Salivary Auto-Antibodies as Noninvasive Diagnostic Markers of Oral Cavity Squamous Cell Carcinoma

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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 males and the seventh highest in women (2). Despite the momentous progress in recent therapeutic approaches, 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, verrucous hyperplasia, 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).

40% to 50% of patients with OSCC 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 the fact 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). Despite 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 in need to ameliorate the treatment regimen of OSCC.

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.

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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-keratin 8 (CK-8; ref. 31), anti-60 kDa heat shock protein (Hsp60; ref. 25), and anti-60S acidic ribosomal protein P0 (RPLP0; ref. 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 an 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.

Because 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 using 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 patients with OSCC. Our data revealed that the levels of salivary anti-p53, anti-survivin, anti-Hsp60, and anti-RPLP0 in patients with OSCC 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 patients with OSCC 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 checkups according to the standard protocol. Individuals with homogeneous leukoplakia, histologic hyperkeratosis, and acanthosis or squamous hyperplasia were classified as the LR-OPMD. The individuals with speckle leukoplakia, erythroplakia, verrucous hyperplasia, OSF, and histologically epithelial dysplasia were classified as the HR-OPMD. Differentiation information of 2 patients is unavailable.

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

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Healthy control</th>
<th>LR-OPMD</th>
<th>HR-OPMD</th>
<th>OSCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of male/female</td>
<td>127/4</td>
<td>42/0</td>
<td>44/0</td>
<td>128/3</td>
</tr>
<tr>
<td>Range/median of age (y)</td>
<td>19–76/45</td>
<td>26–79/50</td>
<td>29–81/50</td>
<td>32–80/53</td>
</tr>
<tr>
<td>Number of individuals with habitual behaviors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Betel nut chewing</td>
<td>72</td>
<td>38</td>
<td>43</td>
<td>117</td>
</tr>
<tr>
<td>Smoking</td>
<td>99</td>
<td>38</td>
<td>41</td>
<td>115</td>
</tr>
<tr>
<td>Alcohol consumption</td>
<td>25</td>
<td>16</td>
<td>26</td>
<td>52</td>
</tr>
<tr>
<td>Site</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buccal mucosa</td>
<td>—</td>
<td>28</td>
<td>24</td>
<td>52</td>
</tr>
<tr>
<td>Gingiva</td>
<td>—</td>
<td>3</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>Lip</td>
<td>—</td>
<td>1</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>Tongue</td>
<td>—</td>
<td>4</td>
<td>11</td>
<td>29</td>
</tr>
<tr>
<td>Palate</td>
<td>—</td>
<td>0</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>Others</td>
<td>—</td>
<td>9</td>
<td>21</td>
<td>6</td>
</tr>
<tr>
<td>Number of patients with well/moderate/poor-differentiated cancers</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>60/62/7</td>
</tr>
<tr>
<td>Number of patients with T1/T2/T3/T4 pT status</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>51/40/10/30</td>
</tr>
<tr>
<td>Number of patients with N0/N1/N2 pN status</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>101/13/17</td>
</tr>
<tr>
<td>Number of patients with overall stages I/II/III/IV</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>49/31/16/35</td>
</tr>
</tbody>
</table>

aThe individuals with homogeneous leukoplakia, histologic hyperkeratosis, and acanthosis or squamous hyperplasia were classified as the LR-OPMD.
bThe individuals with speckle leukoplakia, erythroplakia, verrucous hyperplasia, OSF, and histologically epithelial dysplasia were classified as the HR-OPMD.
cDifferentiation information of 2 patients is unavailable.
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 minutes at 4°C. The supernatants were immediately treated with a protease inhibitor mixture (2 μL/mL; Sigma-Aldrich), 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, Sino Biological Inc., and Abcam, 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). 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 the 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), anti-GST (Santa Cruz Biotechnology), anti-p53 (Santa Cruz Biotechnology), anti-survivin, anti-CK8, anti-Hsp60, or anti-RPLP0 (Abnova) 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) in filter-bottom microplates (Merck Millipore). 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 minutes, followed by incubation with phycoerythrin-conjugated streptavidin (Jackson ImmunoResearch Laboratories) for another 20 minutes 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). 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) for 1 hour. The substrate NeA-Blue (100 μL/well; Clinical Science Products) was added. After 1 hour, the reaction was stopped with 2N H2SO4 (50 μL/well) and optical density (OD) was measured at 450 nm 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 the control group was set as the cut-off value to obtain the sensitivity and specificity of each auto-Ab in the other groups. The χ2 test was used for determining the significant differences of sensitivities between groups. Logistic regression was performed to calculate the OR that defines the relation between biomarkers and cases or control subjects. ORs were calculated on MFI of biomarkers and were represented with their 95% confidence interval 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.).

**Results**

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

Although the serum anti-p53, anti-survivin, anti-CK8, anti-Hsp60, and anti-RPLP0 have been reported as biomarker candidates of OSCC (25–32), the investigation on their salivary levels in patients with OSCC 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 interassay precisions of this platform were evaluated using the saliva samples. The measurement variations (mean of the coefficient of variation, mean CV) of intra- and interassays 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 patients with OSCC 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 patients with OSCC compared with the healthy controls (Fig. 1 and Supplementary Table S1). Elevation of the four auto-Abs was associated with the 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). Noteworthy, the salivary level of anti-RPLP0 was only elevated in the patients with OSCC 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 patients with OSCC compared with the controls (Fig. 1F). To exclude the possibility that the elevated levels of salivary auto-Abs in patients with OSCC 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 patients with OSCC compared with the controls (Supplementary Fig. S2). These findings suggested that the four salivary auto-Abs were useful for OSCC detection.

Effectiveness of using salivary auto-Abs for screening

To evaluate whether salivary auto-Abs are effective OSCC biomarkers, we analyzed the sensitivities of using the auto-Abs for OSCC detection. With a given specificity of 90%, the sensitivities (positive rates) of using anti-p53, anti-survivin, anti-CK8, 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, using 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 using 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 (stages I–II). Compared with the healthy group, the salivary levels of the anti-p53, anti-survivin, anti-Hsp60, anti-RPLP0, and anti-CK8 were significantly elevated in patients at stages 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-RPLP0, and anti-CK8 were all significantly elevated in the patients with well-differentiated OSCC, either compared with the healthy individuals or compared with 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 using anti-p53, anti-survivin, anti-CK8, anti-Hsp60, and anti-RPLP0 for detection of well-differentiated OSCC was 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 patients with OSCC with lymph node metastasis (pN-N1/N2) compared with those without lymph node metastasis (pN-N0; Table 3 and Supplementary Fig. S4). A similar phenomenon was observed in the patients with OSCC at overall tumor stages III–IV compared with 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 patients with OSCC with smoking.
habits compared with those without smoking (Table 3), whereas an expanded number of the nonsmoking group is needed to confirm this significance. Moreover, the salivary levels of auto-Abs were not statistically correlated with age, habitual behaviors (betel nut chewing and alcohol consumption), cancer site, pT status, pN status.
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-CK8, anti-Hsp60, and anti-RPLP0, 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 patients with OSCC 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 LR- and HR-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 patients with OSCC 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 patients with early-stage OSCC compared with healthy controls (Fig. 2 and Supplementary Figs. S3 and S4). These data indicate that the elevated levels of salivary auto-Abs seem 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 with 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.

Because saliva are readily available, large quantities and numbers of salivary specimens from patients with OSCC 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 seems to be more effective than the use of serum auto-Abs (IgG). Despite a high specificity of using serum auto-Abs for distinguishing OSCC from the healthy group, using most of them for OSCC detection are not sensitive enough. For instance, the sensitivities of using 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,

Table 2. Sensitivities of five auto-Ab biomarkers for OSCC detection

<table>
<thead>
<tr>
<th>Groups (n)</th>
<th>p53 auto-Ab</th>
<th>Survivin auto-Ab</th>
<th>CK-8 auto-Ab</th>
<th>Hsp60 auto-Ab</th>
<th>RPLP0 auto-Ab</th>
<th>Four-auto-Ab panel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy control (131)</td>
<td>13 (9.9%)</td>
<td>13 (9.9%)</td>
<td>13 (9.9%)</td>
<td>13 (9.9%)</td>
<td>13 (9.9%)</td>
<td>35 (26.7%)</td>
</tr>
<tr>
<td>LR-OPMD (42)</td>
<td>5 (11.9%)</td>
<td>5 (11.9%)</td>
<td>3 (7.1%)</td>
<td>6 (14.3%)</td>
<td>4 (9.5%)</td>
<td>13 (31.0%)</td>
</tr>
<tr>
<td>HR-OPMD (44)</td>
<td>11 (25.0%)</td>
<td>9 (20.5%)</td>
<td>9 (20.5%)</td>
<td>11 (25.0%)</td>
<td>10 (22.7%)</td>
<td>17 (38.6%)</td>
</tr>
<tr>
<td>OSCC (131)</td>
<td>31 (23.7%)</td>
<td>27 (20.6%)</td>
<td>23 (17.6%)</td>
<td>31 (23.7%)</td>
<td>38 (29.0%)</td>
<td>57 (43.5%)</td>
</tr>
<tr>
<td>Well-differentiated OSCC (60)</td>
<td>18 (30%)</td>
<td>19 (31.7%)</td>
<td>14 (23.3%)</td>
<td>20 (33.3%)</td>
<td>23 (38.3%)</td>
<td>34 (56.7%)</td>
</tr>
</tbody>
</table>

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

The difference between the disease and the healthy groups was determined by the $\chi^2$ analysis.

A combined panel of anti-p53, anti-survivin, anti-Hsp60, and anti-RPLP0.

$^aP < 0.05$.

$^bP < 0.01$.
the sensitivities of using 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 patients with OSCC at later stages (pN-N1/N2 or overall stages III–IV) were lower than those 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 the level of VEGF may correlate with prognosis of OSCC. In line with this, we observe that the patients with OSCC, 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.
Table 3. Correlation between clinicopathologic features and salivary levels of auto-Abs in patients with OSCC

<table>
<thead>
<tr>
<th>Characteristics (n)</th>
<th>p53 auto-Ab*</th>
<th>P</th>
<th>Survivin auto-Ab*</th>
<th>PB</th>
<th>CK-8 auto-Ab*</th>
<th>PB</th>
<th>Hsp60 auto-Ab*</th>
<th>PB</th>
<th>RPLP0 auto-Ab*</th>
<th>PB</th>
<th>Saliva IgA</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y &lt;53 (66)</td>
<td>4.389 ± 5.754 0.2225 2.773 ± 5.601 0.3868 2.371 ± 4.564 0.1811 1.443 ± 1.565 0.5268 1.138 ± 1.551 0.3805 1.87 ± 0.94 0.0124</td>
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<tr>
<td>≥53 (69)</td>
<td>4.562 ± 6.104 2.885 ± 4.448 2.691 ± 4.427 1.850 ± 5.326 1.726 ± 3.857 2.29 ± 0.82</td>
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<tr>
<td>Betel nut chewing</td>
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<tr>
<td>No (14)</td>
<td>2.993 ± 3.782 0.2333 1.696 ± 2.086 0.4385 1.885 ± 2.186 0.7431 1.012 ± 0.889 0.2970 0.843 ± 0.997 0.3634 1.95 ± 1.15 0.9051</td>
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<td>Yes (177)</td>
<td>4.652 ± 6.101 2.964 ± 5.276 2.607 ± 4.682 1.721 ± 2.852 1.500 ± 3.081 2.09 ± 0.87</td>
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<td>No (16)</td>
<td>2.789 ± 4.205 0.0429 1.778 ± 3.136 0.0156 2.403 ± 5.454 0.0876 1.267 ± 1.986 0.0301 0.935 ± 1.445 0.0635 1.94 ± 1.14 0.7278</td>
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<td>Yes (115)</td>
<td>4.710 ± 6.085 2.975 ± 5.247 2.548 ± 4.358 1.698 ± 2.807 1.499 ± 3.083 2.09 ± 0.87</td>
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<td>No (79)</td>
<td>3.815 ± 5.263 0.1076 2.818 ± 5.302 0.2231 2.397 ± 4.607 0.1534 1.718 ± 3.270 0.5377 1.278 ± 2.812 0.1441 1.98 ± 0.94 0.1876</td>
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<td>Yes (52)</td>
<td>5.478 ± 6.701 2.845 ± 4.673 2.732 ± 4.323 1.534 ± 1.565 1.660 ± 3.125 2.23 ± 0.82</td>
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<td>Buccal mucosa (52)</td>
<td>4.068 ± 4.756 0.1830 2.862 ± 5.152 0.1323 2.287 ± 4.315 0.0894 1.469 ± 1.764 0.0808 1.113 ± 1.440 0.4919 1.81 ± 0.97 0.0402</td>
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<td>Gingival (21)</td>
<td>2.763 ± 4.296 1.500 ± 2.561 1.426 ± 1.526 1.055 ± 9.030 733 ± 769 1.85 ± 1.08</td>
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<td>Lip (12)</td>
<td>5.844 ± 6.230 3.141 ± 2.285 4.500 ± 6.201 2.430 ± 2.014 1.540 ± 1.556 2.67 ± 0.45</td>
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<td>Tongue (29)</td>
<td>5.768 ± 7.311 3.071 ± 5.197 2.843 ± 4.957 2.319 ± 4.888 2.496 ± 5.566 2.38 ± 0.52</td>
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<td>Others (6)</td>
<td>3.551 ± 3.499 909 ± 442 1.121 ± 0.619 808 ± 473 1.013 ± 960 2.52 ± 0.61</td>
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<td>Well (60)</td>
<td>5.634 ± 6.415 0.0057 4.206 ± 6.837 0.0586 3.627 ± 6.179 0.1140 2.283 ± 3.777 0.0571 2.066 ± 4.095 0.0150 2.21 ± 0.89 0.0763</td>
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<td>Moderate (62)</td>
<td>3.010 ± 4.758 1.640 ± 2.149 1.568 ± 1.671 1.073 ± 890 340 ± 998 1.92 ± 0.90</td>
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<td>Poor (7)</td>
<td>7.541 ± 8.399 2.228 ± 2.914 2.072 ± 2.950 1.601 ± 1.909 1.174 ± 1.793 2.48 ± 0.58</td>
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<td>T1 (51)</td>
<td>4.561 ± 5.688 0.2655 2.753 ± 4.747 0.3900 2.872 ± 5.075 0.1856 1.691 ± 1.839 0.1788 1.281 ± 1.668 0.2943 2.05 ± 0.98 0.1725</td>
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<td>T2 (40)</td>
<td>4.663 ± 5.855 3.207 ± 5.192 2.908 ± 5.344 2.026 ± 4.300 1.654 ± 3.697 2.33 ± 0.68</td>
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<td>T3 (10)</td>
<td>3.308 ± 6.222 1.275 ± 1.767 1.543 ± 2.801 949 ± 1.315 895 ± 1.283 1.80 ± 0.87</td>
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<td>T4 (30)</td>
<td>4.467 ± 6.391 2.970 ± 6.072 1.774 ± 1.868 1.291 ± 1.173 1.562 ± 3.833 1.86 ± 0.98</td>
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<td>N0 (101)</td>
<td>4.572 ± 6.043 0.7014 3.048 ± 5.525 0.5801 2.793 ± 4.988 0.3974 1.766 ± 3.012 0.3215 1.369 ± 2.629 0.7218 2.06 ± 0.92 0.8436</td>
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<td>N1 and N2 (30)</td>
<td>4.148 ± 5.515 2.089 ± 2.827 1.645 ± 1.796 1.238 ± 1.265 1.833 ± 3.835 2.14 ± 0.86</td>
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<td>Stages I–II (80)</td>
<td>4.447 ± 5.431 0.2342 2.975 ± 5.073 0.3981 2.999 ± 6.432 0.1309 1.877 ± 3.305 0.1638 1.486 ± 2.870 0.0721 2.16 ± 0.87 0.1885</td>
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<td>Stages III–IV (61)</td>
<td>4.518 ± 6.645 2.988 ± 5.037 1.794 ± 2.170 1.281 ± 1.312 1.341 ± 3.060 1.95 ± 0.95</td>
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aData, mean ± SD of MFI.

bThe P values between two and more than two groups were determined by the Mann–Whitney U test and Kruskal–Wallis H test, respectively.

cData, mean ± SD of OD at 450 nm with 540 nm as the wavelength correction.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: C.-C. Wu, Y.-T. Chang, J.-S. Yu, W.-F. Chiang
Development of methodology: Y.-T. Chang, Y.-L. Liu, W.-F. Chiang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.-P. Chang, I.-L. Lee, W.-F. Chiang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y.-T. Chang
Writing, review, and/or revision of the manuscript: C.-C. Wu, Y.-T. Chang, H.-P. Liu, J.-S. Yu, W.-F. Chiang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y.-T. Chang, K.-P. Chang, J.-L. Lee, J.-S. Yu
Study supervision: C.-C. Wu, W.-F. Chiang

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References

Figure 3. Efficacy of using the salivary auto-Ab for detection of well-differentiated OSCC. The levels of auto-Ab in saliva samples collected from healthy controls (Ctrl; n = 131), patients with well-differentiated OSCC (n = 60), and patients with moderately differentiated OSCC (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 90% distribution (whiskers), and the middle 95% distribution (filled circles) of median fluorescence intensity (MFI) are shown; *, P < 0.05; **, P < 0.01.
quid chewing and tobacco smoking in Taiwan. Oral Oncol 2010; 46:276–82.
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.

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