states and

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Received 11 May 2009; revision 14 September 2009; accepted 22 September 2009.

can be easily measured are necessary to accurately diagnose NASH or CHC.

In recent years, proteomic techniques, including 2-D gel electrophoresis (2-DE), have been commonly used to explore novel biomarkers. However, traditional 2-DE-based proteomic approaches are tedious and have several limitations, including reduced sensitivity and lack of quantitative results. Isotope-coded affinity tagging (ICAT) and isotope tagging for relative and absolute quantitation (iTRAQ) are the most commonly used chemical isotope labeling methods and can be used to address many of the limitations of 2-DE. In this report, we examined a novel stable isotope labeling method, the 2-nitrobenzenesulfenyl (NBS) labeling method developed by Kuyama *et al.*[7] The NBS labeling method is based on the specific binding reaction of the NBS reagent to tryptophan residues within a protein, and the 6-Da mass difference between [^{12}C]-NBS-labeled and [^{13}C]-NBS-labeled peptides generates a mass signature for all tryptophan-containing peptides.[7,8]

Here, we explored novel oxidative stress marker candidates using the NBS labeling method and identified four candidate oxidative stress markers in human primary hepatocytes including MnSOD. Furthermore, we verified the clinical significance of MnSOD as a diagnostic marker for NASH.

METHODS

Chemicals and materials

THE ^{13}CNBS® STABLE isotope labeling kit-N was purchased from Shimadzu Biotech (Kyoto, Japan). Human primary hepatocytes (a monolayer of human long-term hepatocytes), which were isolated from a 77-year-old woman, were purchased from Biopredic International (Rennes, France). 4-Hydroxycinnamic acid (CHCA) was obtained from Bruker Daltonics (Bremen, Germany) and 3-hydroxy-4-nitrobenzoic acid (3H4NBA) was purchased from Sigma Chemical (St Louis, MO, USA). Sequencing-grade modified trypsin was from Promega (Madison, WI, USA), and the protease inhibitor cocktail set III was from Calbiochem (Darmstadt, Germany).

Cell culture, NBS labeling and identification of NBS-labeled peptides

Human primary hepatocytes were cultured in a long-term culture medium.[9] Confluent human primary hepatocytes (~2×10^6 cells/12.5 cm^2 flask) were incubated for 24 h with phosphate buffered saline (PBS) or 200 μM hydrogen peroxide (H_2O_2).[10,11] Cells were washed and homogenized in 50 mM phosphate buffer, pH 8.0, containing 1% protease inhibitor cocktail set III. The NBS labeling was performed as previously described.[12,13] Briefly, both cell lysates (100 μg) treated with PBS or H_2O_2 were labeled with [^{12}C]- or [^{13}C]-NBS under acidic conditions, respectively. After labeling, the two respective conditioned protein mixtures were denatured with urea and reduced with tris(2-carboxyethyl)phosphine (TCEP) followed by alkylation with iodoacetamide. NBS-labeled proteins were digested with trypsin and eluted through phenyl sepharose using a stepwise gradient of increasing acetonitrile (10%, 15%, 20%, 25%, 30%, 35%, 40%, 45% and 50%) containing 0.1% trifluoro acetate. Next, the NBS-labeled peptides were ionized by a combined application of CHCA and 3H4NBA as described.[14,15] The mass spectral data were obtained by MALDI-TOF-TOF-MS, Autoflex II TOF/TOF (Bruker Daltonics) in positive-ion and reflectron mode. Pairs of peaks with a 6-Da difference were identified by MS/MS ion searching using tandem MS. The data set from the MS/MS ion was analyzed using the database search engine, Mascot (www.matrixscience.com), to find the closest match with known proteins/peptides in the database from the Swiss-Prot website.

Western blot analysis

Equal amounts of cell lysates from human primary hepatocytes (4 μg) were run on sodium dodecylsulfate polyacrylamide gels and electroblotted onto polyvinylidene fluoride membranes. The blots were probed with anti-cytochrome *c* oxidase VIb isoform 1 (anti-COX6B), anti-liver carboxylesterase 1 (anti-CES1), anti-carbamoyl-phosphate synthase [ammonia] mitochondrial 1 (anti-CPS1) and anti-MnSOD antibodies. After incubating the membrane with the appropriate horseradish peroxidase-conjugated secondary antibody, the reactivity was visualized using an ECL chemiluminescent detection kit (GE Healthcare Biosciences, Tokyo, Japan).

Real-time reverse transcription polymerase chain reaction (RT–PCR)

Total RNA was extracted from cells using ISOGEN (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Samples were reverse-transcribed using the PrimeScript RT reagent Kit (TAKARA Bio, Shiga, Japan). Synthesized cDNA was amplified using SYBR Premix Ex Taq II (TAKARA Bio) and analyzed by StepOnePlus Real-Time PCR Systems and StepOne

Table 1 Identification and quantification of 2-nitrobenzenesulfenyl-labeled peak pairs

Accession no.	Protein name	Peak pairs (^{12}C–^{13}C, m/z)	Identified sequences
P14853	Cytochrome *c* oxidase subunit VIb isoform 1	1705–1711	NC**W**QNYLDFHR
P23141	Liver carboxylesterase 1 precursor	1783–1789	FTP**W**PQPAEP**W**SFVK
P31327	Carbamoyl-phosphate synthase [ammonia], mitochondrial precursor	1902–1908	GAEVHLVP**W**NHDFTK
P04179	Superoxide dismutase [Mn], mitochondrial precursor	2790–2796	FNGGGHINHSIF**W**TNLSPNGGGEPK

Bold and underlined characters highlight the tryptophan (W) residues in the identified peptide sequences.

Software ver. 2.0 (Applied Biosystems, Foster City, CA, USA). The cycle conditions were as follows: one cycle at 95°C for 30 s followed by 35 cycles each at 95°C for 5 s and 60°C for 34 s. To normalize the amount of total RNA present in each reaction, the glyceraldehydes 3-phosphate dehydrogenase (GAPDH) gene was used as an internal standard.

Serum samples and MnSOD enzyme-linked immunosorbent assay (ELISA)

Serum samples were obtained from 20 healthy subjects, 15 simple steatosis (SS) patients and 29 NASH patients after a thorough clinical evaluation. Signed informed consent was obtained from each patient. The patients were diagnosed at University Hospital, Kyoto Prefectural University of Medicine (Kyoto, Japan) and Kagoshima University (Kagoshima, Japan). The study protocol was approved by the Ethics Committee of the Kagoshima University Hospital, the Kyoto Prefectural University of Medicine and the Miyazaki Prefectural Industrial Support Foundation. Serum MnSOD levels were measured by a Human Superoxide Dismutase 2 ELISA (AbFRONTIER, Seoul, Korea).

Statistical analysis

Differences among three groups were evaluated using Kruskal–Wallis test followed by Dunn's multiple comparison test. Correlation coefficients were calculated by Spearman's rank correlation analysis. A receiver-operator curve (ROC) was constructed by plotting the sensitivity and specificity (100 – specificity) for each value.

RESULTS

THE NBS-LABELED peptides from human primary hepatocytes were analyzed by MALDI-TOF/MS, and 73 pairs of peaks with 6-Da differences were detected in all mass spectra. Among these pairs of peaks, 44 pairs had a greater signal intensity in the H_2O_2-loaded sample compared to the PBS-loaded sample (data not shown). Among these 44 pairs of peaks, four peak pairs, m/z 1705–1711, m/z 1783–1789, m/z 1902–1908 and m/z 2790–2796, were identified as COX6B, CES1, CPS1 and superoxide dismutase (Mn), mitochondrial (MnSOD), respectively, by MS/MS ion searching (Table 1). The MS spectrum of the m/z 2790–2796 pair and the MS/MS spectrum of 2796 m/z ([^{13}C]-NBS labeled; MnSOD) are shown in Figure 1(a,b), respectively. Western blotting and real-time RT–PCR revealed that the protein and mRNA expression for each of these molecules increased in human primary hepatocytes after H_2O_2 loading (Fig. 1c–e).

Figure 1 Typical MS spectrum and MS/MS spectra from a proteomic analysis. (a) MALDI-TOF/MS spectra of a pair of peaks, 2790–2796 m/z, and the relative intensities of the [^{13}C]-2-nitrobenzenesulfenyl (NBS)-labeled peak compared to the [^{12}C]-NBS-labeled peak. Relative intensities are the means of two independent values analyzed by Autoflex II TOF/TOF. (b) MS/MS spectra of 2796 m/z ([13C]-NBS-labeled). From the detected MS/MS spectra, superoxide dismutase (Mn) mitochondrial was identified. (c) Equal amounts of cell extracts (4 μg) from human primary hepatocytes loaded with 200 μM hydrogen peroxide (H_2O_2) for 24 h were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis and then immunoblotted with cytochrome *c* oxidase VIb isoform 1 (COX6B)-, liver carboxylesterase 1 (CES1)-, carbamoyl-phosphate synthase (ammonia), mitochondrial 1 (CPS1)-, superoxide dismutase [Mn], mitochondrial (MnSOD)- or β-actin-specific antibodies. (d) Quantitative representation of the western blot data. The results have been normalized to β-actin levels and are expressed as the levels relative to untreated cells. The data are the means of duplicate cultures. (e) The mRNA expression levels of COX6B, CES1, CPS1, MnSOD and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were measured by real-time polymerase chain reaction. The results have been normalized to GAPDH and are expressed as the levels relative to untreated cells. The data are the means of duplicate cultures. PBS, phosphate buffered saline.

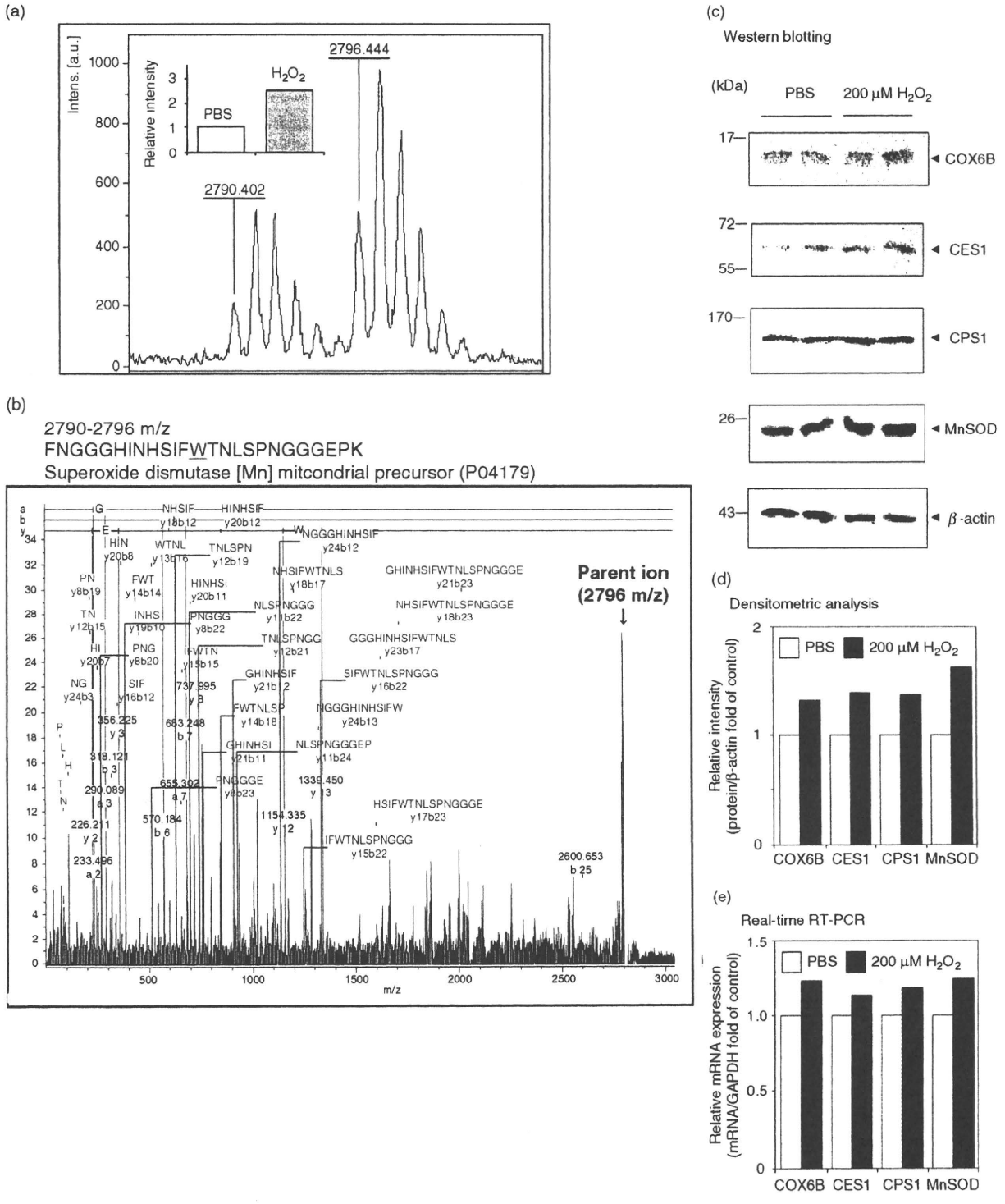

Table 2 Characteristics of study subjects

	Simple steatosis ($n = 15$)	NASH ($n = 29$)	*P*-value
Age (years)	43.2 ± 14.0	60.8 ± 14.9	**<0.001**
Sex (male/female)	11/4	11/18	**<0.05**
Height (cm)	162.5 ± 11.2	156.5 ± 8.7 (28)	0.05
Bodyweight (kg)	69.3 ± 11.8	69.2 ± 15.2 (28)	0.58
BMI (kg/m^2)	26.3 ± 3.6	28.1 ± 4.4 (28)	0.23
Diabetes (yes/no)	5/10	13/15 (28)	0.52
Hyperlipidemia (yes/no)	10/5	16/12 (28)	0.74
Hypertension (yes/no)	4/11	10/18 (28)	0.74
Hb (g/dL)	15.1 ± 1.8	14.4 ± 1.5	0.07
Plt (×10^4/μL)	23.8 ± 7.5	18.8 ± 7.0	**<0.05**
AST (IU/L)	41.6 ± 20.2	69.4 ± 46.5	**<0.05**
ALT (IU/L)	83.1 ± 53.1	94.6 ± 96.0	0.89
γ-GTP (U/L)	75.3 ± 52.4	155.6 ± 303.1	0.40
ChE (IU/L)	417.9 ± 97.5	352.8 ± 135.5	**<0.05**
γ-Glob (g/dL)	1.27 ± 0.40	1.50 ± 0.44 (24)	0.06
Total cholesterol (mg/dL)	209.2 ± 45.8	204.8 ± 52.1	0.97
Triglyceride (mg/dL)	168.3 ± 65.8	184.8 ± 168.3	0.45
BS (mg/dL)	119.1 ± 48.5	112.3 ± 34.3	0.61
Ferritin (mg/dL)	190.0 ± 112.7 (14)	239.8 ± 234.3 (22)	0.75

Values represent means ± standard deviation for the indicated number of subjects. Significant differences between the mean values ($P < 0.05$) were assessed by Fisher's exact probability test (sex, diabetes, hyperlipidemia and hypertension) or Mann–Whitney's *U*-test (other items).
Values in parentheses indicate the number of samples. Bold characters highlight statistically significant *P*-values.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; BS, blood sugar; ChE, choline esterase; γ-Glob, γ-globulin; γ-GTP, γ-glutamyl transpeptidase; Hb, hemoglobin; NASH, non-alcoholic steatohepatitis; Plt, platelet count.

The clinical characteristics of the SS and NASH groups were not significantly different except for the average age, platelet count (Plt), aspartate aminotransferase (AST) and choline esterase (ChE) (Table 2). We examined the serum MnSOD levels in healthy subjects ($n = 20$), SS patients ($n = 15$) and NASH patients ($n = 29$). There were no significant differences in MnSOD serum levels between healthy subjects and SS patients (Fig. 2a). In contrast, NASH patients had significantly higher serum MnSOD levels than both healthy subjects and SS patients (Fig. 2a). In addition, as shown in Figure 2(b), the serum levels of MnSOD tended to increase in parallel with the fibrosis stage. In contrast, there was no correlation between the levels of MnSOD and the activity grade of NASH (Fig. 2c). ROC of MnSOD levels were constructed to distinguish NASH (29 patients) from SS (15 patients) (Fig. 2d). The serum MnSOD threshold level that was used to predict NASH was calculated to be 71.8 ng/mL. At this threshold, the sensitivity was 69.0% and the specificity was 80.0%. The area under the ROC (AUC) for serum MnSOD levels was 0.760 ($P = 0.010$). The ROC curves for Plt, AST and ChE, which were significantly different between SS and NASH (Table 2), were also constructed. As a result, the AUC (*P*-value, threshold, sensitivity [%], specificity [%]) for Plt, serum AST and ChE were 0.733 (0.012, 19.4, 65.5, 86.7), 0.726 (0.015, 42.0, 65.5, 73.3) and 0.687 (0.044, 317.5, 48.3, 86.7), respectively.

DISCUSSION

IN THIS REPORT, we used the NBS labeling method to identify novel oxidative stress markers in hepatocytes that can be used as diagnostic markers for NASH and identified four candidate markers, COX6B, CES1, CPS1 and MnSOD, that were upregulated with H_2O_2 loading (Table 1, Fig. 1). Several recent studies have reported novel approaches that combine the NBS labeling method with 2-DE, high-performance liquid chromatography (HPLC) and lectin column chromatographic techniques.[13–16] In our present study, we identified only four proteins, indicating that it may be necessary to modify the current method by 2-DE and column chromatographic techniques to identify additional NBS-

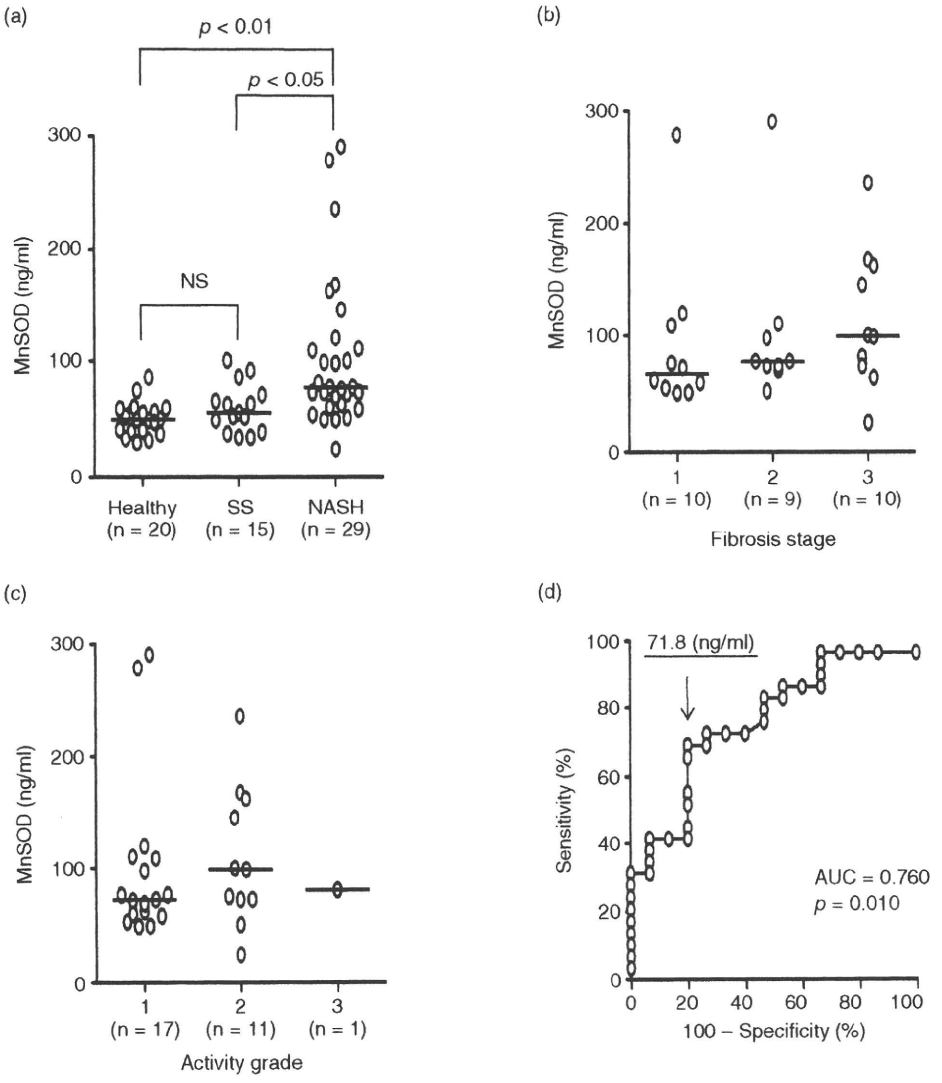

Figure 2 Clinical significance of serum MnSOD levels. (a) Serum MnSOD levels in healthy subjects and patients with SS or non-alcoholic steatohepatitis (NASH). Serum MnSOD levels were measured by enzyme-linked immunosorbent assay. (b) Comparison between serum MnSOD levels and the fibrosis stage in SS and NASH patients. (c) Comparison between serum MnSOD levels and the activity grade in SS and NASH patients. (d) Receiver–operator curve for MnSOD. The differences among three groups were evaluated using Kruskal–Wallis test followed by Dunn's multiple comparison test. Correlation coefficients were calculated by Spearman's rank correlation analysis. Bars indicate the median in the respective groups. AUC, area under the curve.

labeled peptides. In addition, further studies are needed to identify novel biomarkers by other proteomic techniques using serum samples from SS and NASH patients.

COX6B, CPS1 and MnSOD are mitochondrial proteins, and therefore may be indicators of mitochondrial disorders that are induced by oxidative stress. CPS1 is expressed primarily in the liver and small intestine and is involved in the urea cycle.[17] In galactosamine-induced rat acute hepatitis, plasma concentrations of CPS1 increase up to approximately 100-fold for 24 h after treatment.[18] This may indicate that secreted CPS1 is a serum marker for acute hepatitis. CES1, which is responsible for detoxification of exogenous compounds such

as esters, amides and thioesters, is also known to exist in the serum. Therefore, CES1, like CPS1, may be a serum oxidative stress marker.[19,20] Additional studies are needed to further evaluate the serum levels of these identified proteins.

MnSOD primarily exists in the mitochondrial matrix and eliminates reactive oxygen species (ROS) by catalyzing the dismutation of superoxide radicals and hydrogen peroxide.[19] Furthermore, MnSOD expression was previously shown to increase after exposure to hydrogen peroxide in rat hepatocytes.[21] In addition, obese mice were previously reported to have increased hepatic H_2O_2 levels and necrosis following an imbalance between increased MnSOD, which forms H_2O_2, and decreased glutathione activity, which detoxifies H_2O_2.[22] We found that MnSOD is potentially a novel diagnostic marker of NASH that can be used to distinguish between SS and NASH. One of the mechanisms contributing to increased MnSOD serum levels in NASH might be the discharge of MnSOD from necrotic hepatocytes. On the other hand, in the liver, several pro-inflammatory cytokines, such as tumor necrosis factor-α, interleukin-6 and interleukin-1β can act as common inducers of NASH.[23,24] Such pro-inflammatory cytokines have been shown to induce MnSOD expression in liver tissues.[25] Furthermore, pro-inflammatory cytokines induced the expression and secretion of MnSOD in several cancer cell lines including hepatoma cells.[26,27] In our present study, the origin of MnSOD produced in NASH and the precise mechanism of increased serum levels of MnSOD in NASH patients remain unclear. However, these reports may partially support the mechanism of MnSOD production in NASH. Further elucidation is necessary to clarify the mechanism of MnSOD expression and production in NASH.

Several reports have shown a relationship between the enzymatic activity of MnSOD and non-alcoholic fatty liver disease, NASH, liver cirrhosis and hepatocellular carcinoma.[28–31] Ono *et al.* showed that MnSOD serum levels were significantly increased in patients with primary biliary cirrhosis compared to patients with other liver diseases.[32] However, the serum protein levels of MnSOD in liver diseases have not been fully evaluated. In addition, the enzymatic activity of serum MnSOD was not different among the three groups and this activity did not correlate with serum MnSOD levels in our study (data not shown). The reasons for these results are unclear. However, as shown in Figure 2(b), serum MnSOD levels increased in parallel with the stage of fibrosis in NASH. The increase in serum MnSOD levels also significantly correlated with the serum AST levels (data not shown). These results indicate that serum MnSOD might be a biomarker that reflects hepatitic and fibrotic pathology. In addition, although MnSOD levels should increase in patients with other diseases including CHC, ROC analysis revealed that serum MnSOD may be a more sensitive biomarker than Plt, AST and ChE. We concluded that serum MnSOD is a useful biomarker that can distinguish SS and NASH.

ACKNOWLEDGMENTS

THIS WORK WAS supported in part by Grants-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science (JSPS), Research for Promoting Technological Seeds (A) from the Japan Science and Technology Agency (JST), the Collaboration of Regional Entities for the Advancement of Technological Excellence (CREATE) from the JST, and a Grant-in-Aid (Research on Hepatitis and BSE) from the Ministry of Health, Labor and Welfare of Japan.

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トピックス

飲酒とNAFLD

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飲酒，AFLD，NAFLD，内臓肥満，
メタボリック症候群

はじめに

飲酒は生活習慣の1つの因子であり，脂肪肝の原因になることもよく知られている．アルコール性肝障害の診断基準では，日本酒換算で1日平均3合以上，5年以上継続が1つの目安となっているが，この条件を満たす飲酒者は男性でも全体の5%に満たない．多量飲酒が肝硬変をはじめとした種々の肝障害を起こすことは知られていても，大多数の飲酒者における脂肪肝との関係は必ずしも明らかにされていない．本稿では，地域住民を対象とした健診の成績を中心に，飲酒と脂肪肝の関連について述べる．

アルコール性脂肪肝と非アルコール性脂肪肝

脂肪肝はたんに肝細胞の脂肪変性（steatosis）と炎症性細胞浸潤を主体とする単純性脂肪肝（simple steatosis）とそれに肝細胞の風船化や線維化が加わった脂肪性肝炎（steatohepatitis）に分けられる．また，原因によりアルコール性脂肪性肝疾患と（alcoholic fatty liver disease；AFLD）と非アルコール性脂肪性肝疾患（nonalcoholic fatty liver disease；NAFLD）に分けられ，後者に非アルコール性脂肪性肝炎（nonalcoholic steatohepatitis；NASH）が含まれる[1]．

さて，非アルコール性とアルコール性を分けるのは，もちろんアルコールを摂取しているかどうかであるが，一般にはアルコール摂取量20g/日以下の脂肪肝をNAFLD，20g/日超をAFLDとしている．鹿児島県の30～60歳代男性の人間ドック受診者の場合，約6割が飲まないか飲んでも1合（約20 g）/日未満であり，そこに発生する脂肪肝はおおむねNAFLDである．一方，3合以上/日の飲酒者は約4%である．残りの受診者にみられる脂肪肝は，飲酒量は中等度でAFLDと分類されることになるが，おもな原因が「アルコール性」に限られるわけではない．

アルコール代謝と脂肪肝

アルコールの代謝（酸化）はおもに肝臓で行われる．細胞質のアルコール脱水素酵素でアセトアルデヒドに変換され，ミトコンドリアのアセトアルデヒド脱水素酵素で酢酸となる（**図1**）．どちらの反応も，nicotinamide adenine dinucleotide（NAD^+）が利用（還元）されてNADHとなる反応が共役しているため，アルコールを摂取するとNAD^+/NADH比の低下，すなわちレドックス・シフトが生じ，恒常性維持のためにはNADHをNAD^+に酸化する必要がある．肝臓の細胞質でNADH酸化の中心的な役割を担うのがリンゴ酸脱水素酵素の反応であり，オキザロ酢酸がリンゴ酸に変換される過程でNADHは酸化される．消費されるオキザロ酢酸を供給するのは，ミトコンドリアの電子伝達系と協調して働くリンゴ酸/アスパラギン酸シャトルである（**図2A**）．多量飲酒によってレドックス・シフトが進行するときには，オキザロ酢酸はATPクエン酸リアーゼによってクエン酸からも供給されると考えられている．この酵素反応こそ，細胞質における脂肪酸合成のファースト・ステップである（**図2B**）．また，グリセロール3リン酸脱水素酵素の反応

図 1　アルコールの代謝（酸化）

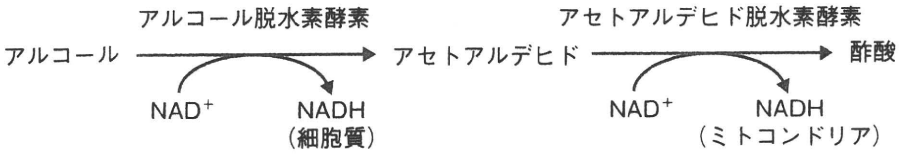
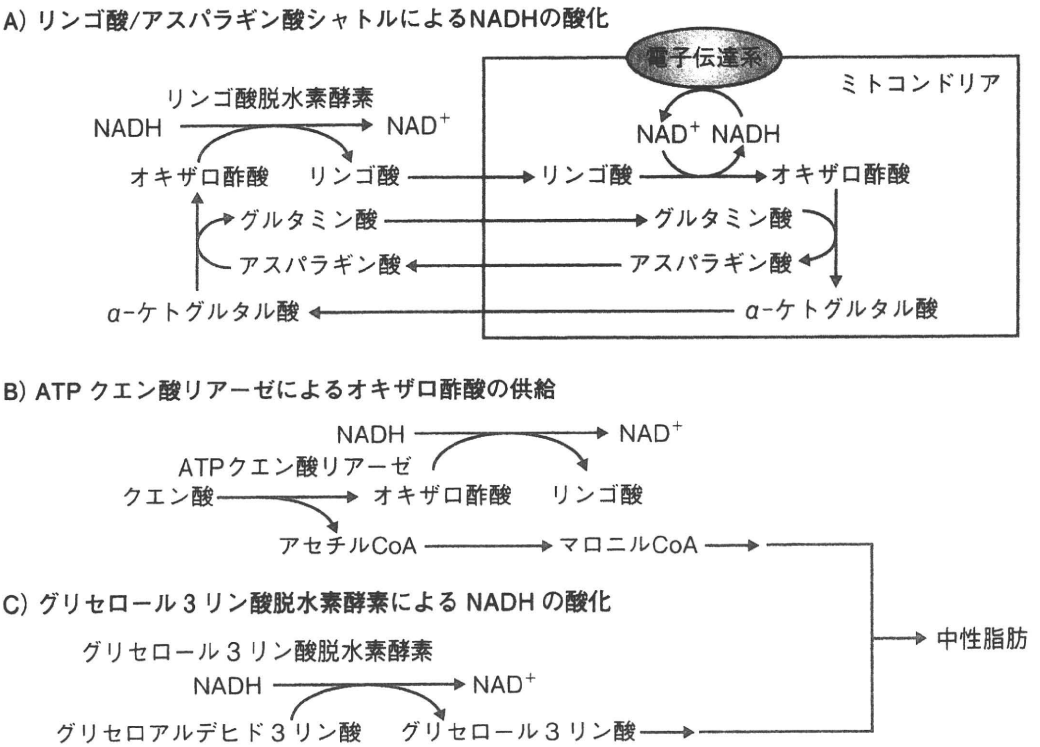


図 2　肝細胞の細胞質における NADH 酸化のしくみ



も進み，NADH を酸化すると同時にグリセロール 3 リン酸も過剰に生成され（**図 2C**），中性脂肪合成の材料となる．しかも，アルコールによる sterol regulatory element binding protein-1 (SREBP-1)の増加[2]や AMP-dependent protein kinase 活性の低下などが知られており，脂肪合成は基質の流れだけでなく，遺伝子や酵素レベルでも活性化されることになる．アセトアルデヒド脱水素酵素の反応でも NAD^+ を利用するため，NADH 再酸化のためにミトコンドリアにはさらに負担がかかることになる．

一方，アルコールは脂肪酸のβ酸化を抑制すると考えられている．また tumor necrosis factor-α（TNF-α）など炎症性サイトカインの増加[3]なども報告されており，それによるインスリン感受性の低下も脂質代謝異常に関与すると考えられている．ALD と NAFLD には共通の病態があり，このため飲酒は，ALFD はもちろん，NAFLD を悪化させる可能性がある．

脂肪肝発生の危険因子

脂肪肝発生の危険因子としては，肥満や糖尿病などの生活習慣病がもっとも重要な因子として考えられている[4]．また，われわれは，BMI は変わらなくても体脂肪率が増加するような身体組成の変化も最近の脂肪肝の増加に寄与している可能性を指摘した[5]．一方，飲酒の影響を評価する報告は少ない．