Proteomics-based identification of haptoglobin as a novel plasma biomarker in oral squamous cell carcinoma

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ABSTRACT

Background: Identification of tumor biomarkers to assist early diagnosis and monitoring of disease progression may potentially decrease the mortality and morbidity associated with oral cancer.
Methods: A mouse model with oral squamous cell carcinoma (OSCC) induced by 4-nitroquinoline 1-oxide (4-NQO)/arecoline in drinking water was established to discover stage-associated biomarkers. A proteomics approach, immunoblot and immunohistochemical analysis were used to validate the expressed biomarkers in mice with OSCC. Human plasma samples were also collected and candidate biomarkers were evaluated using enzyme-linked immunosorbent assay.
Results: Proteomic profiling of mouse plasma samples indicated that haptoglobin and apolipoprotein A1 precursor were up-regulated in the mice with OSCC. Immunoblotting of plasma samples and immunohistochemical analysis of oral tissues showed a significantly higher level of haptoglobin in the OSCC mice than in the control mice. The expression of haptoglobin in human plasma samples from 52 patients with OSCC indicated a strong correlation between the increasing levels of haptoglobin and the clinical stages of OSCC ($P < 0.01$).
Conclusions: These results suggest that haptoglobin has a great potential as a sensitive plasma biomarker for early detection of patients with OSCC.

1. Introduction

Oral cancer is one of the most common neoplasms, which accounts for 2–3% of all human malignancies [1]. Oral squamous cell carcinoma (OSCC) constitutes 90% of oral cancer [2]. Some environmental factors such as tobacco, alcohol and betel quid chewing contribute to the development of oral cancer, have a synergistic effect and may result in tumorigenesis in the oral cavity [3,4]. According to the multi-step theory of carcinogenesis, oral cancer develops from premalignant mucosal lesions to invasive malignant changes, which cause serial histological and clinical changes [5]. Common premalignant lesions present clinically as leukoplakia or erythroplakia, but these lesions may have histologically diverse manifestations such as hyperkeratosis, dysplasia, or even carcinoma [5].

Treatment modalities, including surgery, radiotherapy, or a combination of the 2, have improved in recent years; however, the overall 5-y survival rate for oral cancer is still as low as 50% [6–8]. Patients with advanced oral cancer may achieve complete clinical remission after localized therapy, but the long-term survival rate for these patients is still far lower than that in other major head and neck cancers such as laryngeal or nasopharyngeal cancer [9]. Early detection followed by appropriate treatment can improve the survival rates up to 80–90% and decrease the morbidity associated with OSCC [7].

Clinical examination and biopsy are still the two standard methods to determine the nature of suspicious oral mucosa lesions. Clinical examination should include a comprehensive head and neck examination as well as intraoral visual examination and digital

palpation of the oral cavity. The oral cavity is indeed amenable to direct examination, but oral cancer is still often not detected until the later stages [10,11]. Biopsy of a suspicious mucosal lesion still remains the gold standard to determine the nature of an oral lesion. However, the patient may refrain from biopsy due to the discomfort associated with this invasive procedure. Moreover, the non-uniform appearance of premalignant and malignant lesions may complicate the localization of the biopsy site, which is crucial in the histopathological verification of oral cancer. Other available clinical techniques, such as vital tissue staining with toluidine blue, cytological observation of collected exfoliative cell or molecular analysis, have been developed as additional tools for the early recognition of malignant lesions. These techniques require more advanced technical training and skill to prevent false-positive and false-negative results [2]. Therefore, development of suitable biomarkers needs to be improved to identify early malignant oral lesions, especially in at-risk populations.

Plasma biomarkers are useful for prognostic, diagnostic, and therapeutic determination of various cancers. Detection of tumors at earlier stages would improve the survival rates of OSCC patients [12–14]. However, the complexity of tumor progression and the variability of plasma proteins in different patients make the identification of such markers more difficult. Currently, using traditional methods for analysis and treatment, patients are often diagnosed at later stages and the survival rates are substantially lower than for those diagnosed at an earlier stage. Thus, identification of plasma or serum biomarkers as the means of early diagnosis and the therapeutic target of cancers is important in clinical research.

2. Materials and methods

2.1. Preparation of animals and samples

The 4-NQO/arecoline-induced tongue OSCC experiments were performed in 6-week-old male C57BL/6J mice purchased from the National Laboratory Animal Center in Taiwan [15–17]. All mice were manipulated in accordance with the Animal Care and Use Guidelines for China Medical University under a protocol approved by the Institutional Animal Care Use Committee. Mice ($n = 200$) were randomized into 2 groups: group 1 received only drinking water (control group) and group 2 received both 4-NQO (200 µg/ml) and arecoline (500 µg/ml). Mice were maintained on a normal chow. The carcinogen 4-NQO/arecoline hydrobromide was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in drinking water, which was refreshed once a week. Mice were allowed to drink water at all times during the treatment. Oral lesions were induced by 4-NQO/arecoline after 8 weeks of treatment. The plasma and oral biomarker samples were collected for analysis at different times up to 18 weeks of treatment. Twenty mice in each group were sacrificed by cervical dislocation at weeks 0, 8, 14, 20, and 26. Mouse blood samples were collected in microcentrifuge tubes containing heparin. Plasma samples were separated by centrifugation at 1000 ×g for 10 min at room temperature. The aliquot plasma samples were stored at −80 °C until analysis. All of the tissue dissections were obtained from both normal and OSCC mice and subjected to pathological examination and immunohistochemical staining.

2.2. Pathological examination of mice tongue specimens

Tongue specimens were obtained from mice immediately after cervical dislocation. The specimens were grossly examined and then fixed, embedded in paraffin and sectioned into 8-µm sections. These sections were deparaffinized and stained with hematoxylin and eosin (H & E) for histopathological analysis. The observations of the lesions were classified into five types: epithelial hyperplasia, low-grade dysplasia, high-grade dysplasia, papilloma, and invasive squamous cell carcinoma as described previously [15–17].

2.3. Plasma sample preparation for 2-dimensional gel electrophoresis (2-DE)

A serum protein purification kit was used to deplete albumin and γ-globulin from plasma samples according to the manufacturer's protocol (Bio-Rad, Munich, Germany) [18]. The preparation was then resolved in a sample rehydration buffer and subjected to first-dimension of gel electrophoresis.

2.4. Proteomics analysis, in-gel digestion and liquid chromatography/mass spectrometry (LC–MS)

To identify which tumor-associated proteins differed between OSCC and healthy mice, we analyzed the plasma samples using a proteomics approach according to the manufacturer's instructions and subjected the samples to 2-DE on a Pharmacia Multiphor II electrophoresis apparatus (GE Healthcare, Little Chalfont, UK) [19]. After 2-DE, gels were fixed (40% ethanol and 10% glacial acetic acid), stained with silver nitrate solution, and then scanned by a GS-800 imaging densitometer with PDQuest software version 7.1.1 (Bio-Rad). Data from three independently stained gels from each sample were analyzed by Microsoft Excel to create correction graphs, spot intensity graphs and statistical analysis.

In-gel digestion was performed as described with slight modifications [19]. Briefly, the excised gel pieces were soaked in 100% acetonitrile for 5 min, dried in a lyophilizer for 30 min and rehydrated in 50 mmol/l ammonium bicarbonate buffer (pH 8.0) containing 10 µg/ml trypsin at 30 °C for 16 h. The proteins were identified using the Ultimate capillary LC system (LC Packings, Amsterdam, The Netherlands) coupled with a QSTARXL quadrupole-time of flight (Q-TOF) mass spectrometer (Applied Biosystems/MDS Sciex, Foster, CA). Data acquisition from Q-TOF was performed using automatic Information Dependent Acquisition (IDA; Applied Biosystems/MDS Sciex). Proteins were identified using the nanoLC-MS spectra by searching against NCBI databases for exact matches using the ProID program (Applied Biosystems/MDS Sciex) and the MASCOT search program (http://www.matrixscience.com). A *Homo sapiens* taxonomy restriction was used, and the mass tolerance of both precursor ion and fragment ions was set to ±0.3 Da. Carbamidomethyl cysteine was set as a fixed modification, whereas serine, threonine, tyrosine phosphorylation and other modifications were set as variable modifications.

2.5. Immunoblot analysis of mouse plasma samples

Plasma proteins from 12 mice with OSCC and 6 mice as control were prepared in SDS-PAGE sample buffer. The samples (10 µg) were
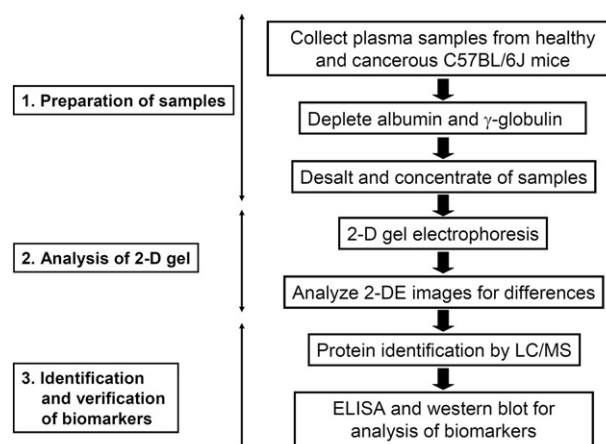

Fig. 1. Schematic flowchart of strategy using a comprehensive proteomics approach to discover plasma biomarkers in oral cancer.

then resolved by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were incubated overnight with anti-mouse haptoglobin (Abcam, Cambridge, UK). The membranes were then incubated with horseradish peroxidase-conjugated anti-mouse IgG (Invitrogen, Carlsbad, CA). Proteins of interest were visualized using the ECL western blotting detection reagents (GE Healthcare) and exposure of the autoradiograph to X-ray film (Kodak, Rochester, NY).

2.6. Immunohistochemical staining of mouse tissue

After a 26-week carcinogen treatment, tissues from five mice with OSCC were analyzed by immunohistochemical staining. Three mice without OSCC from the control group were stained under the same conditions as mice with OSCC. Tissue sections from mice with or without OSCC were deparaffinized and rehydrated. After blocking with 1% normal goat serum, anti-mouse haptoglobin (Abcam) was added to the tissue sections and incubated for 1 h, followed by horseradish peroxidase-conjugated anti-mouse IgG and then 3,3′-diaminobenzidine (Sigma-Aldrich) as the substrate.

2.7. Human subjects

All patients enrolled in this study with or without OSCC were treated at China Medical University Hospital at Taichung in Taiwan from 2007 to 2008. The blood collection protocol was approved by the Institutional Review Board of China Medical University Hospital. All of the enrolled patients provided informed consent forms before beginning the experimental protocol. The clinical staging for OSCC patients was reviewed according to the universal TNM staging system of the International Union against Cancer (UICC)—tumor size (T), nodal metastasis (N) and distant metastasis (M) [20]. The OSCC group consisted of 52 patients with a mean age of 49.8 ± 11.2 (50 male, 2 female): stage I = 21; stage II = 13; stage III = 11; and stage IV = 7. The OSCC-free normal control group included 30 subjects (mean age, 44.9 ± 10.1; 20 male, 10 female) without oral cancer. Plasma samples were collected from the OSCC patients and OSCC-free group. These samples were obtained from peripheral venous blood and stored at −80 °C until analysis. The diagnosis of OSCC was based on clinical

Table 1
Tumorigenesis in mice treated with 4-NQO[a]/arecoline.

Weeks of treatment	Incidence of oral cavity lesions[b] (%)				
	0	8	14	20	26
Control	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
4-NQO/arecoline	0 (0)	3 (15)	8 (40)	17 (85)	20 (100)

[a] 4-NQO, 4-nitroquinoline 1-oxide.
[b] Oral cavity lesions: including obvious papilloma, papilloma-carcinoma, and invasive squamous carcinoma.

examination and verified by pathological examination via biopsy. The inclusion criteria for the OSCC and OSCC-free patients were that they were age ≥20 y and immunocompetent. The exclusion criteria for the OSCC patients were that they had no previous history of chemotherapy, irradiation, immunocompromise or could not properly consent. For the OSCC-free control group, subjects with systemic conditions associated with immune dysfunction such as diabetes, previous chemotherapy, irradiation, and the presence of any oral mucosal lesions, pregnancy or lactation were criteria for exclusion.

### 2.8. Enzyme-linked immunosorbent assay (ELISA) for haptoglobin in human plasma samples

To validate the biomarker expression in human plasma, the plasma samples from OSCC patients with different stages of disease (stages I–IV) [20] and OSCC-free patients (normal control) were analyzed by ELISA. The concentrations of haptoglobin in human plasma were then quantified by ELISA according to the manufacturer's protocol (Roche, Indianapolis, IN).

2.9. Statistical analysis

Haptoglobin concentration levels in plasma between patients with OSCC staging and the OSCC-free control group were analyzed using the Chi-square test with Yates's correction or by ANOVA using the SPSS program (version 10.1, SPSS Inc., IL). Differences in the mean values between the two groups were compared using the Student's t-test. A $P < 0.05$ was considered statistically significant. The linear
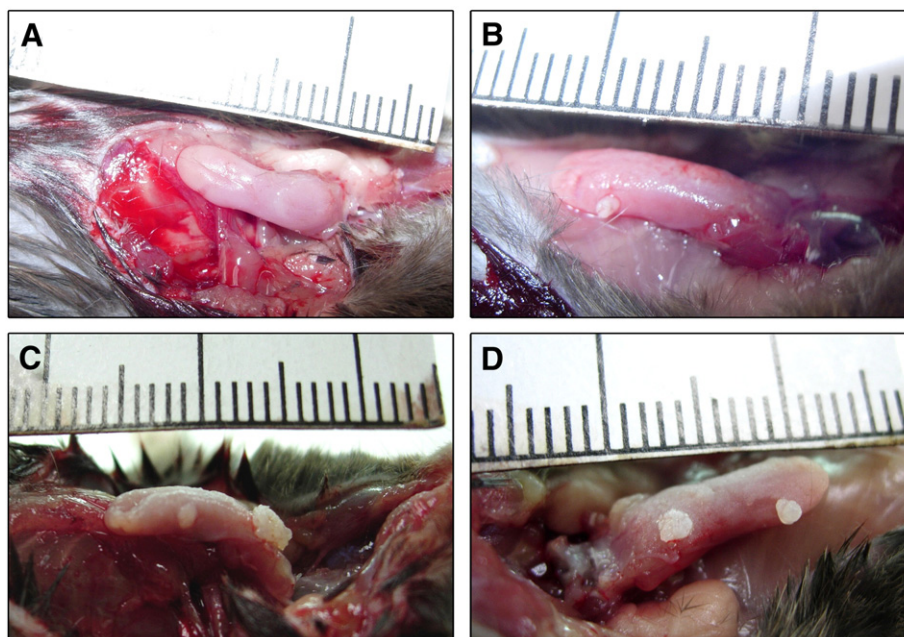
Fig. 2. The progression of oral cavity lesions in mice after treatment with 4-NQO/arecoline. Mice were treated with drinking water containing 4-NQO/arecoline. Mice were sacrificed after treatment with the carcinogen for (A) 8 weeks, (B) 14 weeks, (C) 20 weeks, and (D) 26 weeks. Tongues were removed for observation of morphological changes and lesions. Scales shown in the photographs indicated as centimeter.
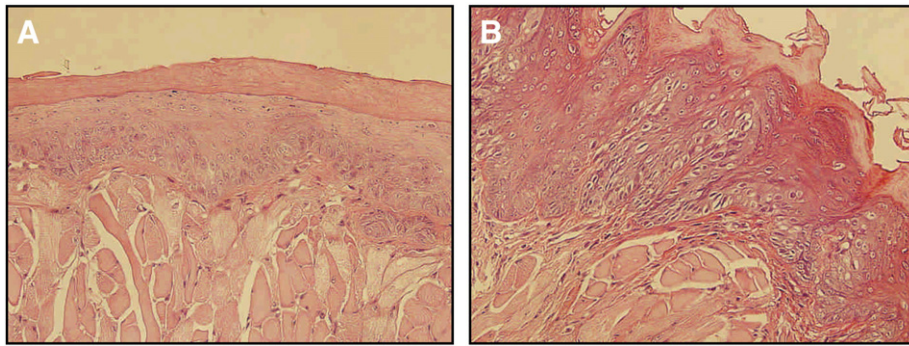
Fig. 3. Pathological examination of tissue sections from mouse tongue after treatment with 4-NQO/arecoline. Mice were given drinking water with or without 4-NQO/arecoline for 26 weeks. The tissue sections of tongue were then stained using H & E. Photographs show (A) healthy control and (B) epithelium with significant dysplasia from 4-NQO/arecoline-exposed mouse tongue. Compared to normal tissue, carcinogen-treated samples showed an increase in the basal layer thickness, neoplastic squamous lesions, and loss of the epithelium organization. The figures were photographed at 400× magnification with H & E staining.

relationship between OSCC staging and haptoglobin concentrations was analyzed using Pearson correlation coefficients.

3. Results

### 3.1. Induction of tongue tumorigenesis in mouse

To investigate potential biomarkers in patients with OSCC, we established a tongue OSCC mouse model using 4-NQO/arecoline in drinking water. Two hundred C57BL/6J mice were divided into 2 groups. Mice in the experimental group were given drinking water containing 4-NQO/arecoline, whereas normal drinking water without the carcinogen was offered to the control group. Twenty mice from each group were sacrificed at weeks 0, 8, 14, 20, and 26. Plasma samples were then collected and purified with the depletion of albumin and γ-globulin. The prepared samples were concentrated, desalted, and subsequently analyzed as shown in Fig. 1.

In the experimental group exposed to 4-NQO/arecoline, 15% of mice developed oral cavity lesions and tongue OSCC at 8 weeks. Gross lesions developed over a 14- to 20-week period. The incidence of oral cavity lesion was 100% after treatment with the carcinogen for 26 weeks (Table 1). Papilloma formation on the tongue surface was observed after treatment with the carcinogen for 8 weeks and the tumor mass gradually increased between 14 and 26 weeks of exposure (Fig. 2). Tissue sections from mice treated with 4-NQO/arecoline in the drinking water were stained using H & E for pathological examination. The tongue tissue sections from the control group showed a clearly defined, single-layer epithelium. As compared to normal stratification of oral epithelium in the control, a marked

elongation and irregular shape of rete ridges, basal cell hyperplasia and pronounced dyskeratosis were observed in the oral epithelium of the 4-NQO/arecoline-treated mice. Furthermore, disrupted epithelial stratification, basal cell hyperplasia, increased nucleocytoplasmic ratio, loss of polarity and pleomorphous cellular morphology were also identified in 4-NQO/arecoline-exposed mice (Fig. 3). This observation revealed that 4-NQO/arecoline-induced tongue OSCC in mice exhibits similar morphological changes to those identified in OSCC human subjects.

3.2. Discovery of biomarkers in plasma of tongue OSCC mice

We compared the plasma protein profiles of control mice and tongue OSCC mice using 2-DE/MS analysis. The intensity of each protein spot was quantified and analyzed simultaneously using a GS-800 imaging densitometer with PDQuest software. Coomassie blue-stained gels showed that the intensity of six protein spots was significantly higher in plasma from the tongue OSCC mice than in the control mice (Fig. 4). These protein spots were selected for in-gel tryptic digestion, followed by peptide mass fingerprinting using the NanoLC Trap Q MS. Five of these six up-regulated protein spots were identified as haptoglobin (spots a to e) using Mascot search parameters (Table 2). The other protein spot (spot f) was identified as the apolipoprotein A1 precursor (Table 2). Haptoglobin (spots a to e) had Mascot scores of 142 to 421, a sequence coverage of 21% to 32%, and one to seven matched peptides, whereas apolipoprotein A1 precursor (spot f) showed a Mascot score of 144, sequence coverage of 16%, and four matched peptides. The results suggest that haptoglobin
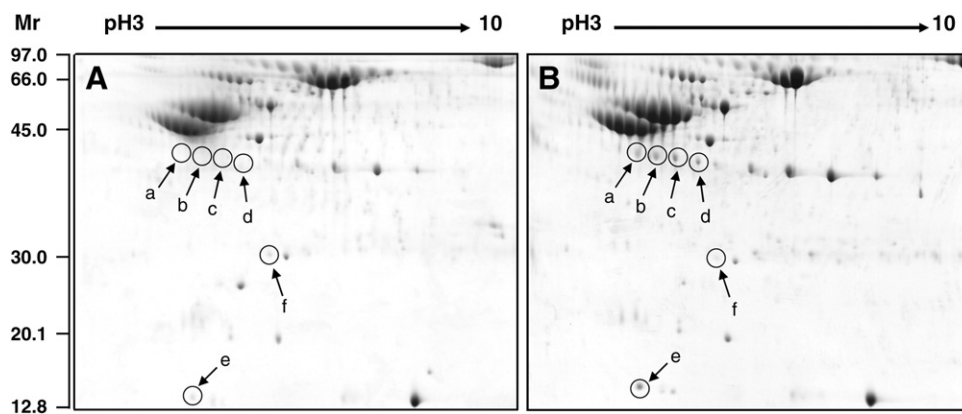
Fig. 4. 2-DE images of mouse plasma from (A) healthy control mice and (B) mice with OSCC. The mice were given drinking water with or without 4-NQO/arecoline for 26 weeks. After depletion of albumin and γ-globulin, the prepared samples were subjected to 2-DE analysis. The IEF was pH 3 to 10. The lowercase letters (a–e) indicate up-regulated protein expression in the cancerous versus normal mouse plasma. Molecular mass markers (Mr) are indicated at the left.

Table 2
Identification of up-regulated proteins in plasma of mice with tongue OSCC compared to control mice using Q-TOF.

| Spot	Protein identification	Accession number	pI	MW (Da)	Mascot score	Matched peptides	Sequence coverage
a	Haptoglobin	gi|8850219	5.88	38,727	392	7	27%
b	Haptoglobin	gi|8850219	5.88	38,727	389	7	27%
c	Haptoglobin	gi|8850219	5.88	38,727	421	6	32%
d	Haptoglobin	gi|8850219	5.88	38,727	397	7	27%
e	Haptoglobin isoform CRA_b	gi|148679477	5.12	13,082	142	1	21%
f	Apolipoprotein A1	gi|109571	5.52	30,358	144	4	16%

may be a potential plasma biomarker for the diagnosis of tongue OSCC in mice.

3.3. Immunoblotting and immunohistochemical analysis of haptoglobin expression

We further validated haptoglobin protein levels in plasma from 4-NQO/arecoline-exposed and control mice at week 26 of treatment. The plasma samples were then subjected to western blotting with anti-haptoglobin. The plasma samples from the OSCC mice showed a significant increase in haptoglobin expression compared with those of control mice ($P<0.05$) (Fig. 5A).
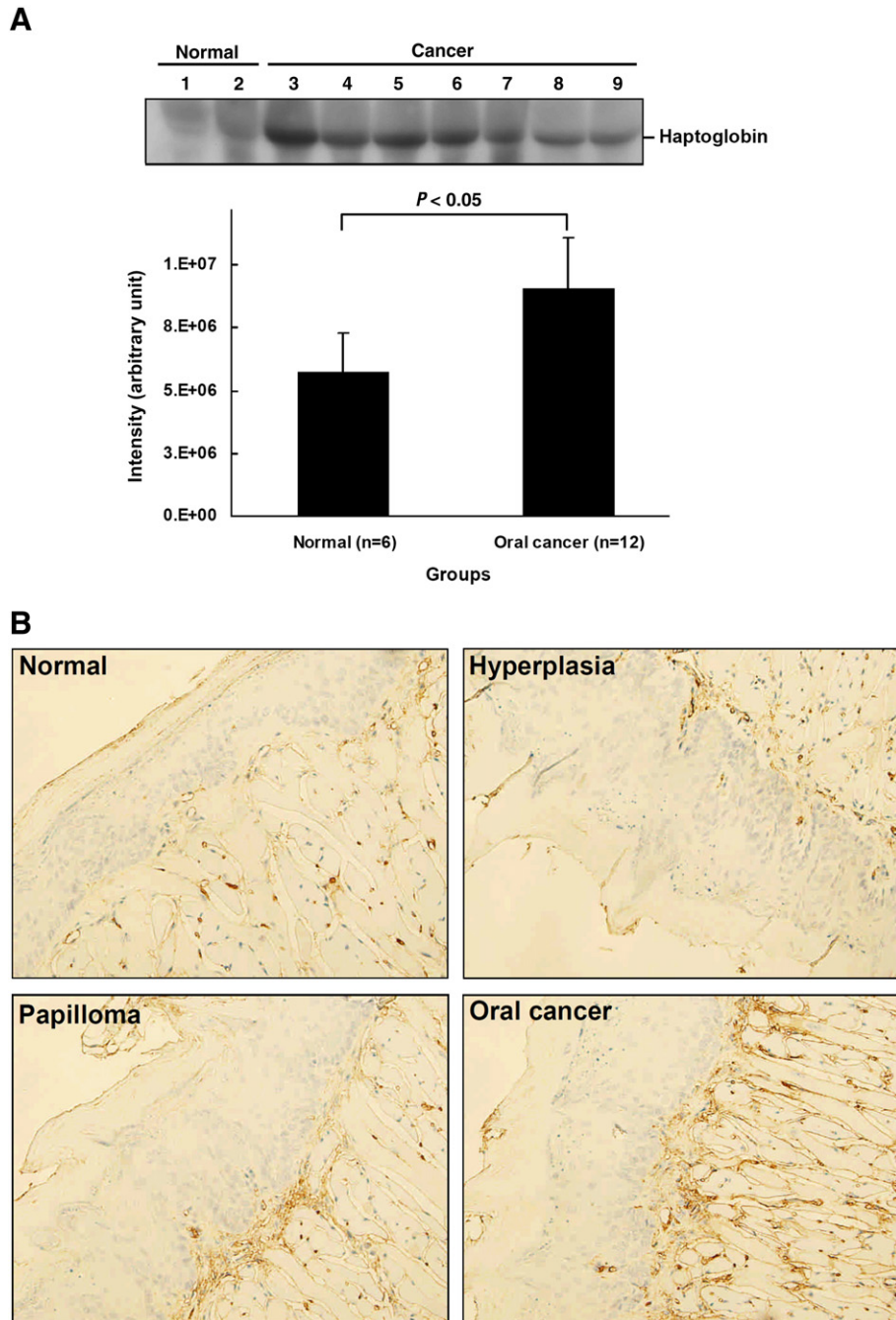
Fig. 5. Expression of haptoglobin correlates with OSCC histology. (A) Immunoblot of mouse plasma samples (upper panel); quantitative analysis of haptoglobin protein expression (lower panel). Lanes 1 and 2 indicated samples from OSCC-free groups, lanes 3 to 9 indicated samples from mice with OSCC. The immunoblot analysis on mouse plasma samples was performed in triplicate. P-values were determined by Student's t-test. $P<0.05$ was considered statistically significant. (B) Immunohistochemical detection of haptoglobin expression in tissue sections from healthy control mice and mice with hyperplasia, papilloma, or invasive squamous cell carcinoma. Immunohistochemical staining demonstrated an obvious immunoreactivity of haptoglobin on the tissue section of tongue with OSCC histology compared to healthy control. The figures were photographed at 400× magnification.
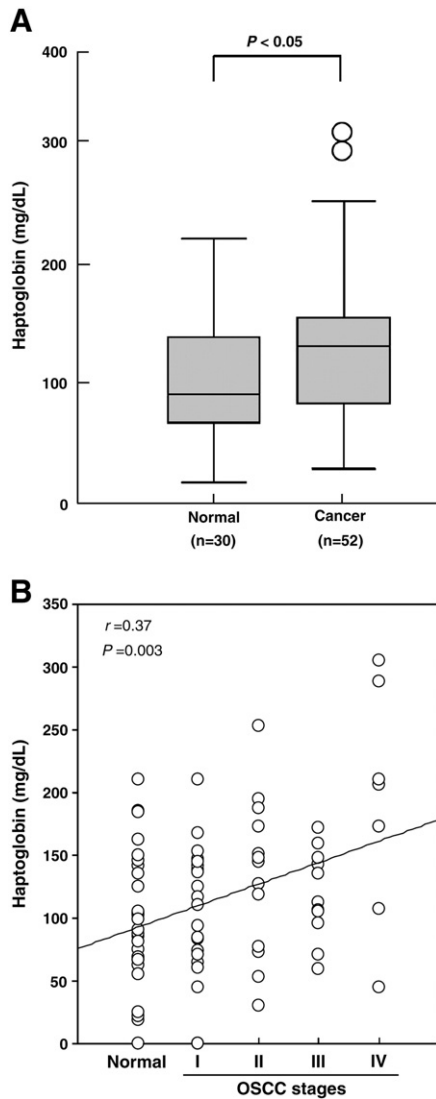
Fig. 6. Validation of plasma haptoglobin as a biomarker in patients with OSCC. Concentrations of plasma haptoglobin protein were determined in healthy control patients or patients with OSCC using ELISA. (A) Box-plot comparison of haptoglobin expression between healthy control and OSCC patients. The box plot (A) is used to show the statistical summary for the distribution of the data. The ends of the boxes define the 25th and the 75th percentiles. Lines drawn from the ends of the box represent the largest and the smallest values that are not outliers. Each extreme value (more than three box lengths) is indicated by an open circle. Each outlier value (between 1.5 and 3.0 box lengths) is represented by an open circle. (B) Linear regression analysis represents the correlation of haptoglobin concentration with OSCC TNM staging. For each marker: r, Pearson correlation coefficients; P-values were determined by Student's t-test. $P < 0.05$ was considered statistically significant.

Haptoglobin protein expression in tongue tissue samples that was analyzed by immunohistochemistry staining was then employed to analyze the up-regulation of haptoglobin protein expressed in the samples of mice with tongue OSCC. As shown in Fig. 5B, faint haptoglobin expression was observed in the tongue tissue from control mice and was not evident in the vascular endothelium region. However, a strong expression of haptoglobin was identified in both the interstitial tissue and vascular endothelium, especially near the basal layer in the oral dysplasia tissue of mice with tongue OSCC (Fig. 5B). Thus, immunoblotting and immunohistochemistry analysis of plasma and tissue samples from tongue OSCC and control mice were consistent with the 2-DE analysis. These results suggest that haptoglobin in plasma has a potential as a biomarker for tongue OSCC progression from early hyperplasia to late carcinomas in mice.

3.4. Correlation of plasma haptoglobin with human OSCC staging

To further validate the potential for haptoglobin as a diagnostic tool in human OSCC, plasma samples from OSCC patients ($n = 52$) and OSCC-free individuals ($n = 30$) were analyzed using ELISA. The results demonstrated that the haptoglobin protein levels in patients with OSCC were significantly higher than those from OSCC-free individuals (Fig. 6A). The mean plasma haptoglobin concentration was 125.8 mg/dl in OSCC patients versus 97.6 mg/dl in OSCC-free individuals ($P < 0.05$). In order to assess the specificity of this biomarker for patients with OSCC but not inflammation status, we further divided the OSCC-free group ($n = 30$) into another two subgroups: normal ($n = 12$) and patients with oral-nasopharyngeal inflammation ($n = 18$). The subgroup of oral-nasopharyngeal inflammation included: chronic otitis media ($n = 2$), chronic paranasal sinusitis ($n = 7$), deep neck infection ($n = 2$), and sudden deafness ($n = 7$). The mean concentrations of plasma haptoglobin of these two OSCC-free subgroups were 96.6 mg/dl and 100.8 mg/dl, respectively. This result indicated that there were no significant differences between healthy individuals and inflammation patients. Nevertheless, the mean concentration of plasma haptoglobin increased to 125.8 mg/dl in OSCC patients. This analysis provided evidence that the level of plasma haptoglobin was not changed between normal individuals and inflammation patients, but dramatically elevated in patients with OSCC.

We then assessed the haptoglobin concentration and TNM stage using linear regression analysis. Patients with OSCC were classified according to UICC TNM staging [20]. Our data showed that haptoglobin levels strongly correlated with OSCC staging (Fig. 6B). The Pearson correlation coefficient (r) for haptoglobin was 0.37 ($P = 0.003$). These data indicated that the identification of the plasma biomarker, haptoglobin, can potentially be used to diagnose patients with OSCC and to identify tumor stages in OSCC.

4. Discussion

Oral cancer is a major worldwide health problem. The prevalence of oral cancer is substantially higher in Asian countries as compared with other industrialized nations [21–24]. In Taiwan, oral cancer is the fourth most common cause of death from cancer in males, behind hepatoma, lung cancer and colorectal cancer (Department of Health, the Executive Yuan, Taiwan, 2006) [25]. More than 90% of oral cancer cases are categorized as OSCC [13,26,27]. In addition, this type of oral cancer causes a high degree of local invasiveness and a high rate of metastasis, which leads to its high incidence of mortality [28]. Early detection of oral cancer would greatly improve long-term survival rates. Plasma biomarkers are thought to have a great potential for assisting the early detection of oral cancer and monitoring cancer progression or recurrence. Investigating the plasma proteome in patients with OSCC could identify potential biomarkers. For OSCC diagnosis, this development may facilitate early detection, improve disease progression surveillance, and provide more information regarding pathophysiological research.

The investigation of biomarkers in patients with OSCC is complex and difficult than the identification of biomarkers in other types of cancer. An animal model is necessary to develop biomarkers for early OSCC detection, progression, and recurrence. Several studies have reported that treatment of mice with 4-NQO could induce OSCC [15–17,29,30]. Adding 4-NQO to the drinking water induces premalignant and malignant lesions in mouse oral cavities, particularly in the tongues and the esophagi [16]. Mice treated with 4-NQO (200 μg/ml) and arecoline (500 μg/ml) for 26 weeks led to 100% neoplasm occurrence in their tongues (Table 1). Gross observation showed that the lesions progressed over time after the 4-NQO treatment was initiated (Fig. 2). Pathological examination demonstrated that the 4-NQO-induced cancer progression ranged from hyperplasia, dysplasia,

papilloma to invasive carcinomas, which is consistent with previous reports by Chang et al. [17] and Sheu et al. [15]. In addition, as shown in Fig. 5B, no inflammatory response such as leukocyte infiltration was observed in hyperplasia, papilloma, or even in cancer. Inconsistently, previous report also indicated that there was no apparent local inflammation in 4-NQO/arecoline-induced mouse oral cancer [17]. Thus, it may explain that the expression of biomarker is associated with oral tumorigenesis but not inflammations. Collectively, this model is potentially useful for the discovery of plasma biomarkers in mice with OSCC and also for staging tumor progression.

Many recent reports using a proteomics approach have suggested a relationship between biomarkers and oral cancer [31,32]. In addition, squamous cell carcinoma antigen-1 was reported to be over-expressed in tumor-bearing mice [18]. Because oral cancer is a heterogeneous disease, multiple cellular pathways may be responsible for tumorigenesis. Thus, we attempted to identify useful biomarkers for the rapid and accurate diagnosis of plasma samples that are also able to differentiate the stages of oral cancer progression. In the present study, we developed a plasma proteomics strategy to discover potential biomarkers for OSCC and used this information to investigate the expression of haptoglobin in plasma from patients with different stages of OSCC.

Haptoglobin is an acute phase protein, which circulates in blood vessels and is associated with infectious disease and trauma [33,34]. Acute phase proteins are mainly biosynthesized by the liver and their expression levels are regulated by cytokines, such as interleukin-1, interleukin-6, and tumor necrosis factor-α [33]. These pro-inflammatory cytokines are primarily secreted by macrophages at the site of inflammatory response. The abundance of haptoglobin in plasma samples implies that the tumor can induce acute phase responses through the activation of the cytokine-mediated pathway. Haptoglobin is also involved in the immunoregulation of tumor growth and mediates a nonspecific immunosuppression *in vitro* and *in vivo* in animals [35]. Haptoglobin can bind free hemoglobin to form a haptoglobin–hemoglobin complex, which prevents free radical damage caused by the heme–iron-mediated generation of free hydroxyl radicals [36]. Haptoglobin is also important in cell migration and extracellular matrix degradation, which is crucial for the development of cancer or other inflammatory diseases such as arthritis [37]. The involvement of haptoglobin with cell migration may contribute to cancer angiogenesis or metastasis [38]. Haptoglobin is also a strong angiogenic factor [39], which is important in cancer progression and metastasis. Cancer cell lines from ovarian cancer, squamous cell carcinoma and small cell lung cancer release a covalent complex containing haptoglobin, which may interact with cellular adhesion factors and regulate the immunosuppression of lymphocytes [40]. In this study, we also demonstrated that haptoglobin was over-expressed in the cancerous tongue region of the mouse model (Fig. 5B). Expression of haptoglobin in the oral lesions of mice with cancer was particularly evident around the vessels, suggesting a possible angiogenic role for haptoglobin in tumorigenesis.

There are two alleles for haptoglobin, which are denoted as 1 and 2. The haptoglobin gene locus is located on chromosome 16q22. The haptoglobin 1 allele is found in all animals, and the conservation between murine and human is more than 90%. However, the haptoglobin 2 allele only exists in human [41]. It has been identified that haptoglobin composes two different types of α-chain and one type of β-chain, which were linked by disulfide bonds [42]. The Hp1-1 genotype produces a single $(\alpha\beta)_2$ homodimer. On the other hand, Hp2 monomer can bind two monomers to form a variety of cyclic multimers in Hp2-2 and a variety of linear complexes in Hp2-1 [43]. The polymorphism of haptoglobin was thought to be a common polymorphism in human subjects and correlated with various diseases. In a very recent report, it has been shown that the frequency of the haptoglobin 2 allele was higher than that of the haptoglobin 1 allele in patients with head and neck cancer [44]. Another report which analyzed nasopharyngeal carcinoma has found that patients with haptoglobin 2-2 genotype had advanced T stages and tumor volumes than those with haptoglobin 2-1 or 1-1 [45]. Thus, the previous studies suggested that haptoglobin 2-2 may be a negative prognostic factor in nasopharyngeal carcinoma. Additionally, Hp 2-2 or Hp 2-0 has also been demonstrated to associate with the recurrence rate in patients with head and neck squamous cell carcinoma [46]. These evidence indicated that patients with haptoglobin 2 allele were associated with various types of cancers. However, in the present study, we did not demonstrate which genotype of haptoglobin was correlated with patients with OSCC. The precise correlation concerned in this regard needs further investigation.

In the present study, we identified haptoglobin as a potential plasma biomarker for oral cancer using 2-DE/LC-MS in a mouse model of oral cancer and in humans with OSCC. Western blot analysis of mouse plasma revealed a significantly higher level of haptoglobin in the cancer samples as compared with normal samples. Further evaluation of the concentration of haptoglobin in plasma from patients with OSCC revealed higher levels than the healthy control group. Moreover, we also found a strong correlation between haptoglobin up-regulation and tumor staging in patients with OSCC. In summary, these findings confirm that plasma concentrations of haptoglobin may serve as a useful plasma tumor stage-associated biomarker in patients with OSCC.

Acknowledgments

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