Salivary auto-antibodies as noninvasive diagnostic markers of oral cavity squamous cell carcinoma

Chih-Ching Wu\textsuperscript{a,c,1,*}, Ya-Ting Chang\textsuperscript{c,1}, Kai-Ping Chang\textsuperscript{e,d}, Yu-Ling Liu\textsuperscript{c}, Hao-Ping Liu\textsuperscript{e}, I-Ling Lee\textsuperscript{f}, Jau-Song Yu\textsuperscript{b,e}, and Wei-Fan Chiang\textsuperscript{f,g,#}

\textsuperscript{a} Department of Medical Biotechnology and Laboratory Science, College of Medicine, \textsuperscript{b} Department of Cell and Molecular Biology, College of Medicine, \textsuperscript{c} Molecular Medicine Research Center, Chang Gung University, Tao-Yuan, Taiwan; \textsuperscript{d} Department of Otolaryngology-Head & Neck Surgery, Chang Gung Memorial Hospital, Lin-Kou, Taiwan; \textsuperscript{e} Department of Veterinary Medicine, National Chung Hsing University, Taichung, Taiwan; \textsuperscript{f} Department of Oral and Maxillofacial Surgery, Chi-Mei Medical Center, Liouying, Taiwan; \textsuperscript{g} School of Dentistry, National Yang-Ming University, Taipei, Taiwan

\textsuperscript{1} These authors contributed equally to this paper.

Conflicts of interest: No potential conflicts of interest were disclosed.

Financial support: All authors received the grant from the Ministry of Education, Taiwan (EMRPD1C0021). C. C. Wu received grants from the Ministry of Science and Technology (MOST), Taiwan (102-2325-B-182-014 and 102-2320-B-182-029-MY3). J. S. Yu received grants from the MOST (102-2325-B-182-010) and Chang Gung Memorial Hospital (CLRPD190013), Taiwan. K. P. Chang received grants from the Chang Gung Memorial Hospital (CMRPG1B0551 and CMRPG3D0261).

* Correspondence: Dr. Chih-Ching Wu, Department of Medical Biotechnology and Laboratory Science, College of Medicine, Chang Gung University, Tao-Yuan 333, Taiwan. Phone: 886-3-2118800, ext. 5093. Fax: 886-3-2118891. e-mail: luckywu@mail.cgu.edu.tw.

# Additional corresponding author: Dr. Wei-Fan Chiang, Department of Oral and Maxillofacial Surgery, Chi-Mei Medical Center, Liouying, Tainan 735, Taiwan. Phone: 886-6-6226999, ext. 73158. Fax: 886-3-2118891. e-mail: bigfanfan@yahoo.com.tw.

Running title: Salivary auto-Ab biomarkers for oral cancer detection
Abstract

**Background:** Oral cavity squamous cell carcinoma (OSCC) is one of the most common cancers worldwide, and its incidence is still increasing. Approximately 50% of OSCC patients die within 5 years after diagnosis, mostly ascribed to that the majority of patients present advanced stages of OSCC at the time of diagnosis.

**Methods:** To discover salivary biomarkers for ameliorating the detection of OSCC, herein we developed a multiplexed bead-based platform to simultaneously detect auto-antibodies (auto-Abs) in salivary samples.

**Results:** Compared with healthy individuals, the salivary levels of anti-p53, anti-survivin, anti-Hsp60, and anti-RPLP0 were significantly elevated in OSCC patients. Noteworthily, the elevated levels of anti-p53, anti-survivin, and anti-Hsp60 were already observed in individuals with oral potentially malignant disorder (OPMD). Moreover, the salivary levels of anti-p53, anti-survivin, anti-Hsp60, anti-RPLP0, and anti-CK-8 were significantly elevated in early-stage OSCC patients compared with those in healthy individuals. Most importantly, the use of a combined panel of salivary anti-p53, anti-survivin, anti-Hsp60, and anti-RPLP0 largely improves the detection of OSCC.

**Conclusion:** Collectively, our results reveal that the salivary auto-Abs are effective OSCC biomarkers and the four-auto-Ab panel provides a novel and practicable approach for OSCC screening.

**Impact:** This study provides the first evidence for the potential clinical application of salivary auto-Abs in OSCC diagnosis.
Introduction

Oral cavity squamous cell carcinoma (OSCC) is a destructive disease, accounting for 3% of all cancer cases estimated each year in the United States (1). In Taiwan, this disease is the fourth most prevalent malignancy in male and the seventh highest in women (2). Despite momentous progress in recent therapeutic approaches, 40%-50% of OSCC patients die within 5 years after being diagnosed (3, 4). The poor prognosis of OSCC and a low 5-year survival rate of patients are mainly attributed to that most patients present advanced stages of OSCC at the time of diagnosis, suggesting that early detection of the disease is needed to improve the treatment outcome and reduce the growing burden of OSCC (3, 4). In spite of increasing numbers of protein biomarkers identified for detection of OSCC (5), most of them are not clinically accessible nor possess sufficient efficacy for early diagnosis of OSCC. Accordingly, discovery of effective biomarkers for early diagnosis is still need to ameliorate the treatment regimen of OSCC.

The majority of OSCC are thought to be developed from oral premalignant lesions, namely the oral potentially malignant disorder (OPMD), which includes epithelial dysplasia, hyperkeratosis or epithelial hyperplasia, erythroplakia, and oral submucous fibrosis (6, 7). The malignant transformation rates of epithelial dysplasia, verrucous hyperplasia, and hyperkeratosis or epithelial hyperplasia are 7.62, 5.21, and
3.26 per 100 person-year, respectively (8). Conceivably, comparative analyses of the clinical specimens from OPMD individuals and those from OSCC patients should lead to discovery of promising biomarkers for early detection of OSCC (9).

Auto-antibodies (auto-Abs) to tumor-associated antigens possess benefits over other body fluid-accessible molecules as cancer biomarker candidates. Auto-Abs are highly specific, stable, and handily detectable in small volumes of specimens with well-established secondary reagents. These advantages lead many efforts into discovery of auto-Abs as biomarkers for detection of various types of cancers, such as colorectal (10-12), breast (13-15), ovarian (16, 17), lung (18-20), liver (21, 22), and pancreatic (23, 24) cancers. As for OSCC, five auto-Abs have been individually identified as serum biomarker candidates, including anti-p53 (25-28), anti-survivin (29, 30), anti-cytokeratin 8 (CK-8) (31), anti-60 kDa heat shock protein (Hsp60) (25), and anti-60S acidic ribosomal protein P0 (RPLP0) (32). Although each by itself has a limited specificity and/or sensitivity for OSCC detection, it is uncertain whether a combined panel of these auto-Abs would be more efficacious than individual auto-Ab is. In other words, further validation is required for these auto-Abs in combination using clinical specimens from identical patient cohorts with large numbers.

Since OSCC cells are immersed in the salivary milieu, it is practicable to detect salivary auto-Abs for OSCC diagnosis. In this study, we aim to determine the
effectiveness of utilizing salivary auto-Abs as OSCC biomarkers. Toward this end, we established a multiplexed bead-based system to simultaneously analyze the levels of the above five auto-Abs in salivary samples collected from healthy volunteers, OPMD individuals, and OSCC patients. Our data revealed that the levels of salivary anti-p53, anti-survivin, anti-Hsp60, and anti-RPLP0 in OSCC patients were significantly higher than those in healthy controls. Furthermore, this four-marker panel performs a better efficacy for early detection of OSCC.

**Materials and Methods**

**Patient populations**

Saliva samples were collected from 131 healthy volunteers, 42 individuals with low-risk OPMD (LR-OPMD), 44 individuals with high-risk OPMD (HR-OPMD), and 131 OSCC patients in Chi-Mei Medical Center, Liouying, Taiwan from January 2005 to August 2012 (Table 1). All volunteers were examined by an oral mucosal screening test. The individuals with LR-OPMD, HR-OPMD, or OSCC were biopsy-proven and underwent routine check-ups according to the standard protocol. Individuals with homogeneous leukoplakia, histological hyperkeratosis, acanthosis or squamous hyperplasia were classified as the LR-OPMD (33). The individuals with speckle
leukoplakia, erythroplakia, verrucous hyperplasia, oral submucous fibrosis (OSF), and histologically epithelial dysplasia were classified as the HR-OPMD (33). Among the OSCCs, 103, 8, and 20 are primary cancers, second primary cancers, and recurrent tumors, respectively. This research followed the tenets of the Declaration of Helsinki, and all subjects signed an informed consent form approved by the Institutional Review Board of Chi-Mei Medical Center before participation and permitting the use of saliva samples collected before treatment.

**Saliva collection and processing**

During oral mucosal examination, unstimulated whole saliva was obtained after mouth rinsing with water (34, 35). The donors avoided eating, drinking, smoking, and using oral hygiene products for at least 1 hour before collection. The collected samples were then centrifuged at 3,000 × g for 15 min at 4°C. The supernatants were immediately treated with a protease inhibitor mixture (2 μl/ml; Sigma-Aldrich, St. Louis, MO, USA), then aliquoted into smaller volumes, and stored at -80°C. The saliva samples that had been thawed were not reused to avoid protein degradation.

**Recombinant proteins for auto-Ab detection**

The recombinant fusion proteins GST-CK-8, His-Hsp60, and His-RPLP0 were
purchased from Creative BioMart (NY, USA), Sino Biological Inc. (Beijing, P. R. China), and Abcam (Cambridge, UK), respectively. The His-tagged p53 and survivin proteins were expressed in *Escherichia coli*. The purification of His-tagged proteins was performed as previously described (5).

**Establishment of a multiplexed system for auto-Ab detection**

Auto-Ab detection was performed by a multiplexed bead-based suspension array system, Bio-Plex (Bio-Rad Laboratories, Taipei, Taiwan). For generating the immunobeads used in the Bio-Plex system, each recombinant protein was covalently conjugated to COOH beads with unique identity (Bio-Rad Laboratories) using Amine Coupling Kit (Bio-Rad Laboratories) according to the instruction manual (36, 37). The coupling efficiency of each protein was verified with anti-His (AbD Serotec, NC, USA), anti-GST (Santa Cruz Biotechnology, CA, USA), anti-p53 (Santa Cruz Biotechnology), anti-survivin, anti-CK8, anti-Hsp60, or anti-RPLP0 (Abnova, Taipei, Taiwan) using the Bio-Plex system.

**Detection of salivary IgA auto-Abs with the Bio-Plex system**

The assays were performed in a 96-well microplate format (9, 36, 37). The beads conjugated with the recombinant proteins were washed with wash buffer (R&D
Systems, Minneapolis, MN, USA) in filter-bottom microplates (Merck Millipore, Taipei, Taiwan). The commercialized Abs or saliva samples (25-fold dilutions) in PBS containing 1% BSA (50 μl/well) were then added into the microplates and incubated for 1 hour at room temperature in the dark. After washing, 1 μg/ml biotin-conjugated anti-human IgA (AbD Serotec) in 1%-BSA PBS (50 μl/well) was applied and incubated for 40 min, followed by incubation with phycoerythrin-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for another 20 min at room temperature in the dark. After washing, the fluorescence intensities of phycoerythrin and bead identities were analyzed using the Bio-Plex 200 system and the Bio-Plex Manager software version 4.2 (Bio-Rad Laboratories).

**ELISA for detection of salivary IgA**

For measuring the salivary levels of IgA, saliva samples were diluted 2,000-fold with PBS and coated on polystyrene microplates (50 μl/well; Corning, NY, USA). After 2 hours, plates were washed with wash buffer (R&D Systems) and blocked with 200 μl PBS containing 1% BSA. Subsequently, 50 ng/ml biotin-conjugated anti-human IgA (AbD Serotec) in PBS containing 1% BSA (50 μl/well) was applied. After 1 hour, samples were incubated with 50 μl of horseradish peroxidase-conjugated streptavidin (diluted 3,000-fold in 1%-BSA PBS; BioLegend, San Diego, CA, USA)
for 1 hour. The substrate NeA-Blue (100 μl/well; Clinical Science Products, Mansfield, MA, USA) was added. After 1 hour, the reaction was stopped with 2N H₂SO₄ (50 μl/well) and OD was measured at 450nm by the SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). OD₅₄₀ was used as the wavelength correction.

**Statistical analysis**

The Mann-Whitney *U* test or Kruskal-Wallis *H* test were used for comparing the levels of auto-Abs between groups. The 90th percentile of mean fluorescence intensities (MFI) in control group was set as the cut-off value to obtain the sensitivity and specificity of each auto-Ab in the other groups. Chi-square test was used for determining the significant differences of sensitivities between groups. Logistic regression was performed to calculate the odds ratio (OR) that defines the relation between biomarkers and cases or control subjects. ORs were calculated on log-transformed MFI of biomarkers and were represented with their 95% confidence interval (CI) and two-sided *p* values. *P* values < 0.05 were considered statistically significant. All data were processed by SPSS software version 12.0 (SPSS Inc., Chicago, IL, USA).
Results

Establishment of a multiplexed bead-based system for auto-Ab detection

Although the serum anti-p53, anti-survivin, anti-CK-8, anti-Hsp60, and anti-RPLP0 have been reported as biomarker candidates of OSCC (25-32), the investigation on their salivary levels in OSCC patients is relatively limited. To simultaneously measure the salivary levels of these auto-Abs, we established a platform combining a direct ELISA with a bead-based suspension array system. We first tested the specificity and sensitivity of this platform using a commercial Ab specific to the individual auto-antigen. As shown in Supplementary Fig. S1A, the indicated auto-antigen conjugated with given beads was dose-dependently recognized by its specific Ab. For evaluating the cross-reactivity during multiplexed assays, we detected the auto-Abs in eight saliva samples using the auto-antigen-conjugated beads both in individual (single-plex) and in combination (five-plex). As shown in Supplementary Fig. S1B, in each case, the ratio of the auto-Ab level measured with a single-plex to that with a multiple-plex was near 1 (ranged between 0.95 and 1.14), indicating that this bead-based system performs limited cross-reactivity to salivary Abs. In addition, the intra- and inter-assay precisions of this platform were evaluated using the saliva samples. The measurement variations (mean of the coefficient of variation, mean CV) of intra- and inter-assays ranged from 4.2% to 7.8% and 7.7% to
13.7%, respectively (Supplementary Fig. S1C and S1D).

**Profiling of the salivary auto-Abs in control, OPMD, and OSCC groups**

We then analyzed the levels of the auto-Abs in saliva samples from 131 healthy controls and 131 OSCC patients using this multiplexed bead-based system (Table 1). To evaluate the effectiveness of the auto-Abs for detection of early-stage OSCC, we also analyzed the samples from OPMD groups, in which 42 and 44 individuals were diagnosed as being low-risk and high-risk for OSCC development, respectively (Table 1). The data revealed that the salivary levels of anti-p53, anti-survivin, anti-Hsp60, and anti-RPLP0 were significantly elevated in OSCC patients compared with the healthy controls (Fig. 1 and Supplementary Table S1). Elevation of the four auto-Abs was associated with presence of OSCC, even after adjustment for age, tobacco, betel nut chewing, and alcohol drinking (Supplementary Table S2). Among them, the levels of anti-p53, anti-survivin, and anti-Hsp60 in OPMD individuals were statistically higher than those in the healthy individuals (Fig. 1 and Supplementary Table S1). Noteworthily, the salivary level of anti-RPLP0 was only elevated in the OSCC patients but not in OPMD groups (Fig. 1).

We also analyzed the level of IgA in all the saliva samples. Notably, its level was elevated in OSCC patients compared with the controls (Fig. 1F). To exclude the
possibility that the elevated levels of salivary auto-Abs in OSCC patients were attributed to an elevated level of IgA, we normalized the level of individual auto-Ab with a corresponding level of IgA in each sample. The data confirmed that the salivary levels of anti-p53, anti-survivin, anti-Hsp60, and anti-RPLP0, indeed, were significantly elevated in OSCC patients compared with the controls (Supplementary Fig. S2). These findings suggested that the four salivary auto-Abs were useful for OSCC detection.

Effectiveness of utilizing salivary auto-Abs for OSCC screening

To evaluate whether salivary auto-Abs are effective OSCC biomarkers, we analyzed the sensitivities of utilizing the auto-Abs for OSCC detection. With a given specificity of 90%, the sensitivities (positive rates) of utilizing anti-p53, anti-survivin, anti-CK-8, anti-Hsp60, and anti-RPLP0 for OSCC detection were 23.7%, 20.6%, 17.6%, 23.7%, and 29.0%, respectively (Table 2). Importantly, the use of a panel combining anti-p53, anti-survivin, anti-Hsp60, and anti-RPLP0 largely enhanced the sensitivity for OSCC detection (43.5%; Table 2) compared with the individual marker alone. The detection efficiency of each auto-Ab in saliva samples was comparable between groups. Notably, utilizing this four-auto-Ab panel for OSCC detection was more sensitive than for detection of either type of OPMD (43.5% vs. 31.0% and
38.6% for LR-OPMD and HR-OPMD, respectively; Table 2), indicating that this marker panel was practicable for OSCC detection with saliva samples.

**Effectiveness of utilizing salivary auto-Abs for early detection of OSCC**

To evaluate whether these salivary auto-Abs could be used for early detection of OSCC, we next analyzed their levels in patients with OSCC at distinct stages, including early stage primary tumors (pT-T1), without lymph node metastasis (pN-N0), and at early overall tumor stages (stage I-II). Compared with the healthy group, the salivary levels of the anti-p53, anti-survivin, anti-Hsp60, anti-RPLP70, and anti-CK-8 were significantly elevated in patients at stage I–II and in those at pT-T1 (Fig. 2 and Supplementary Fig. S3). Moreover, the salivary levels of anti-p53, anti-survivin, anti-Hsp60, and anti-RPLP0 were also significantly elevated in patients at pN-N0 compared with the healthy group (Supplementary Fig. S4). Collectively, our results indicate that the combined auto-Ab panel possesses potency as salivary markers for early detection of OSCC.

**Association between clinical characteristics of OSCC and salivary levels of auto-Abs**

Next, we investigated whether the salivary levels of the auto-Abs associated with
the clinical manifestations of OSCC. The salivary levels of anti-p53 and anti-RPLP0 were significantly correlated with the cell differentiation status of OSCC (Table 3). Furthermore, the salivary levels of anti-p53, anti-survivin, anti-Hsp60, anti-RPLP70, and anti-CK-8 were all significantly elevated in the patients with well-differentiated OSCC, either compared to the healthy individuals or compared to the patients with moderately differentiated OSCC (Fig. 3). After adjusting for overall stage, elevation of anti-p53, anti-survivin, anti-Hsp60, and anti-RPLP0 was correlated with well differentiated OSCC (Supplementary Table S2). The results suggested that these auto-Abs were effective for distinguishing the well-differentiated OSCC from other types. Indeed, with a given specificity of 90%, the sensitivity of utilizing anti-p53, anti-survivin, anti-CK-8, anti-Hsp60, and anti-RPLP0 for detection of well-differentiated OSCC were 30.0%, 31.7%, 23.3%, 33.3%, and 38.3%, respectively (Table 2). Again, the use of a combined panel was superior to that of each marker alone (56.7%; Table 2).

Despite a lack of statistical significance, the salivary levels of the auto-Abs were reduced in the OSCC patients with lymph node metastasis (pN-N1/N2) compared to those without lymph node metastasis (pN-N0; Table 3 and Supplementary Fig. S4). A similar phenomenon was observed in the OSCC patients at overall tumor stages III-IV compared to those at overall tumor stages I–II (Table 3 and Fig. 2). In addition, the
salivary levels of anti-p53, anti-survivin, and anti-Hsp60 were elevated in the OSCC patients with smoking habits compared to those without smoking (Table 3), whereas an expanded number of the non-smoking group is needed to confirm this significance. Moreover, the salivary levels of auto-Ab were not statistically correlated with age, habitual behaviors (betel nut chewing and alcohol consumption), cancer site, pT status, pN status or overall tumor stage of OSCC in this case-control study (Table 3).

**Discussion**

OSCC screening nowadays depends on a visual oral examination by a dentist or an otolaryngologist for possible signs or symptoms of the disease. Despite a need of biomarkers for ameliorating OSCC detection, no marker candidates have been approved for a clinical use, mainly attributed to their insufficient effectiveness. Herein we established a multiplexed immunobead-based platform to simultaneously detect five salivary auto-Abs, including anti-p53, anti-survivin, anti-CK-8, anti-Hsp60, and anti-RPLP70, and verified their effectiveness as OSCC biomarkers for the first time. Although these auto-Abs have been individually reported as serum biomarkers in patients with head and neck cancers (25-32), their salivary levels in OSCC patients as well as their diagnostic and prognostic values have not been investigated on a large
scale until now. Importantly, our case cohorts include two groups of noncancerous patients, who are with low- and high-risk OPMD, respectively (Table 1), for additionally evaluating if such chronic inflammatory diseases in the oral cavity may lead to dysregulated levels of salivary auto-Abs.

Our data revealed that the salivary levels of anti-p53, anti-survivin, anti-Hsp60, and anti-RPLP0 were significantly elevated in OSCC patients compared with healthy individuals (Fig. 1). Notably, the salivary levels of anti-p53, anti-survivin, and anti-Hsp60 in patients with HR-OPMD were statistically higher than those in healthy controls (Fig. 1). Moreover, the salivary levels of all the auto-Abs examined were significantly elevated in early-stage OSCC patients compared with healthy controls (Fig. 2, Supplementary Figs. S3 and S4). These data indicate that the elevated levels of salivary auto-Abs appear to correlate with the progression of OSCC. Accordingly, the use of salivary auto-Abs provides a practicable approach for OSCC screening.

Although individual auto-Ab indeed possessed poor sensitivity for detection of OSCC in the present case-control study, the use of a combined panel largely improved the sensitivity for OSCC detection. In the future, a large cohort study will be required to validate the clinical values of the salivary auto-Abs for OSCC screening.

Compared with traditionally protein markers applied for cancer detection, Abs hold several advantages in the light of the nature of its generation and production (38).
Although the tumor antigens (probable tumor markers) are commonly at an undetectable level in body fluids, the Ab response to the tumor antigens, even with relatively small quantities, could be amplified, generally reflected in the Ab levels (38, 39). In addition, the levels of tumor antigens may be transiently elevated in tissues and blood and thus are not effective enough to be the biomarkers for tumor detection. Nevertheless, the Ab response to tumor antigens is relatively lasting (38). Furthermore, in contrast to other forms of biomarkers, Abs are generally stable and readily detected with well-established and commercially available reagents, such as anti-human immunoglobulin (13). These benefits render cancer-associated Abs to be clinically practical biomarkers for cancer screening.

Since saliva are readily available, large quantities and numbers of salivary specimens from OSCC patients can be acquired (35) for an unabridged study aiming to identify and verify salivary auto-Abs as OSCC biomarkers with adequate statistical power. As OSCC is submerged by the saliva, our data showed that the use of salivary auto-Abs (IgA) for OSCC detection appears to be more effective than the use of serum auto-Abs (IgG). Despite a high specificity of utilizing serum auto-Abs for distinguishing OSCC from the healthy group, utilizing most of them for OSCC detection are not sensitive enough. For instance, the sensitivities of utilizing serum anti-p53, anti-Hsp60, and anti-RPLP0 for OSCC detection are 18.7%, 10.3%, and
10.3%, respectively (25, 27, 28, 32). In contrast, the sensitivities of utilizing salivary anti-p53, anti-Hsp60, and anti-RPLP0 are much improved to 23.7%, 23.7%, and 19.0%, respectively, with a given specificity of 90% (Table 2).

Compared with the healthy controls, the salivary levels of auto-Abs were elevated in the patients with well-differentiated OSCC (Figs. 1 and 3) rather than in those with moderately differentiated OSCC (Fig. 3). Likewise, the salivary levels of auto-Abs in the OSCC patients at later stages (pN-N1/N2 or overall stages III-IV) were lower than the levels in those at early stages (pN-N0 or overall stages I-II) despite a lack of statistical significance (Table 3, Fig. 2, and Supplementary Fig. S4). These observations suggest that late-stage OSCCs not only overcome the immunosurveillance, but may also lead to suppression of Ab production and/or antigen recognition, a process called cancer immunosubversion (38). Antigen presenting cells, in particular dendritic cells (DCs), play a pivotal role in induction of anti-tumor immunity (40). An elevated level of vascular endothelial growth factor (VEGF) in the microenvironment of OSCC may result in a functional suppression of DCs (41, 42). VEGF also leads to recruitment of immature DCs into the cancer site and thereby repress the anti-tumor function of T-cells (42, 43). These findings suggest that the level of VEGF may correlate with prognosis of OSCC. In line with this, we observe that the OSCC patients, in whom the higher levels of VEGF are detected
before treatment, indeed have worse prognoses for overall survival after treatment (9).

In sum, we developed a multiplexed platform to simultaneously detect five salivary auto-Abs in OSCC specimens on a large scale. Our study revealed that these salivary auto-Abs are effective biomarkers for early detection of OSCC. Most importantly, the use of a combined panel of salivary auto-Abs largely improves the effectiveness for OSCC detection. Given the diagnostic and prognostic values of salivary auto-Abs, identification of novel auto-Ab biomarkers in saliva should further ameliorate the treatment regimens of OSCC. To this end, a protein array-wide approach should be applied for discovering novel OSCC-associated Abs in a comprehensive fashion.

Acknowledgements

This work was supported by grants to Chang Gung University from the Ministry of Education, Taiwan (EMRPD1C0021), grants to Chih-Ching Wu from the Ministry of Science and Technology (MOST), Taiwan (102-2325-B-182-014 and 102-2320-B-182-029-MY3), grants to Jau-Song Yu from the MOST (102-2325-B-182-010), the Health Promotion Administration, Ministry of Health and Welfare (PMRPD1B0102) and the Chang Gung Memorial Hospital (CLRPD190013),
Taiwan, and grants to Kai-Ping Chang from the Chang Gung Memorial Hospital (CMRPG1B0551 and CMRPG3D0261).

References

23. Bracci PM, Zhou M, Young S, Wiemels J. Serum autoantibodies to pancreatic...


Table 1. Characteristics of saliva samples used for auto-antibody detection

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Groups of saliva samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy control</td>
</tr>
<tr>
<td>No. of male/female</td>
<td>127/4</td>
</tr>
<tr>
<td>Range/median of age (years)</td>
<td>19-76/45</td>
</tr>
<tr>
<td>No. of individuals with habitual behaviors</td>
<td></td>
</tr>
<tr>
<td>betel nut chewing</td>
<td>72</td>
</tr>
<tr>
<td>smoking</td>
<td>99</td>
</tr>
<tr>
<td>alcohol consumption</td>
<td>25</td>
</tr>
<tr>
<td>Site</td>
<td></td>
</tr>
<tr>
<td>buccal mucosa</td>
<td>-</td>
</tr>
<tr>
<td>gingiva</td>
<td>-</td>
</tr>
<tr>
<td>lip</td>
<td>-</td>
</tr>
<tr>
<td>tongue</td>
<td>-</td>
</tr>
<tr>
<td>palate</td>
<td>-</td>
</tr>
<tr>
<td>others</td>
<td>-</td>
</tr>
<tr>
<td>No. of patients with well/moderate/poor differentiated cancers&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>No. of patients with T1/T2/T3/T4 pT status</td>
<td>-</td>
</tr>
<tr>
<td>No. of patients with N0/N1/N2 pN status</td>
<td>-</td>
</tr>
<tr>
<td>No. of patients with overall stage I/II/III/IV</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> The individuals with homogeneous leukoplakia, histological hyperkeratosis, acanthosis or squamous hyperplasia were classified as the low-risk (LR)-oral potential malignant disorder (OPMD).

<sup>b</sup> The individuals with speckle leukoplakia, erythroplakia, verrucous hyperplasia, oral submucous fibrosis (OSF), and histologically epithelial dysplasia were classified as the high-risk (HR)-OPMD.

<sup>c</sup> Differentiation information of two patients is unavailable.
Table 2. Sensitivities of five auto-antibody (auto-Ab) biomarkers for OSCC detection

<table>
<thead>
<tr>
<th>Groups (n)</th>
<th>No. of auto-Ab-positive cases (positive rate or sensitivity)a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p53 auto-Abb</td>
</tr>
<tr>
<td>Healthy control (131)</td>
<td>13 (9.9%)</td>
</tr>
<tr>
<td>LR-OPMD (42)</td>
<td>5 (11.9%)</td>
</tr>
<tr>
<td>HR-OPMD (44)</td>
<td>11 (25.0%)*</td>
</tr>
<tr>
<td>OSCC (131)</td>
<td>31 (23.7%)**</td>
</tr>
<tr>
<td>Well-differentiated OSCC (60)</td>
<td>18 (30%)**</td>
</tr>
</tbody>
</table>

a With a given specificity of 90%, the median fluorescence intensity (MFI) of each auto-Ab was set as the cut-off value.

b The difference between the disease and the healthy groups were determined by the chi-square analysis. *, p < 0.05; **, p < 0.01.

c A combined panel of anti-p53, anti-survivin, anti-Hsp60, and anti-RPLP0.
Table 3. Correlation between clinicopathological features and salivary levels of auto-antibodies (auto-Abs) in OSCC patients

<table>
<thead>
<tr>
<th>Characteristics (n)</th>
<th>p53 auto-Ab&lt;sup&gt;a&lt;/sup&gt;</th>
<th>p value&lt;sup&gt;b&lt;/sup&gt;</th>
<th>survivin auto-Ab&lt;sup&gt;a&lt;/sup&gt;</th>
<th>p value&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CK-8 auto-Ab&lt;sup&gt;a&lt;/sup&gt;</th>
<th>p value&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Hsp60 auto-Ab&lt;sup&gt;a&lt;/sup&gt;</th>
<th>p value&lt;sup&gt;b&lt;/sup&gt;</th>
<th>RPLP0 auto-Ab&lt;sup&gt;a&lt;/sup&gt;</th>
<th>p value&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Saliva IgA&lt;sup&gt;c&lt;/sup&gt;</th>
<th>p value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 53 (66)</td>
<td>4389±5754</td>
<td>0.2225</td>
<td>2773±5601</td>
<td>0.3868</td>
<td>2371±4564</td>
<td>0.1811</td>
<td>1443±1565</td>
<td>0.5268</td>
<td>1138±1551</td>
<td>0.3805</td>
<td>1.87±0.94</td>
<td>0.0124</td>
</tr>
<tr>
<td>≥ 53 (65)</td>
<td>4562±6104</td>
<td></td>
<td>2885±4448</td>
<td></td>
<td>2691±4427</td>
<td></td>
<td>1850±3526</td>
<td></td>
<td>1726±3857</td>
<td></td>
<td>2.29±0.82</td>
<td></td>
</tr>
<tr>
<td>Betel nut chewing</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No (14)</td>
<td>2993±3782</td>
<td>0.2333</td>
<td>1696±2086</td>
<td>0.4385</td>
<td>1885±2186</td>
<td>0.7431</td>
<td>1012±889</td>
<td>0.2970</td>
<td>843±997</td>
<td>0.3634</td>
<td>1.95±1.15</td>
<td>0.9051</td>
</tr>
<tr>
<td>Yes (117)</td>
<td>4652±6101</td>
<td></td>
<td>2964±5276</td>
<td></td>
<td>2607±4682</td>
<td></td>
<td>1721±2852</td>
<td></td>
<td>1500±3081</td>
<td></td>
<td>2.09±0.87</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No (16)</td>
<td>2789±4205</td>
<td>0.0429</td>
<td>1778±3136</td>
<td>0.0156</td>
<td>2403±5454</td>
<td>0.0876</td>
<td>1267±1986</td>
<td>0.0301</td>
<td>935±1445</td>
<td>0.0635</td>
<td>1.94±1.14</td>
<td>0.7278</td>
</tr>
<tr>
<td>Yes (115)</td>
<td>4710±6085</td>
<td></td>
<td>2975±5247</td>
<td></td>
<td>2548±4358</td>
<td></td>
<td>1698±2807</td>
<td></td>
<td>1499±3083</td>
<td></td>
<td>2.09±0.87</td>
<td></td>
</tr>
<tr>
<td>Alcohol consumption</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No (79)</td>
<td>3815±5263</td>
<td>0.1076</td>
<td>2818±5302</td>
<td>0.2231</td>
<td>2397±4607</td>
<td>0.1534</td>
<td>1718±3270</td>
<td>0.5377</td>
<td>1278±2812</td>
<td>0.1441</td>
<td>1.98±0.94</td>
<td>0.1876</td>
</tr>
<tr>
<td>Yes (52)</td>
<td>5478±6701</td>
<td></td>
<td>2845±4673</td>
<td></td>
<td>2732±4323</td>
<td></td>
<td>1534±1565</td>
<td></td>
<td>1660±3125</td>
<td></td>
<td>2.23±0.82</td>
<td></td>
</tr>
<tr>
<td>Site</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>buccal mucosa (52)</td>
<td>4068±4756</td>
<td>0.1830</td>
<td>2862±5152</td>
<td>0.1323</td>
<td>2287±4315</td>
<td>0.0894</td>
<td>1469±1764</td>
<td>0.0808</td>
<td>1113±1440</td>
<td>0.4919</td>
<td>1.81±0.97</td>
<td>0.0402</td>
</tr>
<tr>
<td>gingival (21)</td>
<td>2763±4296</td>
<td></td>
<td>1500±2561</td>
<td></td>
<td>1426±1526</td>
<td></td>
<td>1055±903</td>
<td></td>
<td>733±769</td>
<td></td>
<td>1.85±1.08</td>
<td></td>
</tr>
<tr>
<td>lip (12)</td>
<td>5844±6230</td>
<td></td>
<td>3141±2925</td>
<td></td>
<td>4500±6201</td>
<td></td>
<td>2430±2014</td>
<td></td>
<td>1540±1568</td>
<td></td>
<td>2.67±0.45</td>
<td></td>
</tr>
<tr>
<td>tongue (29)</td>
<td>5768±7311</td>
<td></td>
<td>3071±5197</td>
<td></td>
<td>2843±4957</td>
<td></td>
<td>2319±4868</td>
<td></td>
<td>2469±5566</td>
<td></td>
<td>2.38±0.52</td>
<td></td>
</tr>
<tr>
<td>palate (11)</td>
<td>5267±9365</td>
<td></td>
<td>5276±9176</td>
<td></td>
<td>3579±6329</td>
<td></td>
<td>1429±2038</td>
<td></td>
<td>1625±2338</td>
<td></td>
<td>2.07±1.00</td>
<td></td>
</tr>
<tr>
<td>others (6)</td>
<td>3551±3499</td>
<td></td>
<td>909±442</td>
<td></td>
<td>1121±619</td>
<td></td>
<td>808±473</td>
<td></td>
<td>1013±960</td>
<td></td>
<td>2.52±0.61</td>
<td></td>
</tr>
<tr>
<td>Cell differentiation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>well (60)</td>
<td>5634±6415</td>
<td>0.0057</td>
<td>4206±6837</td>
<td>0.0586</td>
<td>3627±6179</td>
<td>0.1140</td>
<td>2283±3777</td>
<td>0.0571</td>
<td>2066±4095</td>
<td>0.0150</td>
<td>2.21±0.89</td>
<td>0.0763</td>
</tr>
<tr>
<td>moderate (62)</td>
<td>3010±4758</td>
<td></td>
<td>1640±2149</td>
<td></td>
<td>1568±1671</td>
<td></td>
<td>1073±890</td>
<td></td>
<td>340±998</td>
<td></td>
<td>1.92±0.90</td>
<td></td>
</tr>
<tr>
<td>poor (7)</td>
<td>7541±8399</td>
<td></td>
<td>2228±2914</td>
<td></td>
<td>2072±2950</td>
<td></td>
<td>1601±1909</td>
<td></td>
<td>1174±1793</td>
<td></td>
<td>2.48±0.58</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MFI (mean±SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>---------------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 (51)</td>
<td>4561±5668</td>
<td>0.2655</td>
<td>2753±4747</td>
<td>0.3900</td>
<td>2872±5075</td>
<td>0.1856</td>
<td>1691±1839</td>
<td>0.1788</td>
<td>1281±1668</td>
<td>0.2943</td>
<td>2.05±0.98</td>
<td>0.1725</td>
</tr>
<tr>
<td>T2 (40)</td>
<td>4663±5855</td>
<td></td>
<td>3207±5192</td>
<td></td>
<td>2908±5344</td>
<td></td>
<td>2026±4300</td>
<td></td>
<td>1654±3697</td>
<td></td>
<td>2.33±0.68</td>
<td></td>
</tr>
<tr>
<td>T3 (10)</td>
<td>3308±6622</td>
<td></td>
<td>1275±1767</td>
<td></td>
<td>1543±2801</td>
<td></td>
<td>949±1315</td>
<td></td>
<td>895±1283</td>
<td></td>
<td>1.80±0.87</td>
<td></td>
</tr>
<tr>
<td>T4 (30)</td>
<td>4467±6391</td>
<td></td>
<td>2970±6072</td>
<td></td>
<td>1774±1868</td>
<td></td>
<td>1291±1173</td>
<td></td>
<td>1562±3833</td>
<td></td>
<td>1.86±0.98</td>
<td></td>
</tr>
<tr>
<td>pN status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0 (101)</td>
<td>4572±6043</td>
<td>0.7014</td>
<td>3048±5525</td>
<td>0.5801</td>
<td>2793±4988</td>
<td>0.3974</td>
<td>1766±3012</td>
<td>0.3215</td>
<td>1369±2629</td>
<td>0.7218</td>
<td>2.06±0.92</td>
<td>0.8436</td>
</tr>
<tr>
<td>N1 and N2 (30)</td>
<td>4148±5515</td>
<td></td>
<td>2089±2827</td>
<td></td>
<td>1645±1796</td>
<td></td>
<td>1238±1265</td>
<td></td>
<td>1633±3835</td>
<td></td>
<td>2.14±0.86</td>
<td></td>
</tr>
<tr>
<td>Overall stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>stage I-II (80)</td>
<td>4447±5431</td>
<td>0.2342</td>
<td>2975±5073</td>
<td>0.3981</td>
<td>2999±6432</td>
<td>0.1309</td>
<td>1877±3305</td>
<td>0.1638</td>
<td>1486±2870</td>
<td>0.0721</td>
<td>2.16±0.87</td>
<td>0.1885</td>
</tr>
<tr>
<td>Stage III-IV (51)</td>
<td>4518±6645</td>
<td></td>
<td>2598±5037</td>
<td></td>
<td>1794±2170</td>
<td></td>
<td>1281±1312</td>
<td></td>
<td>1341±3060</td>
<td></td>
<td>1.95±0.95</td>
<td></td>
</tr>
</tbody>
</table>

*Data are shown as mean±SD of MFI.*

The *p* values between two and more than two groups were determined by the Mann-Whitney *U* test and Kruskal-Wallis *H* test, respectively.

*Data are shown as mean±SD of optical density at 450nm with 540nm as the wavelength correction.*
Figure Legends

Fig. 1. Salivary levels of auto-Abs in the OSCC patients. The levels of anti-p53 (A), anti-survivin (B), anti-CK-8 (C), anti-Hsp60 (D), and anti-RPLP0 (E) in saliva samples from healthy controls (Ctrl; n = 131), individuals with LR- (LR-OPMD; n = 42) and HR-OPMD (HR-OPMD; n = 46), and OSCC patients (OSCC; n = 131) were measured by multiplexed immunobead-based methods. (F) The salivary level of IgA was measured by a direct ELISA. Data are presented as the upper and lower quartiles (box); the median value (horizontal lines), the middle 80% distribution (dashed line), and the middle 90% distribution (filled circles) of median fluorescence intensity (MFI) are shown. Symbols: *, p < 0.05 and **, p < 0.01.

Fig. 2. Efficacy of utilizing the salivary auto-Abs for early detection of OSCC. The levels of auto-Abs in saliva samples collected from healthy controls (Ctrl; n = 131), OSCC patients at overall stage I-II (Stage I-II; n = 80), and those at overall stage III-IV (Stage III-IV; n = 51) were measured by multiplexed immunobead-based methods. Data are presented as the upper and lower quartiles (box); the median value (horizontal lines), the middle 80% distribution (dashed line), and the middle 90% distribution (filled circles) of median fluorescence intensity (MFI) are shown. Symbols: *, p < 0.05 and **, p < 0.01.
Fig. 3. Efficacy of utilizing the salivary auto-Abs for detection of well-differentiated OSCC. The levels of auto-Abs in saliva samples collected from healthy controls (Ctrl; n = 131), patients with well-differentiated OSCC (Well differ.; n = 60), and patients with moderately differentiated OSCC (mod. differ.; n = 62) were measured by multiplexed immunobead-based methods. Data are presented as the upper and lower quartiles (box); the median value (horizontal lines), the middle 80% distribution (dashed line), and the middle 90% distribution (filled circles) of median fluorescence intensity (MFI) are shown. Symbols: *, p < 0.05 and **, p < 0.01.
Figure 1

(A) Anti-p53

(B) Anti-survivin

(C) Anti-CK-8

(D) Anti-Hsp60

(E) Anti-RPLP0

(F) IgA
Figure 3

**Anti-p53**

**Anti-survivin**

**Anti-CK-8**

**Anti-Hsp60**

**Anti-RPLP0**

MFI (103)

MFI (103)

MFI (103)

MFI (103)
Salivary auto-antibodies as noninvasive diagnostic markers of oral cavity squamous cell carcinoma

Chih-Ching Wu, Ya-Ting Chang, Kai-Ping Chang, et al.

Cancer Epidemiol Biomarkers Prev  Published OnlineFirst May 23, 2014.