Table 2. Serum Proteins Upregulated (A) and Downregulated (B) in Alcoholic Patients on Admission, as Detected by Three-Step Proteome Analysis

No.	Database accession no.	ID	MW	Score	Number of matching peptides	Sequence coverage (%)
A. Upregulated serum proteins						
1	gi2521981	Alpha2-HS glycoprotein	35,641	112	5	8
2	gi90108664	Apolipoprotein A-I	28,061	1,409	43	70
3	gi121672	Glutathione peroxidase 3	25,489	82	2	6
4	gi23200172	Heparin cofactor II	57,034	161	3	5
5	gi189778	PEDF	46,300	491	9	22
B. Downregulated serum proteins						
1	gi224917	Apolipoprotein C-III	8,759	118	3	24

MW, molecular weight; PEDF, pigment epithelial-derived factor.

(using TotalLab TL120 software v2006), the 8 pairs of samples showed a significant difference in the expression level of the 25 kDa band on admission and after 8 weeks of abstinence from drink ($p < 0.05$) (Fig. 1*B*). A comparison of all 40 RP-HPLC fractions revealed that the expression levels of 27 bands at the time of admission changed significantly after 8 weeks abstinence from drink ($p < 0.01$). On admission, 24 bands were upregulated and three bands were downregulated.

Identification of Protein

Of the 27 bands, 6 bands, which demonstrated particularly remarkable changes, were digested by trypsin and were subjected to tandem mass spectrometry for identification. The 6 proteins that were identified are listed in Table 2. The 5 proteins that were upregulated on admission were alpha2-HS glycoprotein, apolipoprotein A-I, glutathione peroxidase 3, heparin cofactor II, and PEDF (Table 2A). The protein band that was downregulated on admission was apolipoprotein C-III (Table 2B).

Western Blotting

Of the 5 proteins upregulated on admission, we focused on PEDF mainly because the alteration in the levels of this protein because of heavy drinking is not well known. The results obtained by SDS–PAGE were confirmed by Western blotting performed on the same 8 pairs of serum samples subjected to the three-step proteome analysis (Fig. 2*A*). A semiquantitative analysis of the results, using the TotaLab TL120 software, revealed a statistically significant difference between the serum PEDF level on admission and after 8 weeks of abstinence from drink ($p < 0.05$) (Fig. 2*B*).

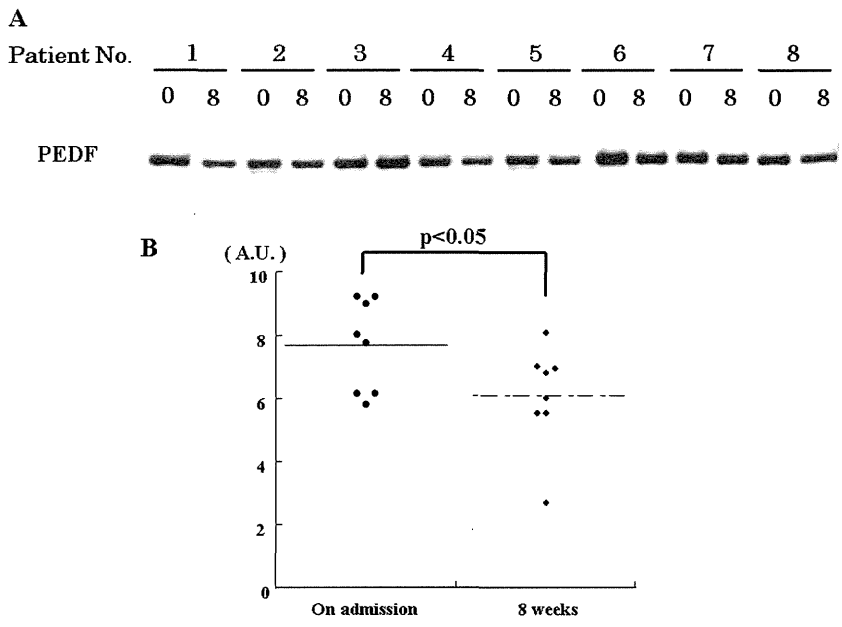

Fig. 2. Western blot analysis of PEDF in the serum samples of the alcoholic patients. (**A**) The 8 sample pairs of the immunodepleted sera, which had been obtained from alcoholic patients on admission (week 0) and after 8 weeks of abstinence (week 8). The serum samples were separated using 10 to 12% SDS–PAGE and probed with anti-PEDF antibody, as described in Methods. (**B**) The densitometric comparison of the bands' intensities. The PEDF expression level is significantly greater on admission than after 8 weeks of abstinence ($p < 0.05$). PEDF, pigment epithelial-derived factor; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

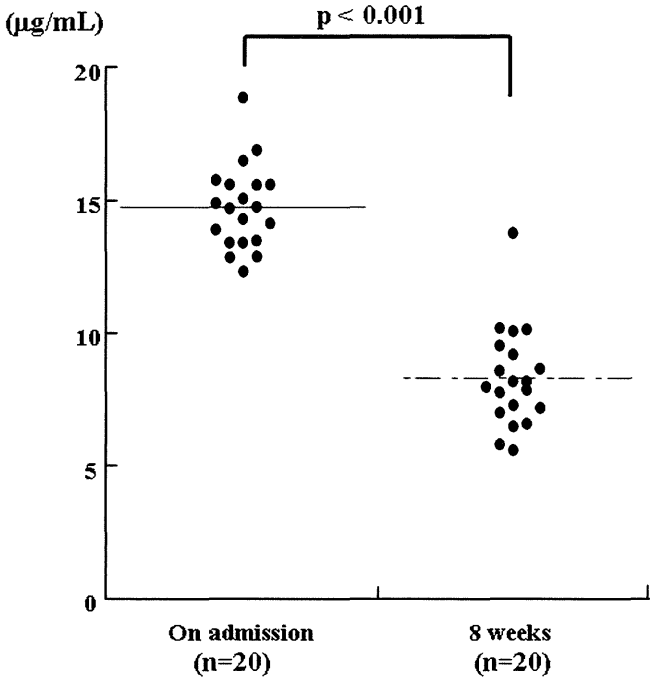

Fig. 3. Serum PEDF levels, as determined by ELISA, in 20 alcoholic patients on admission and after 8 weeks of abstinence. The serum PEDF levels are significantly decreased after 8 weeks of abstinence ($p < 0.001$). ELISA, enzyme-linked immunosorbent assay; PEDF, pigment epithelial-derived factor.

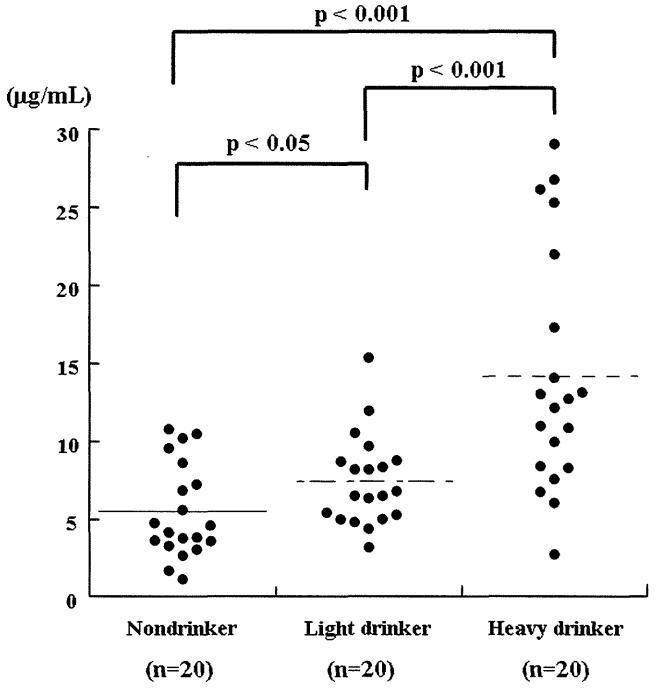

Fig. 4. Serum PEDF levels, as determined by ELISA, in 60 apparently healthy subjects with various drinking habits. The levels are significantly greater in the light habitual drinkers (7.5 ± 2.9 μg/ml) and in the heavy habitual drinkers (14.2 ± 7.7 μg/ml), compared with the nondrinkers (5.5 ± 3.0 μg/ml). ELISA, enzyme-linked immunosorbent assay; PEDF, pigment epithelial-derived factor.

ELISA

ELISA further confirmed an increase in serum PEDF levels in 20 male patients with alcoholic dependency. The PEDF level was 14.6 ± 1.9 μg/ml on admission and decreased significantly to 8.7 ± 2.3 μg/ml after 8 weeks of abstinence from drink ($p < 0.001$) (Fig. 3).

The serum PEDF levels in nondrinkers and habitual drinkers are shown in Fig. 4. The serum PEDF levels in light habitual drinkers (7.5 ± 2.9 μg/ml) and in heavy habitual drinkers (14.2 ± 7.7 μg/ml) were significantly greater than in nondrinkers (5.5 ± 3.0 μg/ml).

Serum PEDF levels were also measured in subjects with nonalcoholic chronic liver diseases of viral and nonviral origin. The PEDF levels in subjects with chronic nonalcoholic liver diseases were comparable to the PEDF levels in normal subjects without a drinking habit, as indicated in Fig. 5.

Serum PEDF levels of the control subjects younger than 50 yrs were 9.4 ± 7.3 μg/ml and the levels in those older than 50 years were 8.7 ± 5.1 μg/ml, indicating that there are no age-related differences in serum PEDF levels. In a total of 120 patients with nonalcoholic liver diseases, there were no gender-related differences (4.2 ± 2.1 μg/ml in males and 4.5 ± 2.4 μg/ml in females).

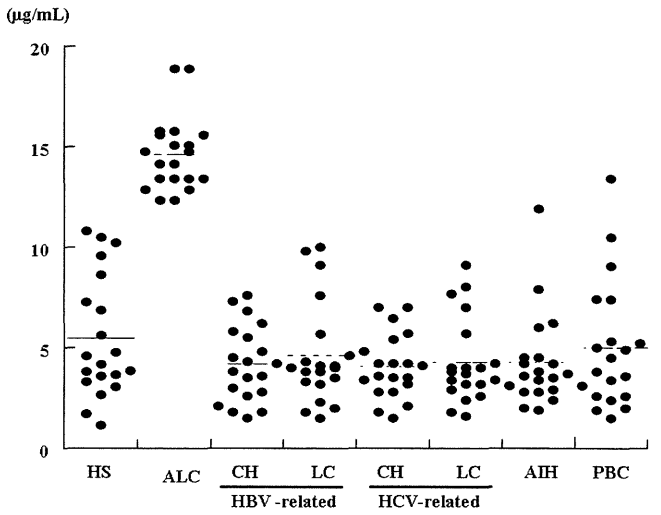

Fig. 5. Serum PEDF levels, as determined by ELISA, in alcoholic patients and in patients with chronic liver diseases of nonalcoholic etiology. The PEDF levels are significantly greater in alcoholic patients than in the healthy subjects. However, the PEDF levels in other patient groups are comparable to those in the healthy controls. AIH, autoimmune hepatitis; ALC, alcohol dependency; CH, chronic hepatitis; ELISA, enzyme-linked immunosorbent assay; HS, healthy subjects; LC, liver cirrhosis; PBC, primary biliary cirrhosis; PEDF, pigment epithelial-derived factor.

DISCUSSION

Recent advances in proteomic technology have provided promising ways to discover and identify novel biomarkers in various fields of clinical medicine. The application of various gel-based and gel-free methods has facilitated the discovery of potential clinical biomarkers, although there has been a long

and uncertain path from marker discovery to clinical utility. We previously discovered a 5.9-kDa peptide as a novel biomarker of alcohol abuse using mass spectrometry–based methods (Nomura et al., 2004; Sogawa et al., 2007). We also used fluorescent two-dimensional difference gel electrophoresis (2D-DIGE) for serum proteome analysis and found a change before and after abstinence in the serum levels of relatively abundant proteins (including alpha 1-antichymotripsin and haptoglobin) in subjects with alcohol abuse (Wu et al., 2007).

A technical challenge in serum proteome analysis has been that serum contains thousands of proteins and peptides that are present in a large dynamic range. Indeed, 22 abundant proteins (e.g., albumin, immunoglobulins, and transferrin) constitute up to 99% of the protein content of plasma (Anderson and Anderson, 2002; Tirumalai et al., 2003). Depletion of these abundant proteins and further fractionation of samples will be necessary in future proteomic studies searching for low-abundant serum proteins or peptides.

We previously used the three-step method and detected 3 proteins, including YKL-50, as promising novel biomarkers of sepsis (Hattori et al., 2009). We assessed reproducibility of the three-step method. To assess between-days precision, 4 aliquots of 40 μl from the same serum samples were subjected to the three-step proteome analyses on 4 different days, and silver-stained SDS–PAGE results of the 4 fractions were compared. As shown in Fig. S1, the between-day differences were minimal. In the present study, we applied the three-step method to search for biomarkers for excessive alcohol drinking. We performed three-step proteome analysis on 2 serum samples collected from each of 8 patients (16 samples in total) with alcohol dependence—one sample was collected on admission and one sample was collected after 8 weeks of abstinence from drink. Three-step serum proteome analysis revealed that the serum levels of 5 proteins—alpha2-HS glycoprotein, apolipoprotein A-I, glutathione peroxidase 3, heparin cofactor II, and PEDF were significantly greater on admission than after 8 weeks of abstinence. On the other hand, serum levels of apolipoprotein III were downregulated on admission. Although the data were shown only for PEDF, the results of Western blotting confirmed the changes in the expression levels of all the 6 proteins by heavy drinking. As alterations of serum levels of apolipoprotein A-I, alpha-2-HS glycoprotein, apolipoprotein C-III, glutathione peroxidase 3, and heparin cofactor II associated excessive alcohol consumption have been reported in the literature (Andersson and Bell, 1988; Kaku et al., 1982; Nanchahal et al., 2000; Peng et al., 2005; Robinson and Quarfordt, 1981), we focused on PEDF, alterations of which by heavy drinking are not well characterized. In the present study, these changes were initially detected by SDS–PAGE, the final step of the three-step proteome analysis. Western blotting and ELISA further confirmed these changes. After patients with alcohol abuse abstained from alcohol for 2 months, their elevated serum PEDF levels (noted on admission) returned to the levels found in the control subjects. This suggests that active and excessive drinking, rather than liver injury per se, could play a role in the upregulation of PEDF.

This notion is partially supported by data showing that the serum PEDF levels in people with nonalcoholic chronic liver diseases, including liver cirrhosis, are comparable to the serum PEDF levels in normal controls without a drinking history. Furthermore, serum PEDF levels in habitual drinkers are significantly greater than serum PEDF levels in nondrinkers.

PEDF is a glycoprotein belonging to the serine protease inhibitor superfamily. It was originally purified from the culture supernatant of retinal pigment epithelial cells as a factor that inhibited vascularization (Leung et al., 1989) and exhibited potent neurosecretory activity for human retinoblastoma cells (Tombran-Tink et al., 1991). The PEDF gene contains 8 exons and 7 introns and is located on chromosome 17p13.1. Its transcript is widely expressed in various tissues such as the eye, brain, spinal cord, skeletal muscle, adipose tissue, liver, and bone (Rychli et al., 2009). PEDF is reportedly also present in human plasma at a concentration of around 5 μg/ml (Petersen et al., 2003), which is very similar to the levels obtained in normal controls without drinking history in the present study.

Serum or plasma PEDF levels have been determined in several pathological conditions. Plasma PEDF levels are significantly elevated in diabetic patients, especially in patients with proliferative diabetic retinopathy (Ogata et al., 2007). Elevated serum levels of PEDF in metabolic syndrome have also been reported (Yamagishi et al., 2006). Matsumoto and colleagues (2004) measured serum PEDF levels in various chronic liver diseases. They reported that serum PEDF levels in patients with liver cirrhosis because of hepatitis C virus are significantly lower than the serum PEDF in controls subjects. These results do not agree with the findings of this study. The reasons for this discrepancy are not clear at the moment. It should be noted, however, that Matsumoto and colleagues used a particular ELISA kit from Chemicon International, which is good for measuring the PEDF level of vitreous fluid, but is reportedly not appropriate for measuring the serum PEDF level (Yamagishi et al., 2006). Indeed, the PEDF levels in the controls in Matsumoto's study were around 6 ng/ml, which is lower by almost three-orders magnitude than the levels obtained in this study (5 μg/ml).

The biological significance of the increase in serum PEDF after excessive alcohol drinking is of great interest. In this context, the antiangiogenic, antitumorigenic, antioxidant, antithrombotic, and anti-inflammatory properties of PEDF have to be considered (Rychli et al., 2009; Tombran-Tink and Barnstable, 2003; Uehara et al., 2004; Yamagishi et al., 2009). It has recently been suggested that PEDF plays a protective role in atherosclerosis and that the antiatherothrombogenic property of PEDF may be a therapeutic target in cardiovascular disease (Rychli et al., 2009; Yamagishi and Matsui, 2010). The reduced risk of fatal coronary diseases in habitual drinkers is well documented, but the underlying

mechanisms are complex (Renaud et al., 2004). It is tempting to speculate that the upregulation of PEDF may (at least partly) play a role in this protective action. Thus, three-step serum proteome analysis reveals that serum PEDF levels are significantly increased after excessive drinking. The exact diagnostic and pathophysiological roles of this phenomenon remain to be investigated.

GRANT SUPPORT

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Silver-stained SDS–PAGE shows the reproducibility of the method. Four aliquots of 40 μl from the same serum sample underwent the three-step method. One-sixteenth of each sample (corresponding to the proteins from 2.5 μl serum) is loaded in each lane. SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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RESEARCH ARTICLE

Proteomic analysis of gingival crevicular fluid for discovery of novel periodontal disease markers

Sachio Tsuchida[1], Mamoru Satoh[1,2,3], Hiroshi Umemura[1,2], Kazuyuki Sogawa[1,2], Yusuke Kawashima[4], Sayaka Kado[3], Setsu Sawai[1,2], Motoi Nishimura[1,2], Yoshio Kodera[2,4], Kazuyuki Matsushita[1,2] and Fumio Nomura[1,2]

[1] Department of Molecular Diagnosis, Graduate School of Medicine, Chiba University, Chiba, Japan
[2] Clinical Proteomics Research Center, Chiba University Hospital, Chiba, Japan
[3] Chemical Analysis Center, Chiba University, Chiba, Japan
[4] Laboratory of Biomolecular Dynamics, Department of Physics, School of Science, Kitasato University, Minato, Tokyo, Japan

The protein composition of gingival crevicular fluid (GCF) may reflect the pathophysiology of periodontal diseases. A standard GCF proteomic pattern of healthy individuals would serve as a reference to identify biomarkers of periodontal diseases by proteome analyses. However, protein profiles of GCF obtained from apparently healthy individuals have not been well explored. As a step toward detection of proteomic biomarkers for periodontal diseases, we applied both gel-based and gel-free methods to analyze GCF obtained from healthy subjects as compared with supragingival saliva. To ensure optimized protein extraction from GCF, a novel protocol was developed. The proteins in GCF were extracted with high yield by urea buffer combined with ultrafiltration and the intensity of spots with supragingival saliva and GCF was compared using agarose two-dimensional electrophoresis. Eight protein spots were found to be significantly more intense in GCF. They included superoxide dismutase 1 (SOD1), apolipoprotein A-I (ApoA-I), and dermcidin (DCD). Moreover, GCF proteins from healthy subjects were broken down into small peptide fragments and then analyzed directly by LC-MS/MS analysis. A total of 327 proteins including ApoA-I, SOD1, and DCD were identified in GCF. These results may serve as reference for future proteomic studies searching for GCF biomarkers of periodontal diseases.

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Biomedicine / Gingival crevicular fluid / Periodontal disease

1 Introduction

Periodontal disease is characterized by destruction of hard tissue and soft connective tissue constituents of the periodontium [1]. Better physiological understanding of periodontal disease would improve the diagnosis, treatment, and prevention of periodontitis [2]. Recent studies suggested that C-reactive protein (CRP) and other systemic markers of inflammation were elevated in periodontal diseases and effective periodontal therapy may decrease CRP values [3–6]. Periodontal disease is an inflammatory lesion initiated by gram-negative periodontal bacterial pathogens such as *Porphyromonas gingivalis* [7]. Therefore, gingival keratinocytes in periodontium are the primary line of defense against bacterial infection.

The components of gingival crevicular fluid (GCF) may include breakdown products of host epithelial and connective tissues, products of host cell in the periodontium, and products derived from the subgingival microbial plaque [8–10]. These factors could be possible candidates for predictors of disease activity. A variety of proteins detected in GCF play a central part in periodontal tissue turnover. Consequently,

Correspondence: Dr. Fumio Nomura, Department of Molecular Diagnosis (F8), Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan
E-mail: fnomura@faculty.chiba-u.jp
Fax: +81-43-226-2169

Abbreviations: **ApoA-I**, apolipoprotein A-I; **AU**, arbitrary units; **CRP**, C-reactive protein; **DCD**, dermcidin; **GCF**, gingival crevicular fluid; **MMP**, matrix metalloproteinase; **SOD1**, superoxide dismutase 1; **WB**, Western blotting

analysis of a biochemical marker in GCF may help in diagnosis as it also predicts the progression of periodontal disease [11]. Therefore, investigations to detect diagnostic markers of periodontal diseases are warranted.

Recently, many studies have applied expression proteomics to identify proteins whose abundance levels were altered by disease. Proteomic analysis is a novel method for detecting biomarkers in tumors and inflammatory diseases. Approaches based on a combination of 2DE and MS analysis for the identification of proteins in a variety of inflammatory diseases and cancer were demonstrated. The technique of proteomic analysis using 2DE and MS has the ability to monitor changes that occur in the protein complement of tissues and subcellular compartments. We have shown that the agarose 2DE method had advantages over the conventional 2DE in that they have a higher loading capacity than 2DE with IPG gel for IEF [12]. This agarose 2DE not only allows for large-scale quantitative comparison of protein but also is able to resolve high molecular mass protein larger than 150 kDa [13].

Compared to other biofluids, such as serum/plasma, urine, and cerebrospinal fluid, the protein profiles of GCF have not yet been explored. The previous report by Bostanci et al. was based on the gel-free method alone [14]. Since diagnostic values of biomarker candidates discovered by proteome analyses are always evaluated in comparison with normal subjects, a standard GCF proteomic pattern from healthy individuals would serve as a reference.

As a step toward future biomarker discovery, we conducted both gel-based and gel-free proteomic analysis of GCF obtained from apparently healthy subjects as compared with supragingival saliva.

2 Materials and methods

2.1 Extraction of GCF

GCF samples were collected from 16 volunteers (ten males, six females, the mean age of 36.0 years). Prior to sample collection, the subjects rinsed their mouths. All volunteers provided informed consent to participate in the procedures. All were in good general health with no history of antimicrobial or antiinflammatory therapy or periodontal treatment for 6 months before the start of the study. Since smoking is a risk factor for periodontal disease, the current study did not include smokers. Clinical parameters such as probing depth, clinical attachment level, gingival bleeding index, full-mouth plaque scores, and bleeding scores of each group were recorded. The probing depth at six sites for each tooth was measured using the PCP-UNC15 probe (Hu-Friedy, Chicago, IL, USA). For recording the loss of clinical attachment level, the cemento-enamel junction was used as a reference. Participants were first examined for periodontal disease. Full-mouth Löe–Silness gingival index and clinical attachment loss were measured at six sites (mesio-, mid-, disto-buccal/palatal, or lingual) per tooth using the PCP-UNC15 probe with a diameter of 0.5 mm at the tip [15, 16]. The periodontal disease subjects were assigned to three groups (moderate, mild, and severe) on the basis of overall clinical diagnostic criteria and the classification of periodontal diseases by the American Academy of Periodontology (AAP) 1999 [17] (Table 1). The study sample included in this study was as follows: healthy; $n = 50$, mild periodontal disease; $n = 30$, moderate periodontal disease; $n = 30$, severe periodontal disease; $n = 50$.

GCF was collected from the labial side of maxillary incisors without crown and restoration. Supra-gingival plaque was carefully removed from the tooth by a curette, the teeth were rinsed with saline, and the sampling sites were isolated with cotton rolls and dried. Each sample site was carefully isolated using cotton rolls to avoid saliva contamination. Absorbent paper points (ZIPPERER®, Munich, Germany) were gently inserted into the gingival crevice and left in place for 30 s. Mechanical irritation was avoided and the absorbent paper points contaminated with blood were discarded. Theses paper points were stored at −80°C for further processing.

2.2 Sample preparation

A mixture of 7 M urea and 2 M thiourea including 2% CAPS and 2 mM DTT was added to GCF in paper points. The paper points were vortexed for 10 min and centrifuged at 20 000 × g for 15 min. After centrifugation, paper points were ultrafiltered using Ultrafree-MC (Millipore, Bedford, MA). The protein concentration of the extract was estimated by the method of Bradford, with bovine serum albumin as a standard.

2.3 2DE

GCF samples from five healthy subjects were applied to agarose IEF gel, and first-dimensional IEF was conducted at 12 000 Vh at 4°C. This was followed by fixation in 10% TCA and 5% sulfosalicylic acid for 45 min at room temperature. After washing in deionized water for 45 min, the agarose gel was transferred to 10–20% gradient of polyacrylamide gel and 2DE was performed. The protein spots on the 2DE gels were stained with silver (2-D silver stain II "DAIICHI," Daiichi Pure Chemicals Co., Ltd., Osaka, Japan).

2.4 SDS-PAGE and Western blot analysis

A total of 10 μg/μL GCF protein was separated by SDS-PAGE on a 10–20% polyacrylamide gradient gel and transferred to PVDF membranes (Millipore, Bedford, MA) in a Bio-Rad Trans-Blot apparatus (Bio-Rad, Hercules, CA). The membranes were blocked at room temperature in PBS with pH 7.5, containing 5% skim milk and 0.05% Tween 20. Anti-Dermicidin mouse monoclonal antibody (G-81: Santa

Table 1. Clinical parameters

Group	PPD (mm)	CAL (mm)	GI	FMPS (%)	FMBS (%)
Healthy ($n = 5$)	1.2 ± 0.2	1.3 ± 0.3	0	38.6 ± 23.5	8.6 ± 20.5
Mild periodontal disease ($n = 3$)	2.7 ± 0.2*	2.9 ± 0.4*	0.7 ± 0.2*	48.8 ± 25.7	46.7 ± 22.5*
Moderate periodontal disease ($n = 3$)	4.7 ± 0.3*	4.6 ± 0.2*	1.3 ± 0.2*	51.7 ± 35.6	61.8 ± 33.7*
Severe periodontal disease ($n = 5$)	7.5 ± 0.3*	7.8 ± 0.3*	1.5 ± 0.3*	60.8 ± 33.5	70.9 ± 23.4*

PPD, probing depth; CAL, clinical attachment level; GI, gingival index; FMPS, full-mouth plaque scores; FMB, full-mouth bleeding scores. The asterisk indicates significant difference from the healthy group (Mann–Whitney *U*-test, $p < 0.05$).

Cruz Biotechnology Inc., Santa Cruz, CA) diluted 1:1000, anti-Apolipoprotein A-I mouse monoclonal antibody (5F4: Cell Signaling Technology, MA) diluted 1:1000, and anti-Superperoxidase 1 rabbit polyclonal antibody in blocking buffer were used as primary antibodies diluted 1:1000 with the same buffer, and the membranes were incubated in it overnight at 4°C. Subsequently, the membranes were washed three times in phosphate-buffered saline (PBS) containing 0.05% Tween 20 and incubated for 1 h with goat anti-mouse IgG HRP (Bio-Rad Laboratories, Hercules, CA) diluted 1:2000, and goat anti-rabbit IgG HRP (Bio-Rad Laboratories, Hercules, CA) diluted 1:2000 in blocking buffer used as secondary antibodies. The bands were visualized by use of ECL (GE Healthcare UK Ltd., Buckinghamshire, UK) Band intensities were quantified using TotalLab TL12 imaging analysis software (Shimadzu Co., Ltd. Kyoto, Japan) and were represented by arbitrary units (AU).

2.5 In-gel digestion of proteins

The gel was cut into small pieces, destained in 50% ACN/50 mM NH_4HCO_3, and washed with deionized water. The gel pieces were dehydrated in 100% ACN for 15 min, and then dried in a SpeedVac Evaporator (Wakenyaku, Kyoto, Japan) for 45 min. The gel pieces were rehydrated in 10–30 μL of 25 mM Tris-HCl/20% CAN containing 25 ng/μL trypsin (Trypsin sequence grade, Roche) for 45 min. After removal of the unabsorbed solution, the gel pieces were incubated in 10–20 μL of 50 mM Tris-HCl/20% ACN for 20 h at 37°C. The solution containing digested fragments of proteins was transferred to a new tube, and the peptide fragments remaining in the gel were extracted in 5% formic acid/50% ACN for 20 min at room temperature.

2.6 In-solution digestion of proteins

A mixture of 4 M urea and 100 mM ammonium bicarbonate were added to the paper point containing GCF. The paper point was vortexed for 10 min and centrifuged at 20 000 × g for 15 min. Then, 2 μL of 200 mM DTT added and incubated at 57°C for 30 min. After incubation, 2 μL of 600 mM iodacetoamide was added and incubated at room temperature for 30 min in dark. Five microliters of trypsin with 26 μL of distilled water was incubated at 37°C for 6 h. The resulting peptides were added to 5 μL of 5% TFA or 5% formic acid.

2.7 Protein identification

In-gel digested peptides were injected into a trap column: 0.3 × 5 mm L-trap column (Chemicals Evaluation and Research Institute, Saitama, Japan), and an analytical column: 0.1 × 50 mm Monolith column (AMR, Tokyo, Japan), which was attached to a HPLC system (Nanospace SI-2; Shiseido Fine Chemicals, Tokyo, Japan). The flow rate of the mobile phase was 1 μL/min. The solvent composition of the mobile phase was programed to change in 35-min cycles with varying mixing ratios of solvent A (2% v/v CH_3CN and 0.1% v/v HCOOH) to solvent B (90% v/v CH_3CN and 0.1% v/v HCOOH): 5–50% B 20 min, 50–95% B 1 min, 95% B 3 min, 95–5% B 1 min, 5% B 10 min. Purified peptides were introduced from HPLC to an LTQ-XL (Thermo Scientific, CA, USA), an ion trap mass spectrometer (ITMS), via an attached Pico Tip (New Objective, MA, USA). The MS and MS/MS peptide spectra were measured in a data-dependent manner according to the manufacturer's operating specifications. The Mascot search engine (Matrix science, London, UK) was used to identify proteins from the mass and tandem mass spectra of peptides. Peptide mass data were matched by searching the Human International Protein Index database (IPI, July 2008, 72 079 entries, European Bioinformatics Institute) using the MASCOT engine. The minimum criterion of the probability-based MASCOT/MOWSE score was set with 5% as the significant threshold level.

In-solution digested peptides were injected into a trap column (C_{18}, 0.3 × 5 mm, DIONEX, CA, USA), and an analytical column (C_{18}, 0.075 × 120 mm, Nikkyo Technos, Tokyo, Japan), which was attached to the Ultimate 3000 (DIONEX, CA, USA). The flow rate of the mobile phase was 300 nL/min. The solvent composition of the mobile phase was programed to change in 120-min cycles with varying mixing ratios of solvent A (2% v/v CH_3CN and 0.1% v/v HCOOH) to solvent B (90% v/v CH_3CN and 0.1% v/v HCOOH): 5–10% B 5 min, 10–13.5% B 35 min, 13.5–35% B 65 min, 35–90% B 4 min, 90% B 0.5 min, 90–5% B 0.5 min, 5% B 10 min. Purified peptides were introduced from HPLC to LTQ-Orbitrap XL (Thermo Scientific, San Jose, CA, USA), a hybrid ion-trap

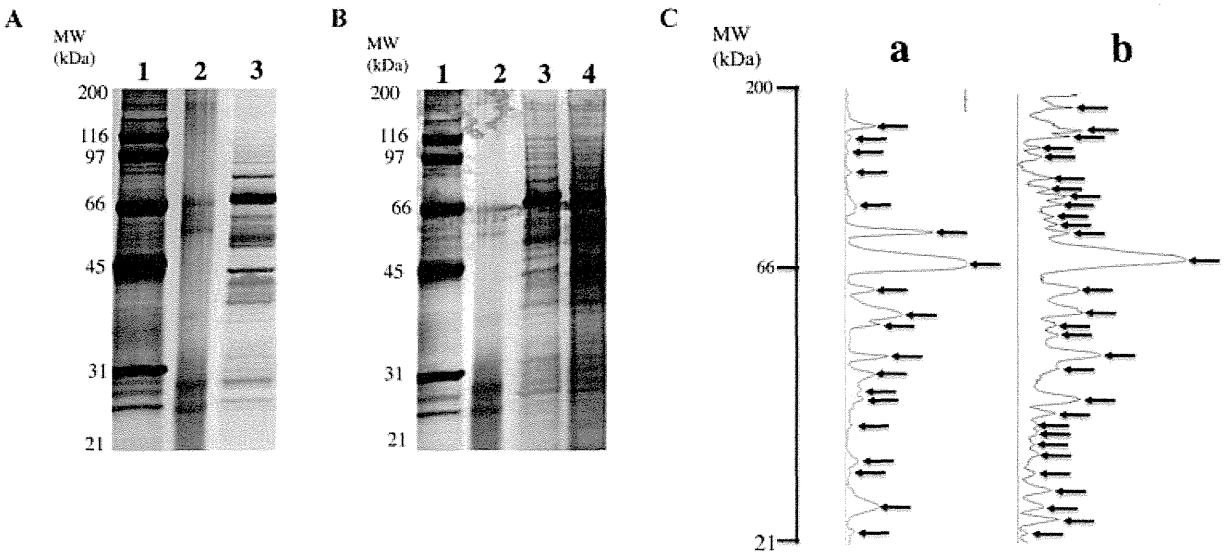

Figure 1. SDS-PAGE gel of GCF proteins extracted by PBS buffer and urea buffer combined with ultrafiltration and stained with Silver. (A) Five microgram of protein was loaded/lane. Lane 1, molecular weight (MW) standard; Lane 2, peptide standard; Lane 3, PBS buffer extractions. Lane 1, MW standard; Lane 2, peptide standard; Lane 3, urea buffer extractions and Lane 4, urea buffer combined with ultrafiltration extractions (B). (C) Densitometric analysis of the gel shown in panels A and B. One peak (arrows) corresponds to one protein band. Lane 3 in panel A corresponds to A. Lane 4 in panel B corresponds to B.

Fourier transform mass spectrometer. The MASCOT search engine (version 2.2.6, Matrixscience, London, UK) was used to identify proteins from the mass and tandem mass spectra of peptides. Peptide mass data were matched by searching the UniProtKB database (SwissProt 2010x, November 2010, 9590 entries). Database search parameters were: peptide mass tolerance, 1.2 Da; fragment tolerance, 0.6 Da; enzyme was set to trypsin, allowing up to one missed cleavage; variable modifications, methionine oxidation. The minimum criteria of protein identification were set as false discovery rate (FDR) $< 1\%$. The FDR was estimated by searching against a randomized decoy database created by the Mascot Perl program supplied by Matrix Science (London, UK).

2.8 Statistical analysis

The differences in values between periodontal and healthy subjects were analyzed by Mann–Whitney *U*-test and Kruskal–Wallis test with the Dwass–Steel–Chritchlow–Flinger method. Differences at $p < 0.05$ were considered statistically significant.

3 Results

3.1 Comparison of two different protein extraction methods

To develop an optimized protein extraction method, either PBS buffer or urea buffer combined with ultrafiltration was employed as an extraction protocol in the first step of this study. We evaluated the extraction ability of the two methods used to extract proteins from GCF by SDS-PAGE, as shown in Fig. 1A and B. Nineteen protein bands (A: marked by arrowheads) were observed in the PBS buffer method and 30 protein bands (B: marked by arrowheads) in the urea buffer combined with ultrafiltration (Fig. 1C). Greater amounts of extracted proteins were obtained using urea buffer combined with ultrafiltration. Proteins in the GCF were extracted with high yield by urea buffer treatment followed by ultrafiltration using an ultrafiltration device.

3.2 Identification of altered expressed protein in GCF

To search for novel GCF markers useful for the diagnosis of periodontal disease, we compared the protein spots of GCF with supragingival saliva using agarose 2DE analysis. Hundred micrograms total protein of GCF and supragingival saliva were separated by agarose 2DE, and proteins were visualized by Silver-staining (Fig. 2A and B). These spots were detected and quantitated with imaging analysis software, and then statistical analysis was performed across the 10 gels. We carefully compared the 2D image with Silver-staining gel and picked altered protein spots manually. When comparing the intensity of spots between supragingival saliva and GCF, eight protein spots were found to be significantly more intense in GCF. In contrast, no protein spots were more intense in supragingival saliva. The proteins in which the expression levels were different are shown in respective gels (Fig. 2A and B).

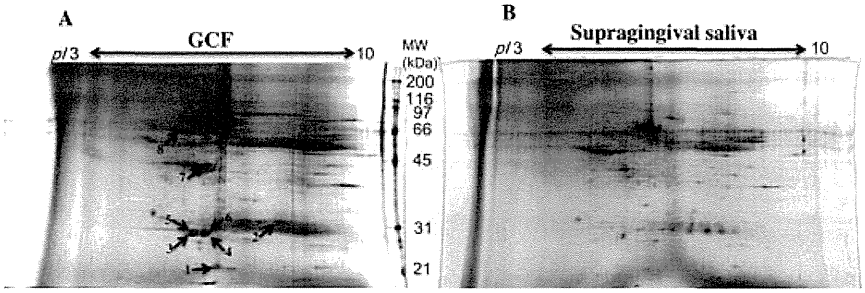

Figure 2. Proteome analysis of GCF and supragingival saliva using agarose 2DE. Conventional agarose 2DE patterns were visualized by Silver-staining. Eight increased protein spots in GCF are displayed with arrows. Eight protein spots cut from this gel were identified by LC-MS/MS and are shown in arrows (increased protein spots).

The increased eight proteins spots in the GCF were cut out from the conventional agarose 2DE gel and were subjected to in gel digestion, followed by LC-MS/MS analysis. They included Haptoglobin, Superoxide dismutase 1(SOD1), ALB protein, Apolipoprotein A-I (ApoA-I), and Dermcidin (DCD) (Table 2).

3.3 Liquid digestion and LC-MS/MS analysis

Whole proteins were enzymatically digested into small peptide fragments with uniform characteristics and then analyzed directly by LC-MS/MS analysis, so-called shotgun proteomics. Shotgun proteomics have been widely used for detection of disease markers, for diseases such as cancer and systemic inflammation. GCF samples from five healthy subjects were pooled and subjected to trypsin digestion, followed by identification by LC-MS/MS. Three hundred twenty-seven proteins including ApoA-I, SOD1, and DCD were identified in GCF by LC-MS/MS analysis. Other identified proteins include cathelicidin, cathepsin G, cystatin-C, interleukin-1 receptor antagonist protein, Lysozyme C, macrophage migration inhibitory factor, matrix metalloproteinase-8, -9 (MMP-8,-9), metalloproteinase inhibitor 1(TIMP-1), neutrophil defensin 1, and vitamin D-binding protein, whose

Table 2. LC-MS/MS identification of increased protein spots on 2DE gels that are differently expressed in GCF

Spot number[a)]	Protein name	MW[b)]	p*I*[c]	Score[d]	Sequence coverage[e)]	MS/MS[f)] (unique)	Fold increase[g)]
1	HP Haptoglobin precorsor	46 693	6.28	118	7%	4	1.58
	SOD1 Superoxide dismutase	15 926	5.7	61	9%	1	
2	ALB ALB protein	45 130	5.77	75	3%	1	1.51
	IGL@ IGL@ protein	24 777	5.93	52	8%	1	
3	APOA1 Apolipoprotein A-I precursor	30 759	5.56	557	50%	12	1.77
	APOA1 Apolipoprotein A-I precursor	30 759	5.56	713	41%	11	
4	IGK@ IGK@ protein	25 757	5.94	204	28%	4	1.77
	DCD Dermcidin precursor	11 277	6.08	74	10%	1	
5	APOA1 Apolipoprotein A-I precursor	30 759	5.56	571	41%	11	1.73
6	APOA1 Apolipoprotein A-I precursor	30 759	5.56	392	43%	11	1.75
	IGKV1-5 IGKV1-5 protein	26 218	6.3	136	15%	2	
7	SERPINA1 Isoform 1 of Alpha-1-antitrypsin precursor	46 707	5.37	241	18%	7	1.62
	IGHV3OR16-13	53 054	6.46	136	8%	3	
8	ALB Uncharacterized protein ALB	71 658	6.33	151	9%	6	1.61

a) Protein spots were described in Fig 2.
b) Theoretical molecular mass (Da) based on NCBI BLAST database.
c) Theoretical p*I* based on Swiss-Prot database.
d) SEQUEST score of candidate proteins.
e) Sequence coverage of MS/MS analysis of protein.
f) MS/MS unique peptide.
g) Intensity of spots in 2D-PAGE gel of 10 matched samples (GCF versus supragingival saliva) was measured.

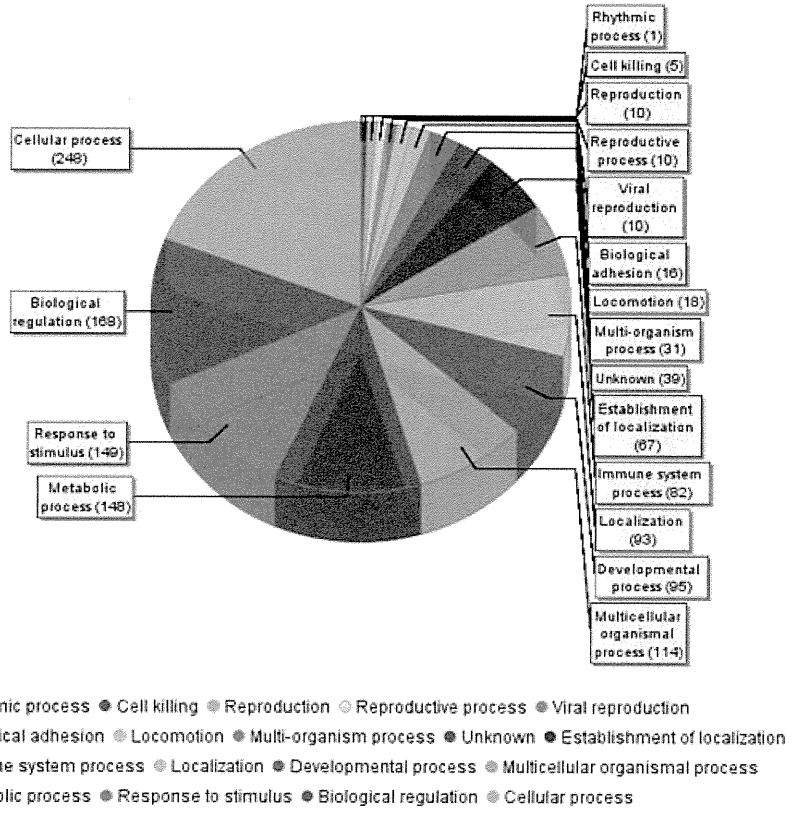

Figure 3. Functional annotation of GCF from healthy subjects using Gene Ontology Annotation Database. Identified proteins have immune system, metabolic function, response to stimulus, and biological regulation.

expression have been previously reported to be related to periodontal disease [18–26].

On the other hand, they included Annexin A5, Annexin A6, Interleukin-36 gamma (IL-36γ), lamin A/C, periplakin, moesin, which are associated with tumors and systemic diseases in human (Supporting Information Table S1).

Figure 3 shows the functional annotation of GCF from healthy subjects using Gene Ontology Annotation Database. Identified proteins in GCF have biological regulation (168 proteins), response to stimulus (149 proteins), metabolic process (148 proteins), and immune system (82 proteins). Figure 4 shows the distribution of proportion of GCF proteins according to their source of origin in healthy subjects. Proteins from bacteria, firmicutes, yeast, and virus are present in the GCF (Table 3). MS/MS Unique peptide of Elongation factor 1-alpha 1 from *Saccharomyces cerervisiae* matched the MS/MS unique peptide from human alone. Moreover, proteins from periodontal pathogenic bacteria such as *P. gingivalis* or *Actinobacillus actinomycetemcomitans* were not detected.

Further investigations are necessary to evaluate whether these proteins in GCF may have a role in diagnosis of various periodontal diseases.

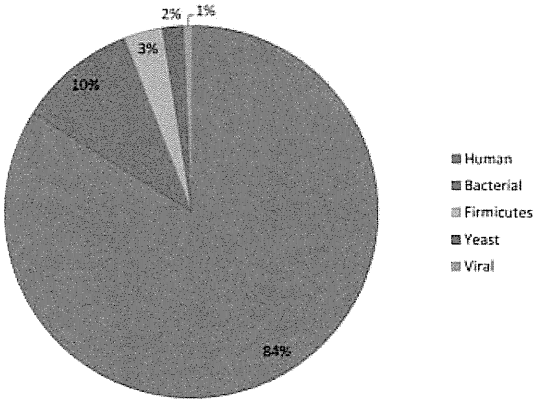

Figure 4. Distribution of the proportion of GCF proteins according to their source of origin in healthy subjects. The proportions are expressed as percentage (%) of the total protein in healthy subjects. Five sources of origin were identified: human, bacterial, firmicutes, yeast, viral.

Table 3. LC-MS/MS identification of GCF proteins derived from nonhuman organisms

Organism	Identified proteins	Accession number[a)]	Molecular weight[b)]	MS/MS unique peptide[c)]	MS/MS unique peptide (HUMAN)[d)]
Bacterial					
Acidovorax ebreus	Methionyl-tRNA synthetase	SYM_ACIET (+1)	77 kDa	1	0
Acinetobacter sp.	Malonyl-CoA O-methyltransferase BioC	BIOC_ACIAD	Unknown	1	0
Acinetobacter sp.	Urease subunit gamma	URE3_ACIAD (+6)	11 kDa	1	0
Agrobacterium rhizogenes	Putative replication protein C	REPC_AGRRH	44 kDa	1	0
Anaeromyxobacter dehalogenans	Methionyl-tRNA formyltransferase	FMT_ANAD2 (+2)	33 kDa	1	0
Blochmannia pennsylvanicus	Glucose-6-phosphate isomeras	G6PI_BLOPB	63 kDa	1	0
Bordetella avium	Serine hydroxymethyltransferase	GLYA_BORA1 (+1)	45 kDa	1	0
Bordetella pertussis	Cyclolysin secretion/processing ATP-binding protein CyaB	CYAB_BORPE	78 kDa	1	0
Bradyrhizobium japonicum	Protein slyX homolog	SLYX_BRAJA	8 kDa	1	0
Bradyrhizobium japonicum	Cytochrome c-type biogenesis protein CycH	CYCH_BRAJA	40 kDa	1	0
Buchnera aphidicola subsp. Acyrthosiphon pisum	Isoleucyl-tRNA synthetase	SYI_BUCA5 (+3)	110 kDa	1	0
Buchnera aphidicola subsp. Baizongia pistaciae	DNA-directed RNA polymerase subunit beta	RPOC_BUCBP	157 kDa	1	0
Buchnera aphidicola subsp. Cinara cedri	50S ribosomal protein L2	RL2_BUCCC	31 kDa	1	0
Coxiella burnetii	Elongation factor Tu	EFTU_COXBN (+15)	44 kDa	1	0
Delftia acidovorans	Probable ubiquinone biosynthesis protein UbiB	UBIB_DELAS	60 kDa	1	0
Desulfobacterium autotrophicum	Threonyl-tRNA synthetase	SYT_DESAH	72 kDa	1	0
Desulfococcus oleovorans	Alanine racemase	ALR_DESOH	41 kDa	1	0
Dichelobacter nodosus	Chaperone protein DnaK	DNAK_DICNV (+2)	70 kDa	1	0
Enterobacter aerogenes	Glyceraldehyde-3-phosphate dehydrogenase	G3P_ENTAE	31 kDa	1	0
Erwinia tasmaniensis	Triosephosphate isomerase	TPIS_ERWT9	27 kDa	1	0
Escherichia coli	Transcriptional activator perA	PERA_ECO27	32 kDa	1	0
Geobacter bemidjiensis	Threonyl-tRNA synthetase	SYT_GEOBB (+3)	72 kDa	1	0
Geobacter sp.	Dihydroorotase	PYRC_GEOSF (+1)	45 kDa	1	0
Helicobacter pylori	Uncharacterized protein jhp_0176	Y190_HELPJ	58 kDa	1	0
Lawsonia intracellularis	Ribonuclease Y	RNY_LAWIP	Unknown	1	0
Magnetococcus sp.	Protein translocase subunit SecF	SECF_MAGSM	Unknown	1	0
Magnetococcus sp.	DNA replication and repair protein recF	RECF_MAGSM	42 kDa	1	0
Methylobacterium nodulans	Chaperone protein DnaK	DNAK_METNO	69 kDa	1	0

Table 3. Continued

Organism	Identified proteins	Accession number[a)]	Molecular weight[b)]	MS/MS unique peptide[c)]	MS/MS unique peptide (HUMAN)[d)]
Methylocella silvestris	ATP synthase subunit delta	ATPD_METSB	20 kDa	1	0
Novosphingobium aromaticivorans	N-Succinylglutamate 5-semialdehyde dehydrogenase	ASTD_NOVAD	51 kDa	1	0
Pseudomonas mendocina	Adenosylhomocysteinase	SAHH_PSEMY	51 kDa	1	0
Psychromonas ingrahamii	Phospho-N-acetylmuramoyl-pentapeptide-transferase	MRAY_PSYIN	40 kDa	1	0
Rhodobacter sphaeroides	Phosphoglycerate kinase	PGK_RHOS5	41 kDa	1	0
Rickettsia bellii	Aconitate hydratase	ACON_RICBR (+2)	98 kDa	1	0
Salmonella agona	Flagellar P-ring protein	FLGI_SALA4 (+13)	38 kDa	1	0
Serratia proteamaculans	Alanyl-tRNA synthetase	SYA_SERP5	96 kDa	1	0
Shewanella oneidensis	Chemotaxis response regulator protein-glutamate methylesterase of group 1 operon	CHEB1_SHEON	39 kDa	1	0
Vibrio vulnificus	3-Octaprenyl-4-hydroxybenzoate carboxy-lyase	UBID_VIBVY	69 kDa	1	0
Wolbachia pipientis	Chromosomal replication initiator protein dnaA	DNAA_WOLPM	53 kDa	1	0
Xylella fastidiosa	Glycyl-tRNA synthetase beta subunit	SYGB_XYLF2 (+3)	80 kDa	1	0
Firmicutes					
Bacillus anthracis	Enolase	ENO_BACAA (+15)	46 kDa	1	0
Bacillus subtilis	Probable glucarate dehydratase	GUDH_BACSU	51 kDa	1	0
Clostridium kluyveri	DNA ligase	DNLJ_CLOK1 (+11)	75 kDa	1	0
Clostridium tetani	Probable 2-phosphosulfolactate phosphatase	COMB_CLOTE	26 kDa	1	0
Enterococcus faecalis	ATP-dependent helicase/ deoxyribonuclease subunit B	ADDB_ENTFA	139 kDa	1	0
Lactobacillus acidophilus	UPF0348 protein LBA1527	Y1527_LACAC	44 kDa	1	0
Listeria innocua	Phosphoribosylamine–glycine ligase	PUR2_LISIN	46 kDa	1	0
Oceanobacillus iheyensis	Chaperone protein DnaK	DNAK_OCEIH	67 kDa	1	0
Staphylococcus aureus	Conserved virulence factor B	CVFB_STAA3 (+8)	34 kDa	1	0
Streptococcus pyogenes serotype M1	Elongation factor Tu	EFTU_STRP1 (+12)	44 kDa	1	0
Streptococcus suis	Ribosomal RNA small subunit methyltransferase H	RSMH_STRS2 (+4)	36 kDa	1	0

Table 3. Continued

Organism	Identified proteins	Accession number[a)]	Molecular weight[b)]	MS/MS unique peptide[c)]	MS/MS unique peptide (HUMAN)[d)]
Symbiobacterium thermophilum	Chorismate synthase	AROC_SYMTH	42 kDa	1	0
Thermoanaerobacter tengcongensis	Triosephosphate isomerase	TPIS_THETN	27 kDa	1	0
Yeast					
Geobacter sp.	Dihydroorotase	PYRC_GEOSF (+1)	45 kDa	1	0
Saccharomyces cerevisiae	Elongation factor 1-alpha 1	EF1A1_HUMAN (+3)	50 kDa	2	2
Saccharomyces cerevisiae	Phosphatidylglycerol phospholipase C	PGC1_YEAST	unknown	1	0
Saccharomyces cerevisiae	Malate dehydrogenase, mitochondrial	MDHM_YEAST	36 kDa	1	0
Saccharomyces cerevisiae	Ras-related protein SEC4	SEC4_YEAST	24 kDa	1	0
Saccharomyces cerevisiae	Histone H2B.1	H2B1_YEAST (+1)	14 kDa	1	0
Saccharomyces cerevisiae	Heat shock protein SSC1, mitochondrial	HSP77_YEAST	71 kDa	1	0
Virus					
Acanthamoeba polyphaga mimivirus	Uncharacterized protein R292	YR292_MIMIV	22 kDa	1	0
Carnation etched ring virus	Virion-associated protein	VAP_CERV	unknown	1	0
Infectious salmon anemia virus	Fusion glycoprotein F0	FUS_ISAVC	49 kDa	1	0

GCF proteins from healthy subjects ($n = 5$) were identified using LC-MS/MS. Identified proteins are listed.
a) Reference for protein identification.
b) Theoretical molecular mass based on Swiss–Prot database.
c) MS/MS unique peptide.
d) MS/MS unique peptide (human database).

3.4 Expression levels of ApoA-I, SOD1, and DCD in healthy subjects and patients with periodontal disease

Among the identified proteins, we looked at the proteins identified by the two methods (gel-based and gel-free) in common and also focused on disease-associated proteins, whose expression levels have not been well studied in periodontal disease. Three proteins, namely SOD1, DCD, ApoA-I met the above conditions.

ApoA-I is the major protein component of high-density lipoprotein (HDL) in plasma. ApoA-I has a specific role in lipid metabolism [27]. The superoxide dismutases (SOD) are the most important line of antioxidant enzyme defense system against reactive oxygen species (ROS). SOD1, one of the human SOD, is also known as superoxide dismutase [Cu-Zn] [28]. DCD was identified as a gene for an antimicrobial peptide DCD-1 in human sweat glands. This peptide has antimicrobial function and a range of biological functions [29].

Western blot analyses of ApoA-I, SOD1, and DCD were performed to compare the expression levels of these proteins in GCF obtained from healthy controls and those from subjects with periodontal disease. The GCF in severe periodontal disease group exhibited higher expression of SOD1 and DCD than the healthy control group ($p = 0.0131$, Fig. 5B, $p = 0.0162$, Fig. 5C). However, the expression level of ApoA-I did not differ between the two groups (Fig. 5A).

4 Discussion

As a fluid lying in close proximity to periodontal tissue, GCF is the principal target in biomarker search for periodontal diseases. However, it is not an easy task to collect GCF samples from periodontal pockets during the active phase of periodontitis without any bleeding. GCF samples are usually collected by periopaper strips. In this study, we collected GCF samples using absorbent paper points. Paper points are widely used for the collection of subgingival plaque or other oral samples to analyze the microbes especially the presence of periodontal pathogenic bacteria [30]. In addition, the very apical portions of the periodontal pocket are accessible, as the niche of front-line species. This procedure using paper points could minimize bleeding from periodontal pocket during GCF sample collection.

Conventionally, protein extraction from GCF in periodontal disease research has been performed with PBS buffer alone [14, 31]. However, we suspected that the capacity of PBS buffer alone was not enough to remove substantial amount of proteins from paper points used for GCF collection. Therefore, as the first step toward biomarker discovery for periodontal diseases, we developed a novel extraction protocol and found that use of urea buffer combined with ultrafiltration with Ultrafree-MC was satisfactory as indicated in Fig. 1. Ultrafiltration is the process of separating ex-

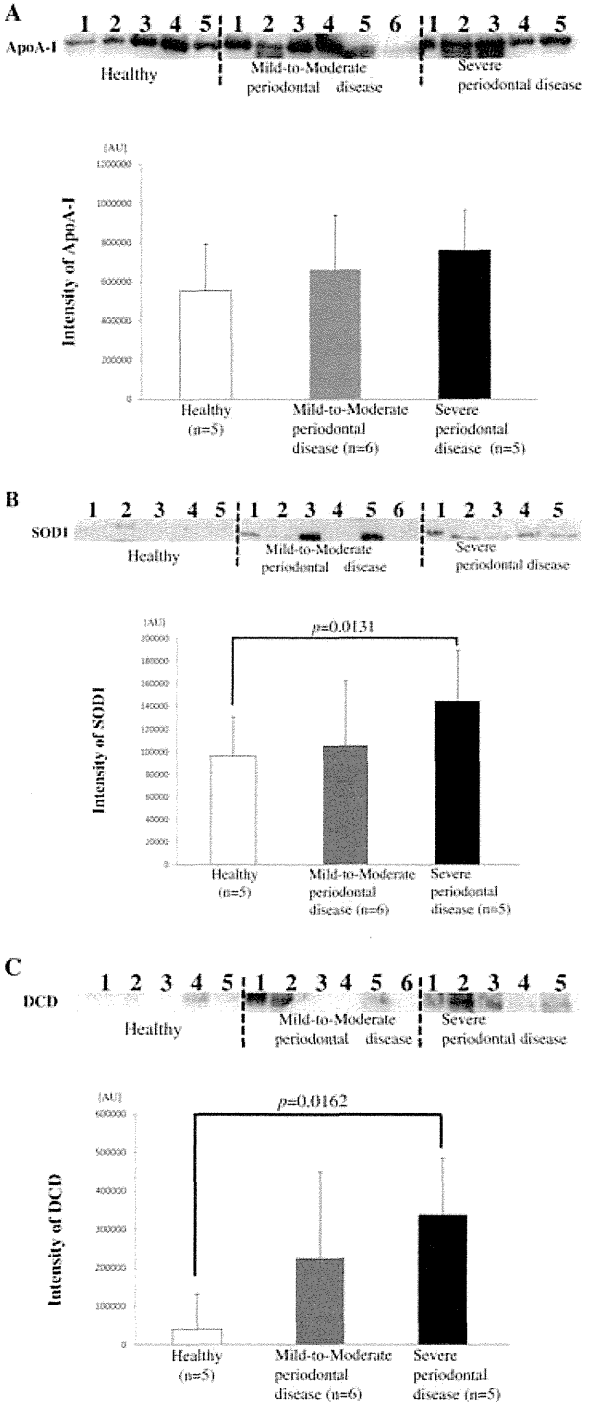

Figure 5. Western blot analysis of ApoA-I, SOD1, and DCD in GCF. Total protein prepared from samples from healthy subjects and mild-to-moderate and severe periodontal patients were separated by electrophoresis on 10% to 20% polyacrylamide gradient gel, and immunoblotted with anti-ApoA-I antibody (A), anti-SOD1 antibody (B), and anti-DCD antibody (C). The intensity of each band was measured using imaging analysis. Data are expressed as the mean ± SD. The SOD1 and DCD band volumes were significantly increased in the severe periodontal disease group when compared with those of the control group ($p < 0.05$).

tremely small particles and dissolving molecules from samples. Ultrafree-MC is available in a range of microporous membranes (http://www.millipore.com/ catalogue/module/c7554) for removing particulates from solutions and from ultrafiltration membranes for protein concentration as well as protein removal prior to HPLC. These methods could remove substantial amount of proteins from paper points and could serve as effective pretreatment of GCF when used as a source for discovery of diagnostic markers for periodontal diseases.

In this study, low-abundance proteins in GCF samples from healthy subjects were separated by agarose 2DE method. The agarose 2DE has a higher loading capacity than the conventional 2DE with IPG gel for IEF [12]. Therefore, the agarose 2DE method would be preferable when GCF is present in relatively small amounts in the body. GCF contains substances from the host as well as those from microorganisms in the subgingival and supragingival plaque. A standard GCF proteomic pattern of healthy individuals would serve as a reference to search for biomarkers of periodontal diseases by proteome analyses. However, protein profiles of GCF obtained from apparently healthy individuals have not been well explored. As a second step, we applied both gel-based and gel-free methods to analyze GCF obtained from healthy subjects as compared with supragingival saliva. With the gel-based method using the agarose 2D-DIGE, eight proteins preferentially expressed in GCF were identified; out of these eight proteins, SOD1, ApoA-I, and DCD were identified by the gel-free method as well. The SOD comprise the most important line of antioxidant enzyme defense system against ROS [32]. Excess production of ROS contributes to tissue damage in a large spectrum of diseases such as diabetes, rheumatoid arthritis, and cancer [33, 34]. ROS has also been implicated in the pathogenesis of periodontal disease. Kimura et al. have shown increased generation rate of ROS from peripheral blood polymorphonuclear leukocytes in chronic adult periodontal disease [35]. In addition, lipopolysaccharide (LPS) induces the production of ROS in organs such as liver and kidney. LPS, the glycolipid of the outer membrane of gram-negative bacteria, elicits inflammatory responses leading to the release of various proinflammatory cytokines and contributes to pathophysiology of periodontal diseases. As the third step, we compared the expression levels of SOD1, Apo-A1 and DCD in GCF in healthy subjects and patients with periodontal diseases. In this study, there was a significant difference in the SOD1 concentration between the two groups (Fig. 4B). This is in agreement with the increased SOD1 activity in GCF reported by Akalin et al. in periodontal disease [36]. However, the exact role of SOD1 in GCF for the pathogenesis of periodontal disease remains to be established in further studies. The SOD1 gene is conserved in *S. cerevisiae* whose proteins were detected in GCF samples in the proteome study performed [14]. Since the sequence coverage is low (9%) and just one MS/MS unique peptide was used to identify the protein spot number 1 (Fig. 2), there is a possibility that the increased amount of SOD1 protein in GCF samples of periodontal disease reflects the existence of *S. cerevisiae*. Therefore, we conducted database homology searches using Blast search. Results indicated that the amino-acid sequence similarity of SOD1 from *S. cerevisiae* and human was very weak (56%). Furthermore, we performed identification of GCF proteins using the yeast (*S. cerevisiae*) database. There was no protein equivalent to SOD1 from *S. cerevisiae*. Therefore, we can conclude that the amino acid sequence of SOD1 from *S. cerevisiae* was not included in the MS/MS unique peptide in this study. Indeed, we confirmed SOD1 expression in Human gingival fibroblast by Western blot analysis (data not shown). It is interesting to note that SOD1 was observed in the Human Gingival Fibroblast. In the future, we plan to explore the mechanism of SOD1 expression in GCF.

We identified an antimicrobial peptide DCD for the first time in GCF using agarose 2DE method. DCD levels were found to be significantly increased in GCF compared with supragingival saliva (Fig. 2). Furthermore, the results of Western blot test clearly showed that DCD levels were significantly greater in GCF obtained from patients with periodontal disease than that from healthy subjects. Periodontal disease is an inflammatory disease initiated by gram-negative bacteria, such as *P. gingivalis* [37]. *Porphyromonas gingivalis* is the bacteria most strongly associated with periodontal disease. DCD including antimicrobial peptides play multiple roles in immune defense. These peptides have been demonstrated to kill gram-negative and gram-positive bacteria, mycobacteria, enveloped viruses, fungi, etc [38]. DCD could defend against inflammatory microbial peptide at the gingival epithelial surface. A detailed analysis to determine the exact roles of DCD in periodontal disease may be required.

On the other hand, using the gel-free method, a total of 327 proteins were identified in GCF samples from healthy subjects (Supporting Information Table S1) [14]. Interestingly, tissue inhibitors of metalloproteinase (TIMP-1) were identified in GCF from healthy subjects, MMPs are able to degrade most proteins of the extracellular matrix. MMPs are counteracted by the TIMPs, which inhibit MMP activity and thereby restrict ECM breakdown. A disturbed balance of MMPs, such as MMP-8, MMP-9, and TIMPs is found in various pathologic conditions, including rheumatoid arthritis, cancer, and periodontal disease [18]. Moreover, there are a number of reports indicating the role of MMPs in health and disease, but information on TIMPs is limited, especially with respect to their role in oral disease including periodontal disease.

The distribution of the proportion of GCF proteins according to their source of origin in healthy subjects is shown in Fig. 4 and Table 3. As a result, proteins from expected periodontal pathogenic bacteria such as, *P. gingivalis* or *A. actinomycetemcomitans* were not detected. *P. gingivalis* and *A. actinomycetemcomitans* are strongly associated with periodontal disease status and disease progression. In this study, subgingival plaque must be collected together with GCF in healthy subjects as well. We suppose that subgingival microbiota from healthy subjects contains just a little percent of periodontal pathogenic bacteria, even if it is beyond the

detectable limit. The major purpose of this investigation was to reveal proteomic patterns of GCF obtained from apparently healthy subjects. Proteins derived from *P. gingivalis* and *A. actinomycetemcomitans* were not included in the results obtained. It is likely that proteins derived from these periodontopathic microbes may be detectable in GCF obtained from patients with periodontitis. In this context, it should be noted that subgingival microbiota composition is different between healthy subjects and subjects with periodontitis [39, 40].

A total of 327 proteins included Annexin A5, Annexin A6, IL-36γ, lamin A/C, periplakin, and moesin, which are reported to be involved in a variety of human diseases. In the present study, we focused on SOD1, apo-A1, and DCD proteins that were identified by the two methods in common. We plan to investigate the association of other proteins and peptides among the 327 candidates with periodontal diseases.

In summary, we first described a novel protocol for effective protein extraction from GCF and then conducted the gel-based and gel-free proteome analyses of GCF proteins in comparison with supragingival saliva. A total of 327 GCF proteins were identified. Also, it was found that SOD1 and DCD were significantly increased in GCF obtained from periodontal patients. It remains to be investigated how other GCF proteins identified in this study are related to periodontal diseases.

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Serum anti-Ku86 is a potential biomarker for early detection of hepatitis C virus-related hepatocellular carcinoma ☆

Fumio Nomura [a,*], Kazuyuki Sogawa [a], Kenta Noda [a], Masanori Seimiya [a], Kazuyuki Matsushita [a], Toshihide Miura [a], Takeshi Tomonaga [b], Hideyuki Yoshitomi [c], Fumio Imazeki [d], Hirotaka Takizawa [e], Kaoru Mogushi [f], Masaru Miyazaki [c], Osamu Yokosuka [d]

[a] Department of Molecular Diagnosis, Graduate School of Medicine, Chiba University and Divisions of Laboratory Medicine, Clinical Genetics and Proteomics, Chiba University Hospital, Chiba, Japan
[b] Laboratory of Proteome Research, National Institute of Biomedical Innovation, Ibaraki, Japan
[c] Department of General Surgery, Graduate School of Medicine, Chiba University, Chiba, Japan
[d] Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, Chiba, Japan
[e] Kashiwado Clinic in Port-Square of the Kashiwado Memorial Foundation, Chiba, Japan
[f] Information Center for Medical Sciences, Tokyo Dental and Medical University, Tokyo, Japan

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ABSTRACT

Hepatocellular carcinoma (HCC), the predominant form of primary liver cancer, is one of the most common cancers worldwide and the third most common cause of cancer-related death. Imaging studies including ultrasound and computed tomography are recommended for early detection of HCC, but they are operator dependent, costly and involve radiation. Therefore, there is a need for simple and sensitive serum markers for the early detection of hepatocellular carcinoma (HCC). In our recent proteomic studies, a number of proteins overexpressed in HCC tissues were identified. We thought if the serum autoantibodies to these overexpressed proteins were detectable in HCC patients. Of these proteins, we focused on Ku86, a nuclear protein involved in multiple biological processes and aimed to assess the diagnostic value of serum anti-Ku86 in the early detection of HCC.

Serum samples were obtained prior to treatment from 58 consecutive patients with early or relatively early hepatitis C virus (HCV)-related HCC and 137 patients with HCV-related liver cirrhosis without evidence of HCC. Enzyme immunoassays were used to measure serum levels of autoantibodies.

Serum levels of anti-Ku86 antibodies were significantly elevated in HCC patients compared to those in liver cirrhosis patients (0.41 ± 0.28 vs. 0.18 ± 0.08 Abs at 450 nm, $P < 0001$). Setting the cut-off level to give 90% specificity, anti-Ku86 was positive in 60.7% of stage I solitary tumor <2 cm in diameter, whereas the sensitivities of alpha-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist II (PIVKA-II) were 17.8% and 21.4%, respectively. The results of ROC analyses indicated the better performance of anti-Ku86 for early detection of HCC. Serum anti-Ku86 levels decreased after surgical resection of the tumors in the 12 HCC cases tested, Elevation of anti-Ku86 in solid tumors other than liver was minimal.

Serum anti-Ku86 is a potential biomarker for early detection of HCV-related HCC. Further studies in a larger number of HCC patients with various etiologies are needed to further evaluate the diagnostic and pathophysiological roles of elevation of serum anti-Ku86 in early HCC.

Abbreviations: 2-DE, two-dimensional gel electrophoresis; 2D-DIGE, two-dimensional fluorescence difference gel electrophoresis; AFP, alpha-fetoprotein; AFP-L3, lectin lens culinaris agglutin bound fraction of AFP; CHC, clathrin heavy chain; CT, computed tomography; DCP, des-gamma-carboxy prothrombin; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MRI, magnetic resonance imaging; NASH, non-alcoholic steatohepatitis; PIVKA-II, protein induced by vitamin K absence or antagonist II; SELDI-TOF MS, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry; MRI, magnetic resonance imaging; US, ultrasonography.

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* Corresponding author at: Department of Molecular Diagnosis, Graduate School of Medicine, Chiba University and Divisions of Clinical Genetics and Proteomics, Chiba University Hospital, 1-8-1 Inohana, Chiba City, Chiba 260-8670, Japan. Fax: +81 43 226 2324.
E-mail address: fnomura@faculty.chiba-u.jp (F. Nomura).

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1. Introduction

Hepatocellular carcinoma (HCC), the predominant form of primary liver cancer, is one of the most common cancers worldwide and the third most common cause of cancer-related death [1]. Chronic infection by hepatitis B virus (HBV) or hepatitis C virus (HCV) and cirrhosis of any cause are major risk factors for HCC development [2,3]. Approximately 80% of HCC cases are derived from HCV-associated chronic liver diseases in Japan [4]. It has been shown that HCC surveillance of subjects at risk can facilitate tumor detection at an early stage, which in turn may improve survival [5,6]. Ultrasound (US) has been recommended for HCC surveillance [7], but the efficacy of this approach is highly operator-dependent. Other imaging modalities such as computed tomography (CT) and magnetic resonance imaging (MRI) may be effective [8,9], but are costly and unsuitable as a first-line examination.

Given this background, there is a need for sensitive serum markers for early detection of HCC. Alpha-fetoprotein (AFP) has been most widely used for this purpose, but many small HCCs do not secrete a diagnostic level of AFP [10,11]. A recent study indicated that AFP lacks adequate sensitivity and specificity for effective early diagnosis of HCC [12]. Measurement of the lectin lens culinaris agglutin-bound fraction of AFP (AFP-L3) can improve the specificity [13]. Protein induced by vitamin K absence or antagonist II (PIVKA-II; also referred to as des-gamma-carboxy prothrombin (DCP)) is a tumor marker complementary to AFP, but elevated PIVKA-II levels are found in only 28–47.6% of HCCs smaller than 3 cm [14,15]. Markers such as glypican-3 have been proposed to be complementary to AFP [16], but this marker has yet to be widely used, as reviewed elsewhere [17,18]. Thus, currently available tumor markers for HCC are not satisfactory in terms of sensitivity and specificity, indicating the need for development of new HCC serum biomarkers.

Recent technological advances have made proteomics useful for discovery of markers in various fields of medicine, including the discovery and identification of biomarkers for HCC [19,20]. Autoantibodies against autologous tumor-associated antigens are also promising targets as biomarkers for early cancer detection [21,22].

We previously conducted proteome analyses to compare protein expression between surgically resected HCC tissues and adjacent nontumor tissues using agarose two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) [23]. Expression levels of 83 proteins were found to differ between tumor and non-tumor tissue, and immunoblot analysis showed significantly increased expression of clathrin heavy chain (CHC) and Ku86, and decreased expression of formiminotransferase cyclodeaminase, rhodanese, and vinculin in the tumor tissue [23]. It is possible that the upregulated proteins can elicit autoantibody formation. Indeed, it has been shown that HCC with higher levels of cyclin B1 expression elicit anti-cyclin B1 antibody levels [24]. To test whether the proteins overexpressed in HCC tissues in our proteomic study elicit autoimmunity in primary liver cancers, we determined the anti-CHC and anti-Ku86 antibody levels in HCV-related HCC patients in comparison with those in patients with chronic liver diseases without HCC or other forms of cancers.

2. Materials and methods

2.1. Subjects

Fifty-eight consecutive patients with early (Stage I, $N = 28$) and relatively early (Stage II, $N = 30$) HCV-related HCC (29 males and 29 females, 69.7 ± 8.6 years old) hospitalized in the Gastroenterology Unit of Chiba University Hospital between January, 2008 and December, 2010 were included in the study (Table 1). For comparison, 137 with liver cirrhosis (56 males and 81 females, 65.8 ± 11.0 years old) we encountered during the same period were also included. Serum samples were obtained prior to initial treatment. Staging of the tumors was based on International Union Against Cancer (UICC)-TNM classification. The diagnosis of HCC was based on typical findings in three-phase dynamic CT or MRI. In cases with inconclusive imaging findings, the diagnosis was confirmed histopathologically. In 12 cases, serum samples were obtained just before and 2 months after surgical resection of the tumors in the Department of Hepatobiliary Surgery at Chiba University Hospital.

For comparison, 48 patients with other gastrointestinal cancers were analyzed in the study, including 16 gastric, 16 colorectal and 16 pancreatic cancers (Table 1). Blood samples obtained at the time of diagnosis were immediately centrifuged and serum was stored at −80 °C until analysis. Blood samples were also obtained from 114 apparently healthy subjects. The mean age of the healthy controls was comparable to that of the HCC patients (Table 1). All the samples were handled and stored essentially with the same protocol.

Written informed consent was obtained from each subject. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethical committee of the Graduate School of Medicine, Chiba University.

2.2. Determination of serum anti-CHC and anti-Ku86 antibody levels

CHC (Sigma–Aldrich Japan, Japan) and Ku86 (Abnova Corporation, Taiwan) proteins dissolved in PBS buffer were dispensed into a 96-well polystyrene microtiter plate (Thermo Fisher Scientific, Japan) at 0.5 μg/well and incubated for 1 day at 4 °C. The plate was washed three times with PBS containing 0.05% Tween 20, coated with 1.5% BSA (Proliant, Ankeny, IA, USA) containing 10% sucrose for 1 day at 4 °C, and then kept at 4 °C until use. After washing the microtiter plate with PBS buffer containing 0.05% Tween 20, 100-μL aliquots of 100-fold-diluted serum samples were added to wells. The plates were incubated at 37 °C for 1 h and then washed three times. Mouse anti-human IgG conjugated to HRP in PBS containing 0.05% Tween 20 (100 μL) was added to

Table 1
Subjects studied.

	Mean age (years)	Sex		Tumor stage (UICC stage[a])			
		M	F	I	II	III	IV
HCC ($N = 58$)	70	29	29	28	30	0	0
Liver cirrhosis ($N = 137$)	66	56	81	–	–	–	–
Others cancers							
Stomach ($N = 16$)	70	8	8	7	4	3	2
Colorectal ($N = 16$)	65	8	8	5	6	4	1
Pancreas ($N = 16$)	68	8	8	1	1	3	11
Healthy controls ($N = 114$)	67	74	40	–	–	–	–

[a] Union for International Cancer Control; TNM Classification of malignant tumors.

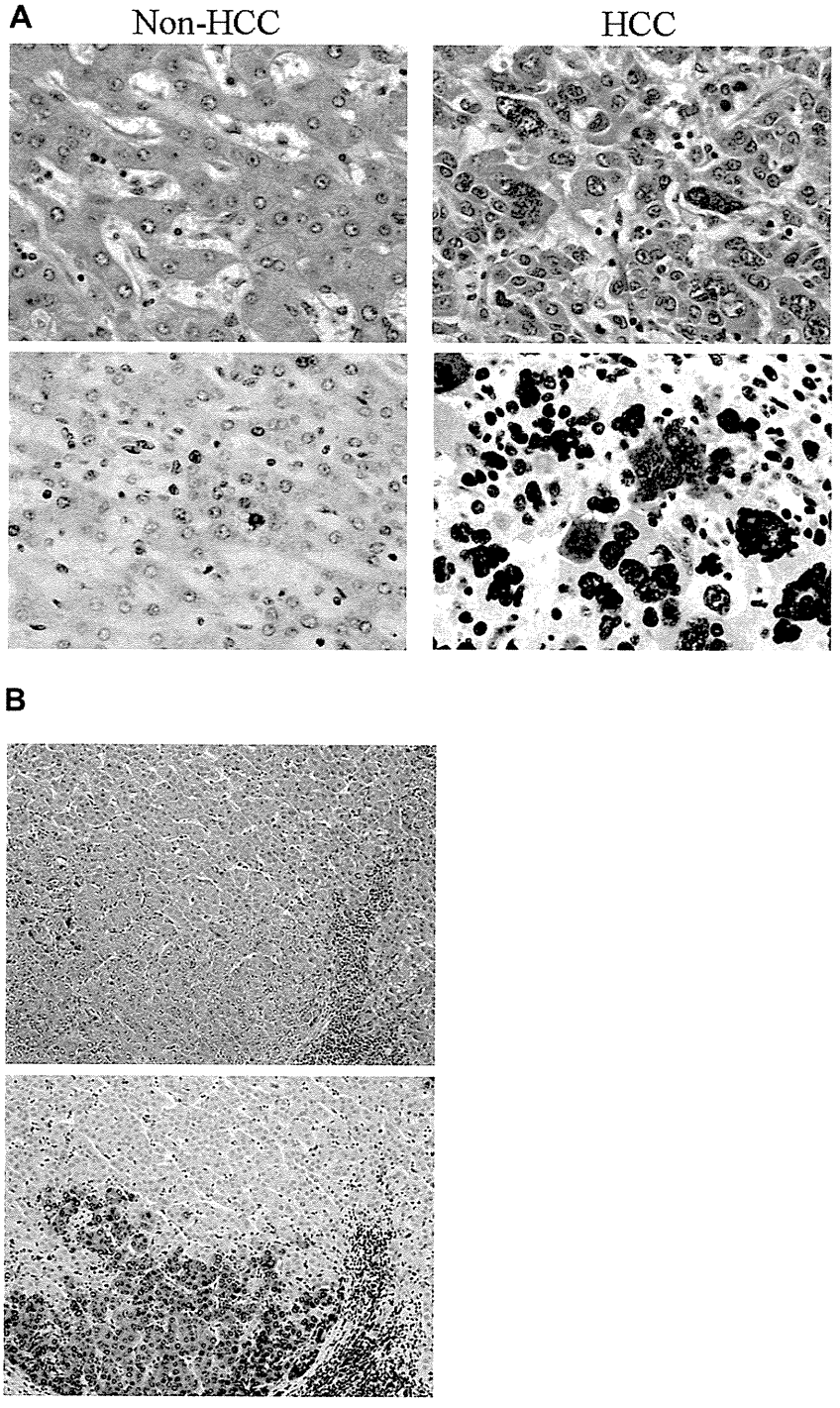

Fig. 1. Immunohistochemical analyses of Ku-86 in HCC and non-HCC tissues. (A) Surgically resected HCC tissues were stained with hematoxylin-eosin (upper panel) or anti-Ku86 antibody (lower panel). Strong staining of Ku86 was noted in the nuclei of tumor cells. In some tumor cells, faint staining was also seen in the cytoplasm. Similar results were obtained in four other comparisons. (B) HCC was distinguished from adjacent nontumor tissue by stronger staining of Ku86; hematoxylin-eosin (upper panel) and immunohistochemistry (lower panel).

each well and the plate was incubated at 37 °C for 1 h. The plate was washed three times and then 100 μL of TMB (3,3′,5,5′-tetramethylbenzidine) solution was added. After incubation at room temperature for 30 min, 100 μL of stop solution was added and absorbance at 450 nm was measured.

2.3. Immunohistochemistry of surgically obtained HCC tissues

Four-μm sections from paraffin tissue were fixed on slide glasses. Tissues were deparaffinized in xylene and rehydrated by reducing the concentration of ethanol (100%, 100%, and 70%, 5 min each). Antigen was unmasked with microwave irradiation for 5 min in pH 6.0 citric buffer three times. Anti-Ku86 antibody (Bio Matrix Research Inc., Chiba, Japan) was diluted 1:50 in blocking buffer (Dako Real™ Antibody Diluent; DAKO Japan, Kyoto, Japan). EnVision + system (DAKO Japan, Kyoto, Japan) was used to visualize tissue antigens. Tissue sections were counterstained with hematoxylin for 1 min. Protein expression, evaluated independently by two pathologists, was scored as negative (0), weak (1), moderate (2), and strong (3).