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Review

Application of proteomic technologies to discover and identify biomarkers for excessive alcohol consumption: A review☆

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Abstract

Since currently available markers of alcohol abuse are not satisfactory, searches for novel markers are warranted. Proteomic analyses are promising tools to discover and identify novel biomarkers. Using two different proteomic technologies, surface enhanced laser desorption/ionization time-of-flight mass spectrometry and agarose fluorescent two-dimensional difference gel electrophoresis, we could detect and identify a total of 11 potential biomarkers of excessive alcohol consumption. It was noteworthy that the down regulation of the 5.9 kDa protein fragment was consistently seen in habitual drinkers and the diagnostic efficiency was greater than those of conventional markers such as gamma glutamyl transferase and carbohydrate deficient transferrin.

Keywords: Alcohol; SELDI; 2D-DIGE; CDT; GGT

Contents

1. Introduction

Excessive consumption of alcohol is a health risk that can lead to a variety of medical and social problems [1,2]. Heavy drinking causes not only alcoholism and alcoholic liver diseases, but aggravates many common medical disorders including hypertension, stroke, diabetes mellitus and gout.

Although the first line of detecting heavy drinking resorts to self reports, heavy drinkers tend to underestimate their alcohol consumption assessed via quantity and frequency questions. This difficulty of obtaining an accurate drinking history may lead to under diagnosis of hazardous alcohol use and related disorders. Indeed, it has been reported that as few as 28% of problem drinkers are recognized by their doctors [3].

Diagnostic indicators of alcohol consumption would be useful in the following medical settings. First of all, they can be indicators of excessive drinking and alcohol related organ damages. Second, these markers can monitor sobriety in patients during alcoholism treatment and prevention programs. Further-

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more, a reliable marker would be valuable for epidemiologic studies investigating the incidence of alcoholism, and to monitor for alcohol abuse in workers involved in areas of public safety. Since currently available alcohol biomarkers are not ideal in terms of sensitivity and specificity, efforts should be directed towards developing satisfactory laboratory markers of alcohol consumption.

Recent advances in sophisticated technologies in proteomics should provide promising ways to discover novel markers in various fields of clinical medicine. In this article, we summarize our recent comprehensive proteomic studies to discover and identify novel biomarkers for excessive alcohol consumption [4–6].

2. Currently available markers and new directions toward the newer markers

Currently available and emerging biomarkers for excessive alcohol consumption are presented in Table 1. The most well-known marker of alcohol abuse is the serum enzyme, gamma glutamyl-transferase (GGT) [7]. More recently, it has also been reported that GGT may explain individual difference in susceptibility to the pressor effects of alcohol. Blood pressure reading, as well as incidence and prevalence of hypertension are significantly higher in subjects with serum GGT levels >50 IU/l than those with <50 U/l [8].

Serum GGT levels are elevated in around 75% of subjects with established alcoholism, indicating that a significant portion of problem drinkers have normal GGT levels (so-called GGT non-responders) [7]. Another disadvantage of GGT is that there are many other causes that raise serum GGT levels, including other hepatobiliary diseases (cholestatic type in particular) and anticonvulsant medication.

Carbohydrate deficient transferrin (CDT) is a collective term referring to isoforms of transferrin deficient in sialic acid residues. CDT has been widely used mainly in Europe [9]. More recently, it has been approved by the U.S. Food and Drug Administration (FDA). This marker is still not commercially available in Japan. Although CDT can detect heavy drinkers with higher specificity than GGT, it is valid only when the daily alcohol intake is greater than 60 g and is less sensitive in women than in men [9]. Recent progress in CDT research has been extensively and critically reviewed [10].

Table 1
Currently available and emerging biomarkers for excessive alcohol consumption

Currently used markers
Gamma glutamyl transferasG (GGT)
Aspartate aminotransferase (AST)
Mean corpuscular volume CMCV)
Carbohydratedeificienttransferrin (CDT)
Emerging markers
Bound acetaldehyde
Betahexosaminidase
Ethyl glucuronide
Phosphatidyl ethanol
Fatty acid ethyl esters
Sialic acid index of plasma apoprotein J
Ratio of urinary serotonin metabolites

Thus, the two representative alcohol markers have some limitations in their clinical efficiency. Indeed, the results of recent multicenter studies indicate that both markers had limited performance in the detection of high-risk drinking [11,12]. Therefore, searches for novel markers are warranted.

Non-oxidative direct metabolites of ethanol are one of the new category of alcohol markers. These markers include ethyl glucuronide (EtG) [13], phosphatidyl ethanol (PEth) [14], fatty acid ethyl esters (FAEEs) [13] and ethyl sulfate (EtS) [15]. Among these, FAEEs and EtG are markers for very recent alcohol consumption. Other new candidates such as bound acetaldehyde [16], serum beta-hexosaminidase [17] and the ratio of urinary serotonin metabolites [14] are also proposed.

Ideal alcohol biomarker should meet the following requirements; first of all, it must reflect the amount of alcohol consumed over a reasonable period of time and return to normal value after several weeks of abstinence. It is also desirable to distinguish harmful drinking from light drinking. The test results should be reproducible and the sample collection must be simple to be accepted by the practitioner and the patient. From these points, the new candidates described above are not necessarily satisfactory. Indeed, a recent survey for use of biomarker screening by general practitioners in US [18] indicated that 85% were very familiar with GGT, but only 6.3% frequently ordered CDT.

3. Alcohol biomarker discovery by proteomic technologies

The sequencing of the human genome has opened the door for comprehensive transcriptome and proteome analysis. Transcriptome analyses utilizing DNA microarrays have revealed unique patterns for gene expression that are clinically informative. Messenger RNA abundances, however, are not necessarily predictive of corresponding protein abundances [19]. Furthermore, DNA microarrays have limited utility for the analysis of clinical specimens such as serum and urine for the purpose of uncovering biomarkers of clinical significance. As a result, there is growing interest in proteomic technologies allowing for the identification of disease specific alterations of proteins.

3.1. Proteomic analyses of liver proteins after long-term ethanol exposure

Since the majority of serum or plasma proteins are synthesized in the liver, proteome analysis of liver proteins should be basis for serum or plasma proteome analyses. In 1988, Wirth and Vesterberg [20] reported on rat liver cytosolic protein changes after ethanol exposure studied by two-dimensional electrophoresis (2-DE). They found pronounced differences in the relative abundance of protein in several spots in ethanol exposed rats as compared with controls. The identity of the majority of the spots were unknown.

Long-term alcohol consumption causes liver damages by a complex process including ethanol's hepatotoxicity linked to its metabolism, up-regulation of proinflammatory cytokines, and oxidative stress [1]. Mitochondria are particularly susceptible to increased formation of reactive oxygen species. Venkatraman

et al. [21] reported the use of proteomics (conventional 2-DE and blue native gel electrophoresis) to evaluate alterations in the levels of mitochondrial proteins following chronic voluntary alcohol consumption in a rat model. A total of 45 proteins showed significant changes as a consequence of alcohol exposure, 25 of which were not previously related to alcohol effects.

Most animals do not voluntarily consume sufficient amount of alcohol to produce pathophysiologically meaningful blood alcohol levels. Through selective breeding, however, the lines of high and low alcohol consuming rats have been produced [22]. Recently, 2-DE based proteome analyses were conducted to determine the alteration of protein expression in the liver of inbred high alcohol consuming rats as compared to control animals [23]. A total of 118 spots were found to be significantly altered after alcohol exposure; the majority of these proteins were enzymes involved in glycolysis, gluconeogenesis, fatty acid oxidation, protein synthesis and antioxidant activity.

3.2. Discovery of serum biomarkers for excessive alcohol consumption by proteomic approaches

More than 20 years ago, Marshall et al. [24] tried to detect alcohol-associated changes in human serum protein patterns by conventional 2-DE. They found elevated levels of IgA, a1-antichymotrypsin, haptoglobins and apo A-1 lipoprotein, whereas a number of proteins or polypeptides were unidentified.

More recently, proteomic study was conducted again by 2-DE to search for potential protein biomarkers for fetal alcohol syndrome (FAS) [25]. Sera from 12 subjects in whom FAS had been diagnosed and 8 sex- and age-matched subjects whose mother did not consume alcohol were analyzed. Eight proteins were found to be candidate biomarkers. No single protein, however, differentiated all case subjects from controls.

The great advantage of proteome analysis is that the identified protein itself is a good candidate as a diagnostic marker. Recent progress in proteomic approaches have been divided into top-down and bottom-up approaches [26]. We took advantage of the two powerful top-down approaches to discover and identify novel biomarkers for excessive alcohol consumption.

3.2.1. Surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS)

Surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) is an innovative approach introduced by Hutchens and Yip [27].

This technology has been used successfully to detect several disease-associated biomarkers and disease related plasma protein fingerprints in complex biological specimens.

The principles and clinical applications of the SELDI-TOF MS have been reviewed [28–30].

Briefly, in this technology, a number of proteins or peptides included in a crude sample such as serum are first retained in a solid-phase chromatographic surface or biochemical molecules. The retained proteins or peptides are subsequently ionized and detected by TOF MS. The use of several different chromatographic arrays and wash conditions enables high-speed, high-resolution chromatographic separations.

In our experiments, an anionic exchanger (SAX2) and a cationic exchanger (WCX2) were used. These chips were chosen because types of ion exchange resin and buffer conditions for purification of the recognized peaks could be based on ProteinChip affinitiy condition during the SELDI analysis.

We used ProteinChip SELDI technology to generate comparative protein profiles of consecutive serum samples obtained during abstinence from alcoholic patients hospitalized for a rehabilitation program [4].

Sixteen patients (15 males and 1 female) with alcohol dependency according to the DSM IV criteria (American Psychiatric Association, 1994) were included. All patients had consumed >100 g of alcohol per day until the day of hospitalization for more than 10 years. Blood samples were taken on the morning of their arrival at the hospital, and also at 7 and 90 days of abstinence.

An aliquot of the sera stored at −80 °C was used for the SELDI-TOF MS analysis. Details of the SEDLDI-TOF MS analysis are described elsewhere [4].

The reproducibility of the SELDI spectra, namely mass location and intensity from array to array on a single chip (intra-assay) and between chips (inter-assay), was determined as described by Adam et al. [31] using a pooled normal serum sample. Seven proteins in the range of 3–10 kDa observed on the spectra were used to calculate the coefficient of variance. The intra-assay and inter-assay coefficient of variance for the peak location was 0.02 and 0.03%, respectively, and the intra- and inter-assay coefficient of variance for normalized intensity (peak height or relative concentration) was 12.1 and 20.5%, respectively.

We obtained mass spectra for a total of 48 serum samples from SELDI analysis using anionic arrays (SAX2) and cationic arrays (WCX2). Fig. 1 shows a representative view of the spectra of proteins retained on the WCX2 protein chips. It was of note that a 5.9 kDa and a 7.8 kDa peaks, down regulated on admission, remarkably increased during abstinence. Similar patterns were seen in 13 out of 16 cases. Analyses were also made using anionic arrays. We recognized one peak (28 kDa), increased on admission, significantly decreased during a 1-week abstinence.

GGT activities, on admission were normal in 4 cases (so-called GGT non-responders), and were elevated in 12 cases. It

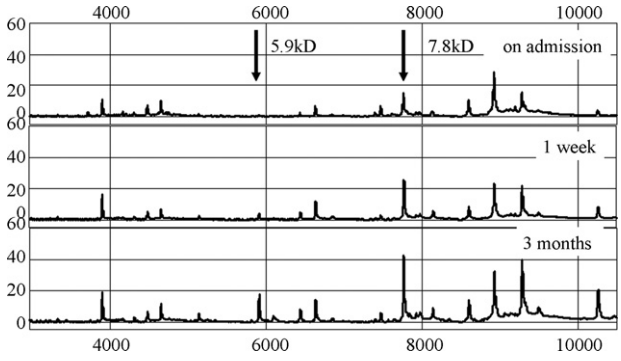

Fig. 1. Representative view of the spectra of proteins retained on the WCX2 protein chips by SELDI-TOF MS analysis. It was of note that the 5.9 and 7.8 kDa peaks, down regulated on admission, remarkably increased during abstinence.

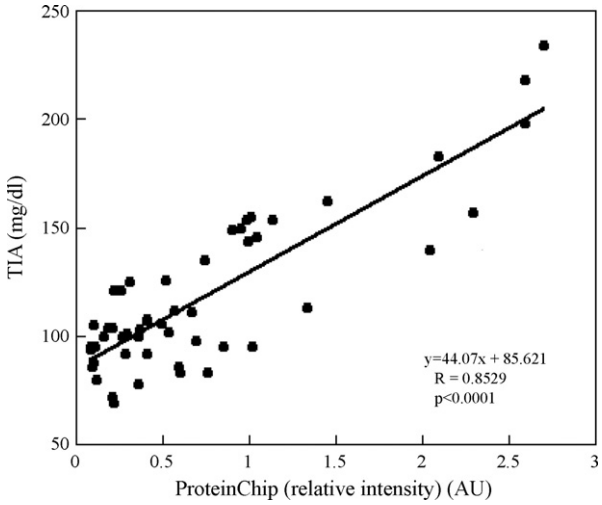

Fig. 2. Correlation between serum apoprotein A-1 levels determined by the immunoassay (TIA) and relative intensity of the 28 kDa peaks in the SELDI-TOF MS analysis.

was of note that the down regulation of the 5.9 kDa protein was found in all four cases of GGT non-responders.

Partial purification of the three proteins (5.9, 7.8 and 28 kDa) were carried out as detailed before [4]. Amino acid sequencing of the trypsin digests of the 28 and 7.8 kDa bands, and the final preparation of the 5.9 kDa protein revealed that the 5.9 kDa was a fragment of fibrinogen αE chain, and that the 28 kDa was apoprotein A-I. The 7.8 kDa protein was identified as a fragment of the apoprotein A-II.

Since established immunoassays are available for serum apoprotein A-I measurement, relative peak heights of the 28 kDa protein in SELDI analysis were compared with apoprotein A-I levels determined by an immunoassay. Positive correlation was found as shown in Fig. 2.

The fact that apoprotein A-I, one of the well-established alcohol-related markers [32], was also screened in the SELDI-TOF MS analysis may substantiate the validity of the ProteinChip technology. The peak heights of the 5.9 and 7.8 kDa peaks in serum samples obtained from patients with liver cirrhosis were similar to those in normal subjects. Therefore, it is unlikely that hepatic dysfunction itself in the alcoholics, rather than heavy alcohol consumption, was responsible for the decreased levels of the two protein fragments. The exact mechanisms by which excessive alcohol consumption decrease serum levels of the 7.8 kDa fragment of apo-AII and the 5.9 kDa fragment of αE chain of fibrinogen remain to be investigated. It is of note that alcohol-induced alteration of fibrinolytic activity has been shown in human monocytes [33].

It is a general view that a large part of the human serum peptidome as detected by SELDI or MALDI-TOF MS is produced ex vivo by degradation of high-abundance endogenous circulating proteins by endogenous proteases. While some investigators have regarded the serum peptidome as noise or non-specific epiphenomenon [34], others have proposed that the serum peptide fingerprint may contain a rich source of disease-specific information [35]. Indeed, recent study by Villanueva et al. [36] demonstrated that a limited subset of serum peptides provides accurate class discrimination between patients with three types of solid tumors and controls without cancer, providing a direct link between peptide marker profiles of diseases and different protease activity. In their study, it was shown that exoprotease activities superimposed on the ex vivo coagulation and complement-degradation pathways contribute to generation of a number of peptides. It is likely that the 5.9 kDa peptide identified in our study was also an ex vivo product. Indeed, the 5.9 kDa peak found in serum sample was minimal when plasma samples were analyzed.

Thus, we identified two novel protein fragments as potential new markers in alcoholic subjects hospitalized for a rehabilitation program. The next question was how these novel markers perform as screening tools when applied to subjects with moderate alcohol consumption in the general population. We determined the diagnostic values of the novel markers in screening male habitual drinkers with moderate alcohol consumption (hereafter habitual drinkers) in the general population as compared with two conventional markers, GGT and CDT [5].

Relative intensities of the 5.9, 7.8 and 28 kDa peaks in non-drinkers and habitual drinkers are presented in Fig. 3. Relative intensities of the 5.9 kDa peak of habitual drinkers were significantly smaller than those in non-drinkers, whereas 28 kDa peaks were significantly greater in habitual drinkers.

Among 55 habitual drinkers, serum GGT activities were within the reference interval in 20 subjects. It was noteworthy that relative intensities of the 5.9 and 28 kDa were significantly different from those in non-drinkers even among GGT non-responders as shown in Fig. 4.

Fig. 5 shows the receiver-operating characteristic (ROC) curve analysis obtained for the 5.9 and 28 kDa peaks and also for serum GGT and CDT activities. The areas under the curves for the 5.9 kDa, GGT, CDT and 28 kDa were, 0.939, 0.661, 0.633, and 0.816, respectively. The differences were statistically significant between 5.9 kDa and CDT ($p < 0.01$), between

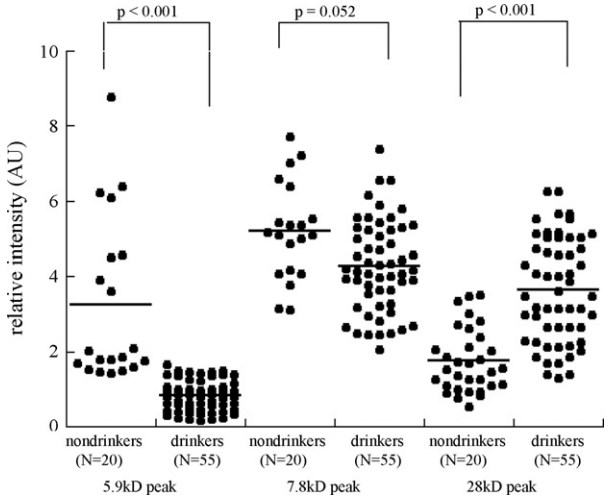

Fig. 3. Relative intensities of the 5.9, 7.8 and 28 kDa peaks obtained by the SELDI-TOF MS analysis in non-drinkers and habitual drinkers (values are mean ± S.D.).

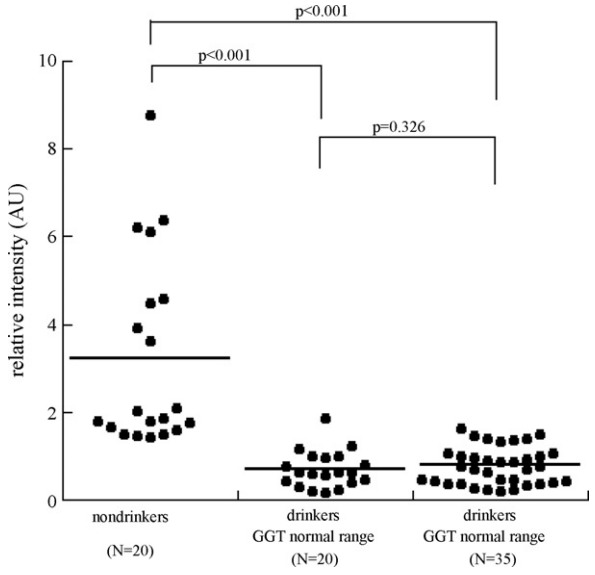

Fig. 4. Relative intensities of the 5.9 kDa peak obtained by the SELDI-TOF MS analysis in non-drinkers, habitual drinkers with or within elevated GGT (values are mean ± S.D.).

5.9 kDa and GGT ($p < 0.05$), between 5.9 and 28 kDa ($p < 0.05$), between GGT and CDT ($p < 0.01$), between 28 kDa and CDT ($p < 0.01$).

Preferably, serum 5.9 kDa peptide levels should be measured by an immunoassay rather than MS-based method from a practical point of view. We constructed an ELISA for the 5.9 kDa fibrinopeptide and established a standard curve using a synthesized peptide [37]. Since human serum contains a number of different fibrinopeptides including the 5.9 kDa, it is not an easy task to measure specifically and accurately the 5.9 kDa peptide alone in human serum samples. Alternatively, refinement of the mass-based method is necessary to obtain better throughput.

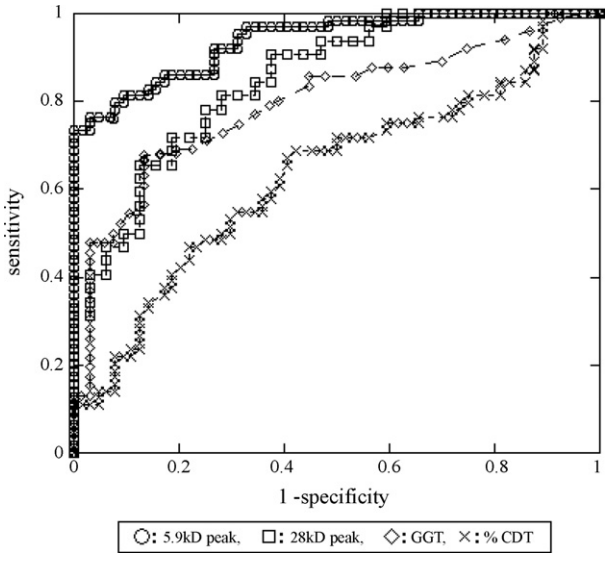

Fig. 5. The receiver-operating characteristic (ROC) curve analysis obtained for the 5.9 and 28 kDa peaks and also for serum GGT and CDT activities.

3.2.2. Fluorescent two-dimensional difference gel electrophoresis (2D-DIGE)

Although the SELDI-TOF MS is suitable for detection of low-molecular-weight proteins, it is difficult to detect higher molecular weight proteins. Therefore, a complementary method that can detect higher molecular weight proteins are warranted. We used agarose two-dimensional differential gel electrophoresis (agarose-2D-DIGE) for this purpose.

The agarose 2-DE method was previously shown to have higher loading capacity of protein and have higher capability in separation of high molecular weight proteins than 2-DE with immobilized pH gradient (IPG) gel for isoelectric focusing (IEF) [38]. We used agarose 2-DE and identified several novel proteins with altered expression in primary colorectal cancer [39]. Recently, fluorescent 2D differential gel electrophoresis (2D-DIGE) was introduced [40] and it was considered as one of the major advances in quantitative proteomics. The basis of this technique is the use of two protein samples that are labeled covalently with fluorescent cyanine dyes, Cy3 and Cy5, respectively. These labeled proteins are mixed and separated in the same 2D gel. This technique appears to have advantages of adequate sensitivity, high reproducibility, and wide dynamic range. Combination of these two methods, agarose 2-DE and 2D-DIGE (agarose 2D-DIGE), would be a powerful new tool for analysis of clinical samples for biomarker discovery.

We have selected matched sample pairs of 12 alcoholism patients before and after 3 months abstinence treatment. In order to improve the detection of proteins, we removed albumin and IgG from serum with an albumin removal kit as a pretreatment [41].

Each sample before abstinence was labeled with Cy5, and sample after abstinence was labeled with Cy3. Internal standard, created by pooling aliquots of all the samples and labeled with Cy2, was used to normalize protein abundance measurements across multiple gels in each experiment [42].

Labeled samples for each patient were subjected to the first-dimensional agarose IEF, followed by the second-dimensional SDS-PAGE. The second-dimensional gel was imaged with the Typhoon 9400™ (Amersham Biosciences) using optimal excitation/emission wavelength for each DIGE fluor: Cy2 (488/520 nm), Cy3 (532/580 nm), and Cy5 (633/670 nm). Shimadzu 2D evolution software (Shimadzu Biotech, Kyoto, Japan) was used to analyze the DIGE images across samples from patients before and after treatment.

A total of eight spots were found to be significantly different in their expression before and after abstinence [6]. These protein spots were cut out from the conventional agarose 2-DE gel and were subjected to in gel digestion, followed by identification by MS as we described before [43]. Changes of the expression levels of these proteins before and after abstinence are summarized in Fig. 6. Among these spots, clusterin (also called apoprotein J) is notable. We found that serum clusterin levels are significantly decreased after long-term alcohol intake. It has been shown that the sialic acid index of apoprotein J is decreased after long-term alcohol intake [44]. Since clusterin has been shown to modulate the vascular smooth muscle cell response to injury [45], it is tempting to speculate that the down

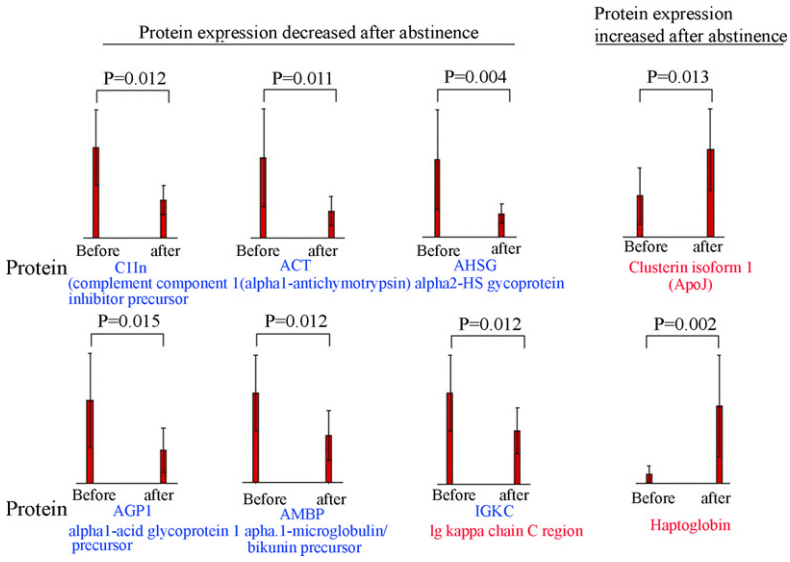

Fig. 6. Proteomic differential displays of serum proteins before and after abstinence by 2D-DIGE analysis revealed eight proteins whose expression was significantly altered.

regulation of clusterin is related to anti-atherogenic effects of habitual alcohol consumption.

4. Conclusion

In summary, using two different proteomic technologies, SELDI-TOF MS and 2D-DIGE, we could detect and identify a total of 11 serum proteins or peptide, potential biomarkers of excessive alcohol consumption. Among them, down regulation of the 5.9 kDa protein fragment was seen in non-responders of GGT as well, indicating that this novel peptide is a promising alternative marker for excessive alcohol consumption.

Since advancement of proteomic technologies is rapid, it is expected that increasing number of new marker candidates will be added to the current list in a near future.

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