Development of a Multiplexed Assay for Oral Cancer Candidate Biomarkers Using Peptide Immunoaffinity Enrichment and Targeted Mass Spectrometry

Yung-Chin Hsiao‡§§, Lang-Ming Chi¶¶, Kun-Yi Chien¶¶¶, Wei-Fan Chiang**‡‡, Szu-Fan Chen‡, Yao-Ning Chuang‡, Shih-Yu Lin‡, Chia-Chun Wu‖, Ya-Ting Chang‡, Lichieh Julie Chu‡§, Yi-Ting Chen†§§¶¶, Shu-Li Chia||, Chih-Yen Chiena, Kai-Ping Changb, Yu-Sun Changb, and Jau-Song Yu‡§

Oral cancer is one of the most common cancers worldwide, and there are currently no biomarkers approved for aiding its management. Although many potential oral cancer biomarkers have been discovered, very few have been verified in body fluid specimens in parallel to evaluate their clinical utility. The lack of appropriate multiplexed assays for chosen targets represents one of the bottlenecks to achieving this goal. In the present study, we develop a peptide immunoaffinity enrichment-coupled multiple reaction monitoring-mass spectrometry (SISCAPA-MRM) assay for verifying multiple reported oral cancer biomarkers in saliva. We successfully produced 363 clones of mouse anti-peptide monoclonal antibodies (mAbs) against 36 of 49 selected targets, and characterized useful mAbs against 24 targets in terms of their binding affinity for peptide antigens and immuno-capture ability. Comparative analyses revealed that an equilibrium dissociation constant (K_D) cut-off value < 2.82 × 10^{-9} M could identify most clones with an immuno-capture recovery rate >5%. Using these mAbs, we assembled a 24-plex SISCAPA-MRM assay and optimized assay conditions in a 25-μg saliva matrix background. This multiplexed assay showed reasonable precision (median coefficient of variation, 7.16 to 32.09%), with lower limits of quantitation (LLOQ) of <10, 10–50, and >50 ng/ml for 14, 7 and 3 targets, respectively. When applied to a model saliva sample pooled from oral cancer patients, this assay could detect 19 targets at higher salivary levels than their LLOQs. Finally, we demonstrated the utility of this assay for quantification of multiple targets in individual saliva samples (20 healthy donors and 21 oral cancer patients), showing that levels of six targets were significantly altered in cancer compared with the control group. We propose that this assay could be used in future studies to compare the clinical utility of multiple oral cancer biomarker candidates in a large cohort of saliva samples. Molecular & Cellular Proteomics 16: 10.1074/mcp.RA117.000147, 1829–1849, 2017.

Oral cavity cancer, one of the most common cancers worldwide, accounts for more than 10,000 deaths annually (1, 2). These cancers can occur at different locations in the oral cavity, including the tongue, buccal area, gingiva, lip, floor of mouth, and hard palate. The main risk factors for oral cavity cancer include alcohol and tobacco use, betel quid chewing, and viral infections (3–6). The World Health Organization predicts that the incidence of oral cavity cancer will continue to increase worldwide for the next several decades, especially in Asia, because of distinct cultural practices such as betel-quid chewing (6, 7). The incidence of oral cavity cancer in Taiwan has increased over the past two decades; between 1996 and 2009, the age-standardized incidence in males reached...
24.64/100,000 annually, which is among the highest in the world (8). The majority (~90%) of oral cavity cancer cases are oral squamous cell carcinomas (OSCCs), which are quite locally aggressive and are characterized by a moderate locoregional recurrence rate and relatively poor survival, with a 5-year overall survival rate of ~60% (9–11). Despite recent advances in therapeutic modalities and strategies, OSCC has maintained one of the lowest 5-year survival rates among all major cancers (1, 2). This low rate is at least partly a reflection of the fact that ~50% of OSCC patients present with late stage (stage III and IV) tumors (12).

Most cases of OSCC develop from potentially malignant oral disorders characterized by visible changes in oral mucosa (13, 14). Currently, visual inspection of oral mucosa combined with pathological examination of lesion showing morphological alternation and/or colored change is the most common strategy for oral cancer detection. However, determining which oral lesions warrant referral to a specialist for further histological confirmation is often challenging for frontline health workers, mainly because early-stage oral cancer is largely indistinguishable from certain benign or inflammatory disorders; moreover, submucous fibrosis may complicate detection. Therefore, identifying effective biomarkers that can serve as more objective molecular tools represents an urgent need for aiding early detection and/or management of OSCC. Currently, no biomarkers that meet this need are approved by official health agencies in endemic areas (15).

Over the past two decades, numerous protein biomarker candidates implicated in the carcinogenesis of OSCC have been discovered through analyses of DNA, RNA and/or protein expression in different specimens, such as cultured cells, tissue, plasma/serum, and saliva (16–19). However, very few of these candidates have been verified in parallel in clinical specimens (plasma/serum and/or saliva) to test and compare their clinical utility. The lack of careful validation that such biomarker candidates warrant and the task of generating appropriate reagents/technology platforms for multiplexed quantification assays of selected targets represent two obvious bottlenecks to the successful translation of biomarker candidates into clinical use (20, 21).

Multiplexed quantification assays for verification of protein biomarker candidates in clinical specimens require high precision, reproducibility, and sensitivity. Some enzyme-linked immunosorbent assay (ELISA)-based multiplexed assays have been successfully applied to quantify multiple targets in clinical specimens (22, 23), but the availability of specific antibodies against selected targets, the inconsistency of quantification results using different antibodies and false-positive or negative interference caused by some endogenous antibodies, such as autoantibodies and heterophilic antibodies, create additional roadblocks in the biomarker verification pipeline (24–27). In the past decade, a multiplexed and targeted quantitation technique based on peptide quantification using liquid chromatography coupled multiple-reaction-monitoring mass spectrometry (LC-MRM-MS) approach has been described for quantifying dozens of proteins in a single assay with acceptable precision in different biofluids, such as plasma (28–30), urine (31, 32), cerebrospinal fluid (33), and saliva (34). In this MRM-MS technique, specific transitions of precursor/product ions are selected for detection using triple quadrupole MS instruments (or QTRAP operating in triple quadrupole mode). The coefficients of variation (CVs) for target protein quantification using well-designed, scheduled MRM coupled with stable isotope-coded standard (SIS) peptides can be less than 15% (35). Protein concentrations in unfractionated plasma in the mid to high nanogram per milliliter range have been reported with high reproducibility within and across laboratories and instrument platforms (28, 35). To further improve the quantitation limit for targeted peptides, researchers have developed a platform that combines immuno-affinity enrichment using antipeptide antibodies and MRM-MS, known as “SISCAPA-MRM” (stable isotope standards with capture by anti-peptide antibodies coupled with multiple reaction monitoring MS) or “immuno-MRM” (36, 37). Recent studies have demonstrated the high precision quantification of human plasma proteins using the automated SISCAPA-MRM (38), as well as acceptable reproducibility across independent laboratories for assaying plasma proteins (39). The feasibility of assembling a single multiplexed assay for measuring more than 100 target peptides in clinical plasma samples was also manifested (40). High-affinity antipeptide antibodies have played a key role in the successful development of these multiplex assays.

As noted above, very few candidate OSCC biomarkers have been verified using multiplexed assays in clinical specimens. Thus, development of effective multiplexed assays for potential OSCC biomarkers should greatly facilitate the future development of clinically useful biomarkers for OSCC detection and/or management. In this study, we report the production and characterization of anti-peptide mouse monoclonal antibodies (mAbs) and the subsequent successful development of a 24-plex SISCAPA-MRM assay for quantifying multiple OSCC candidate biomarkers in saliva samples. In a further application, we used this assay to detect and quantify target proteins in saliva samples from healthy controls and OSCC patients.

**EXPERIMENTAL PROCEDURES**

**Experimental Design and Statistical Rationale** — The purpose of this study is to establish a multiplexed and automated SISCAPA-MRM assay for verification of OSCC biomarker candidates in the saliva...
samples. The overall study design is shown in Fig. 1, which includes selection of 49 candidate proteins and their proteotypic peptides (one peptide for one protein), production of anti-peptide mouse mAbs, screening of useful mAbs, establishment and optimization of the multiplex SISCAPA-MRM assay, and application of the optimized assay to measure candidate proteins in a small set of clinical saliva samples for evaluating the applicability of the developed assay. For screening of useful mAbs, analyses of binding kinetics (using surface plasmon resonance (SPR) technique) and peptide-capture ability (using SISCAPA-MS platform) were performed to characterize the produced mAbs. To assess whether the binding kinetics reflects the peptide-capturing ability of mAbs, the following statistical analyses were employed. Correlation between equilibrium binding constant ($K_d$) and peptide-capture ability of the produced mAbs were analyzed using Spearman’s correlation. The power of kinetic constants ($k_a$, $k_d$, and $K_d$) to predict mAbs with higher peptide-capture ability was estimated by the receiver operating characteristic (ROC) curve analysis using SPSS statistical software. Two kinds of magnetic beads and three different elution solutions were applied to optimize the 24-plex SISCAPA-MRM assay, and the performance characteristics (the linearity of the response curves, and values for LOD, LLOQ, coefficient of variation (CV), and accuracy for all 24 targets) of the optimized assay were evaluated in a background of saliva digest (25 μg protein). Finally, two model saliva samples (pooled from 20 healthy donors and 20 OSCC patients, respectively) and another set of 41 individual saliva samples (20 healthy donors and 21 OSCC patients) were used to evaluate the applicability of the developed assay for quantifying all 24 targets. Three process repeats were performed independently (from digestion to the final LC-MRM/MS step) for each of the model/individual clinical samples, and significance of differences of the quantified target protein levels between two groups (healthy donors and OSCC patients) were analyzed by the Mann Whitney test. In addition, we prepared another model saliva sample (pooled from seven OSCC patients) to evaluate the intra- and interday reproducibility of the 24-plex SISCAPA-MRM assay as well as the stability of the targets in unprocessed or trypsin-digested saliva sample during storage at different conditions (~80, 4, and 25 °C) for various time periods (1–7 or 1–14 days). Each sample was assayed in triplicate (i.e. three process repeats). Our study design for establishing and testing a 24-plex SISCAPA-MRM assay with internal standards for accurate quantification of selected biomarker candidates in a small set of clinical saliva samples belongs to a Tier 2 analysis.

Selection of Candidate Biomarkers and Their Signature Peptides—A total of 49 candidate OSCC biomarkers were selected from review of >1,400 papers related to OSCC or head-and-neck cancer published between 1995 and 2012 in the PubMed database and our previous studies of OSCC/head-and-neck cancer biomarkers using genomic and proteomic approaches (16, 41–46). The procedure used for prioritization and selection of these 49 targets was detailed in our recent work (34). Appropriate signature peptides of these 49 targets (supplemental Table S1) were selected and used for the development of the SISCAPA-MRM assay (see below).

One signature peptide for each target protein was selected for mAb production and MRM detection from the list of tryptic peptides generated in silico by the MRMPlot software (version 2.1; AB-Sciex). The following selection criteria were used: (a) MS-detectable peptides with high identification score in our shotgun MS data sets—secretomes of cancer cell lines and primary cells (16, 47, 48) and tissue proteomes of OSCC (42, 43, 49); (b) peptides without sequences that could potentially lead to missed cleavages, such as RP and KP; (c) unique peptides containing 8 to 23 residues without any known post-translational modification sites, determined from the human protein reference database (HPRD) (50); (d) peptides without chemically reactive amino acids (such as Cys, Met) and unstable sequences (such as NG, DG, QG, and N-ter Q); and (e) peptides containing more hydrophilic, charged, or branched amino acids and/or proline residue. For candidate proteins for which no empirical evidence was available or no suitable peptide was found in the shotgun MS data sets, we obtained all possible tryptic peptides by in silico prediction and selected their signature peptides using the criteria described above but allowing the presence of unstable sequences (NG, DG, QG or N-ter Q).

Peptide Synthesis—Synthetic (light) peptides with or without a C-terminal Cys or GSGC linker were purchased from Kelowna International Scientific (Taipei, Taiwan). Stable isotope-coded peptides, used as internal standards (SIS peptides), were synthesized and purified at the UVic-Genome BC Proteomics Centre, BC Canada, and their concentrations were determined as previously described (28). The purity of synthetic stable isotope-labeled peptides is higher than 90% (most peptides have purity higher than 95%), as evidenced by capillary zone electrophoresis after HPLC purification. The purity of synthetic peptide used as immunogen for antibody production is higher than 75% (the majority are higher than 85%). The inaccuracy of peptide mass was less than 0.1% for the synthetic peptides. Furthermore, the sequence of each synthetic peptide was double-checked in the lab using LC-MS/MS.

Production of Antipeptide mAbs—One tryptic peptide for each of the 49 prioritized target proteins (supplemental Table S1) was selected as an antigen for the production of mAbs in mice. Each synthetic peptide was chemically conjugated to a carrier protein, either bovine serum albumin (BSA) or ovalbumin, via a C-terminal Cys or GSGC linker, as previously described, using Reichlin’s procedure (51). Briefly, 1 ml of dissolved peptide (2 mg/ml) was mixed with 1 ml of BSA or ovalbumin solution (4 mg BSA or ovalbumin in 1 ml of 2x phosphate-buffered saline [PBS]), followed by gentle addition of 2 ml of 2% glutaraldehyde/PBS. After a 2-hour reaction at room temperature, 1 ml of 1 M glycine/PBS was added slowly to stop the reaction. The resulting peptide-BSA or -ovalbumin conjugates were extensively dialyzed against PBS for 3 days. The dialyzed peptide-BSA conjugates were used as immunogens for mAb production in mice, and the peptide-ovalbumin conjugates were used as antigens for evaluating serum titers in mice and for subsequent screening of hybridomas generated from immunized mice that showed good immune responses. The mouse mAbs for these peptide antigens were produced at Abnova Inc. (Taipei, Taiwan). Briefly, 1 to 2 mice were immunized with each target, and spleens of mice with serum titers more than 2-fold greater than background signal at a 16,000× dilution were harvested. Lymphocytes from each mouse were fused with myeloma cells to prepare ten 96-well plates containing hybridomas (~1000 clones). The supernatants of hybridomas were screened by ELISA using peptide-ovalbumin, free peptide, BSA, and ovalbumin as antigens. The clones that have higher signal against peptide-ovalbumin or free peptide than BSA and ovalbumin were defined as positive clones. Up to 20 clones for each target were selected and expanded for further experiments, including antibody-antigen binding affinity and SISCAPA assays. Finally, one clone for each target with the highest capture ability in SISCAPA assays was selected for ascites production in mice, and the resulting monoclonal antibodies were purified by protein-A affinity chromatography.

Isotyping of Mouse Immunoglobulin G (IgG)—The subclasses of the generated mouse mAbs were determined using a rapid ELISA mAb isotyping kit (Thermo Fisher Scientific, San Jose, MA). Briefly, the culture supernatants of monoclonal hybridoma cells were added to 8-well ELISA strip-plates in which different wells were precoated with anti-mouse heavy-chain capture antibodies (anti-lgG1, anti-lgG2a, anti-lgG2b, anti-lgG3, anti-lgA and anti-lgM) or anti-mouse light-chain capture antibodies (kappa or lambda), followed by addition of goat anti-mouse IgG+IgA+IgM conjugated with
horseradish peroxidase (HRP). After a 1-hour incubation, the plates were washed three times with 250 µl wash buffer and reacted with 3.3' ,5',5'-tetramethylbenzidine (TMB) substrate for 10 min. The optical density of each well was measured at 450 nm using a microplate reader.

Quantification of Mouse IgG—The concentrations of mouse IgG in the culture supernatants of monoclonal hybridoma cells were quantified by ELISA using a pair of goat anti-mouse Fab and Fc antibodies (AfiniPure grade) purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Briefly, 96-well plates were coated with 100 µl of the first goat anti-mouse antibody (diluted 1:2000 in PBS) at 4 °C overnight. After washing wells three times with wash buffer (50 mM Tris pH 8.0, 0.14 mM NaCl, 0.05% Tween 20), 200 µl of blocking buffer (50 mM Tris pH 8.0, 0.14 mM NaCl, 3% BSA) was added to each well and plates were incubated for 1 h at room temperature. After repeated washing steps, whole molecule mouse IgG standards (ChrmPure grade) or supernatants of monoclones, serially diluted with diluent buffer (50 µl Tris pH 8.0, 0.14 mM NaCl, 0.05% Tween 20, 1% BSA), were added to wells and incubated for 1 h. The plates were washed five times with wash buffer, refilled with 100 µl of the second HRP-conjugated goat anti-mouse antibody (diluted 1:5000 in PBS) and incubated for an additional 1 h. After washing wells five times with wash buffer, 100 µl of TMB substrate solution was added to each well and plates were incubated at room temperature for 10 min. The reaction was stopped by adding 100 µl of 1 M H2SO4, and the optical density of each well was measured at 450 nm using a microplate reader.

Measurement of Antibody-Peptide Antigen Binding Kinetics Using Surface Plasmon Resonance—Binding kinetics of interactions between antibodies and peptide antigens were quantitatively analyzed by surface plasmon resonance (SPR) using a Biacoore 3000 optical biosensor system (GE Healthcare, Little Chalfont, UK). CM5 sensor chips (GE Healthcare) consisting of four channels were used for these experiments. Channels 1 and 3 were used as reference channels, which were blocked using an amine-coupling kit (GE Healthcare); channels 2 and 4, in which peptide antigens were immobilized, were immobilized by PDEA thiol coupling kit (GE Healthcare), were used as analytic channels. HBS-EP (0.1 mM HEPES, 1.5 mM NaCl, 30 mM EDTA, 0.05% v/v surfactant P20, pH 7.4), vacuum filtered and degassed prior to use, was employed as the flow buffer. CM-dextran surfaces were activated by injecting a freshly prepared solution containing a 1:1 mixture of 0.4 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 0.1 mM N-hydroxysuccinimide (NHS) for 5 min (flow rate, 20 µl/min). After activation, surfaces of reference channels were immediately blocked by injecting 1 mM ethanolamine-HCl (pH 8.5) for 10 min (flow rate, 9 µl/min). For analytic channels, the activated CM-dextran surfaces were further modified by a 10-min injection of a solution containing 120 mM 2-(2-pyridinyl)dithio)ethanamine hydrochloride (PDEA) in ddmH2O2 to conjugate an active thiol-group to the chip surface. Peptide antigens (100 µg/µl) containing a C-terminal cysteine linker were then injected at a flow rate of 10 µl/min for a few minutes until the change in resonance units (ΔRU) value reached 100. The coupling reaction was stopped by injecting a solution containing 50 mM l-cysteine in 0.1 mM sodium acetate (pH 4.0) for 10 min. Finally, the channels were washed twice by injecting 100 mM NaOH (20 µl/min) for 2 min each and re-equilibrated with HBS-EP by flowing the buffer through the channels. Afterward, the protein concentration of culture supernatant of each hybridoma clone was first determined by ELISA, and 1 µg of mouse IgG from each supernatant was captured by 20 µl of sheep anti-mouse IgG magnetic beads (Dynabeads M-280; Invitrogen, Carlsbad, CA) for 1 h. IgG-captured beads were transferred to the next plate, then incubated with 1000 fmol of light peptide in the presence of trypsin-digested plasma background (10 µg protein) for an additional 2 h, and washed three times with PBS (2 min/wash). Thereafter, 50 µl of elution buffer (5% acetic acid or 0.2% triethylamine) containing 20 fmol of heavy peptide was added into the wells and incubated for 8 h. All eluents were then quantified using a C18 column (Synergy Hydro-RP, 75 µm × 12 cm, 2.5 µm particle size; Phenomenex, Torrance, CA) coupled with a qTOF mass spectrometer (Bruker Daltonics, Billerica, MA). The recovery rate of the light peptide in immunoprecipitates was calculated using the following formula: area ratio of L/H x 20 fmol/1000 fmol 100%.

Collection of Saliva Samples—Saliva samples from healthy controls and OSCC patients were collected in two hospitals (Chi-Mei Medical Center, Liouying, Taiwan and Chang Gung Memorial Hospital, Linkou, Taiwan) with Institutional Review Board approval and written informed consent from each patient and processed as described previously (34, 52). The donors avoided eating, drinking, smoking, and using oral hygiene products for at least 1 h before collection. The obtained saliva samples were centrifuged at 3,000 × g for 15 min at 4 °C. The resulting supernatant was collected, treated with a protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO), and stored in aliquots at −80 °C. All subjects signed an informed consent form approved by the Institutional Review Board of Chi-Mei Medical Center and Chang Gung Memorial Hospital permitting the use of saliva samples collected prior to treatment. All experiments were performed in accordance with approved guidelines. The saliva samples collected from Chi-Mei Medical Center (20 healthy controls and 21 OSCC patients) were used to generate two pooled saliva samples (5 µg of salivary protein in each case), whereas those from Chang Gung Memorial Hospital (20 healthy controls and 21 OSCC patients) were used as individual samples for target verification using SISCAPA-MRM assays.

Determination of Total Protein in Saliva Samples—Protein concentration of saliva samples was determined by BCA assay kit (Pierce Chemical, Rockford, IL) according to the manufacturer’s protocol. Briefly, twenty-five µl of the standard samples (0, 0.625, 0.125, 0.25, 0.5, 1 µg/µl of BSA) and saliva samples (diluted 5-fold with ddH2O2) were incubated with the pre-mixed working reagent (50:1 mixture of reagent A and reagent B) for 30 min at 37 °C in eppendorf and then transferred to an EIA/RIA polystyrene ELISA 96-well plate (Cat. No. 9018, Corning Costar, Tewksbury, MA). The absorbance at 562 nm for each well was measured by a spectrophotometer and used to determine the protein concentration of saliva samples.

Tryptic Digestion and Desalting of Saliva Samples—Saliva samples containing equal amounts of protein were subjected to a tryptic digestion protocol as previously described (53). Briefly, 25 µg of protein diluted with an appropriate amount of 25 mM ammonium bicarbonate (Sigma) was mixed with 40 µl of 20% (w/v) sodium deoxycholate (DOC; Sigma) and then boiled for 5 min. The denatured proteins were reduced by incubating with 5 mM Tris(2-carboxyethyl)
phosphinehydrochloride (TCEP; Sigma) at 60 °C for 30 min and then alkylated by incubating with 10 μl iodoacetamide (Sigma) at 37 °C for 30 min in the dark. The sample was then diluted 3-fold with 0.1 M ammonium bicarbonate and digested with 1 μg trypsin (Agilent Technologies, Santa Clara, CA) at 37 °C overnight. Digestion was stopped by boiling for 15 min, followed by addition of 4 μg trypsin inhibitor (Sigma). Afterward, DOC was precipitated by adding 0.1% trifluoroacetic acid (TFA; Alfa Aesar, Tewksbury, MA) and 0.4% formic acid (FA) to the digests, and the precipitated DOC was removed by centrifugation at 15,000 × g for 10 min at room temperature. The supernatants were then desalted with solid-phase extraction (SPE) equipment using Waters Oasis HLB 96-well μElution plates (2 mg) (Waters, Milford, MA). Briefly, supernatants were loaded onto the columns and passed through them twice. After washing columns with 2 ml of 3% acetonitrile (ACN; J.T. Baker Chemical, Phillipsburg, NJ), peptides bound to the columns were eluted with 100 μl of 75% ACN per column, dried using a spin vacuum, and stored at −20 °C until use. Before SISCAPA experiments, the dried samples were rehydrated in 50% ACN and diluted in PBS containing 0.03% CHAPS to achieve a concentration of 0.125 μg peptide/μl and an ACN percentage less than 5%.

Automated and Multiplexed Immuno-affinity Enrichment—To establish a multiplexed SISCAPA-MRM assay using 24 selected mAbs, we adopted a method described by Whiteaker et al. (37), with some modifications. A KingFisher magnetic particle processor was used for automated handling of the transfer of magnetic beads between plates (plates 1 to 7). The wells of plate 1 contained 120 μl of protein G magnetic beads (GE Healthcare) and 80 μl of PBS plus 0.03% 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS; USB Corp., Cleveland, OH). The beads in plate 1 were washed for 5 min, transferred to plate 2 containing 24 mouse monoclonal antibodies against 24 analytes (1 μg antibody for each analyte), and incubated for 1 h. Mouse IgGs bound to protein G magnetic beads were captured by the magnetic probe of the processor, transferred to plate 3 containing 25 μg of trypsin-digested saliva samples and 100 fmol of spiked SIS peptides, and then incubated in plate 3 for 2 h. IgG-captured beads were then washed three times by sequentially transferring to plates 4, 5, 6, and 7, which contained 200 μl of PBS (plates 4 and 5) or 0.1× PBS (plate 6). The total wash time through plates 4 to 6 was about 5 min. The captured peptides were then eluted in plate 7 containing 50 μl of 5% acetic acid (J.T. Baker) and 70% ACN. After elution, the beads were collected from plate 7 and transferred back to plate 6. Finally, all eluants (without beads) were subjected to LC-MRM-MS analysis using a nano-ACUITY UPLC system equipped with a nanoACQUITY UPLC C18 column (100 μm × 100 mm, 1.7-μm particle size; Waters) was used to separate the peptide samples. Samples (4 μl) were injected onto a C18 resolving analytical column at a flow rate of 0.6 μl/min in 99% buffer A (0.1% FA in H2O) (J.T. Baker) and 1% buffer B (0.1% FA in ACN; J.T. Baker) for 15 min. Samples were then separated at a flow rate of 0.4 μl/min with a 38-min linear gradient from 3% to 22% buffer B, a 5-min linear gradient from 22% to 30% buffer B, and a final 2-min linear gradient from 30% to 95% buffer B. The resolved fractions were applied to an AB/MDS Sciex 5500 QTRAP with a nano-electrospray ionization source controlled by Analyst 1.5.1 software (all from AB Sciex, Singapore). MRM acquisition methods were constructed using three selected transitions (Q1/Q3 pairs) per peptide, as shown in supplemental Table S2, and each transition was monitored within a 6-min scheduled MRM detection window. Optimization of data acquisition parameters (collision energy, CE; declustering potential, DP; entrance potential, EP; collision entrance potential, CEP; and collision exit potential, CXP) were essentially performed as previously described (31, 34), with the following modifications: ion spray voltage, 2300 V; curtain gas setting, 20 psi (UHP nitrogen); interface heater temperature, 150 °C; and MS operating pressure, 3.5 × 10⁻⁵ Torr. Q1 and Q3 were set to unit resolution (0.6–0.8 Da full width at half height; detailed in supplemental Table S3).

MRM Data Analysis and Generation of Response Curves—All MRM data were processed based on the preloaded mass list of transitions for the 24 target peptides using Skyline software (54). The integration boundaries were automatically determined by Skyline built-in function (CRAWDAD algorithm with Savitzky–Golay smoothing) and then adjusted manually to avoid possible interference in the spectrum. Once the interference signal was observed in each spectrum, the boundaries were manually narrowed to exclude the interference, where the boundaries of light and heavy peptide were the same. The final peak area is determined by taking trapezoidal area of the unsmoothed chromatogram. Endogenous and analyte peptide-specific peaks were identified based on their co-elution with exogenously added ¹³C/¹⁵N-labeled heavy peptides, which were 8 and 10 Da heavier for Ly- and Arg-containing peptides, respectively. The concentration of each ¹³C/¹⁵N-labeled heavy peptide is known; therefore, the concentration of the target peptide in samples was determined as the ratio of the observed peak area to that of the heavy peptide. Response curves for the 24 peptides were generated from sextuplicate experiments. For each target, serially diluted heavy peptide (0, 0.24, 0.49, 0.98, 1.95, 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250, 500, and 1000 fmol) and a constant amount of light peptide (100 fmol) were spiked into a saliva digest background (25 μg protein) and analyzed by LC-MRM-MS. The limit of detection (LOD) was determined using the MRM statistical software, QuaSAR; this software uses the “blank and low concentration sample” method, in which the mean of the blank samples and the standard deviations of blank and low-concentration samples are estimated (35). The lower limit of quantification (LLOQ) was calculated as the LOD value multiplied by 3 (55).

Preparation of Saliva Samples for Characterizing the Intraday/Interday Reproducibility and the Stability of the Targets Assayed by the 24-plex SISCAPA-MRM Assay—The saliva samples collected from Chang Gung Memorial Hospital (seven OSCC patients) were used to generate another pooled saliva sample (250 μg of salivary protein in each case) for this evaluation. Both unprocessed and trypsin-digested saliva samples were evaluated. Briefly, the pooled saliva sample was divided into two parts. The first part (designed as unprocessed saliva) was further separated into 10 aliquots; one aliquot (labeled as day 0) was subjected to the 24-plex SISCAPA-MRM assay immediately, and the other nine aliquots were divided into three groups, which were stored at −80 °C, 4 °C, and 25 °C for 1, 3, and 7 days, respectively (labeled as day 1, 3, or 7), and then thawed for the 24-plex SISCAPA-MRM assay. Each sample was assayed in triplicate, and the assay results of the day 0 sample and the samples stored at −80 °C for 1, 3, and 7 days were used to evaluate the intra-day/inter-day reproducibility. Additionally, the assay results of the samples stored at −80 °C, 4 °C and 25 °C for different intervals were used to evaluate the stability of the targets in their undigested forms. The second part (designed as digested saliva) was completely digested by trypsin (1 μg trypsin at 37 °C overnight), divided into 13 aliquots, and dried by SpeedVac. The dried samples were then stored at different conditions and processed/assayed as described above except the inclusion of a longer storage time (14 days). The assay results from these samples were used to evaluate the stability of the targets in their trypsin-digested forms.

Bead-based Suspension Immunoassay for the Detection of MMP1, MMP3, and TNC—Protein concentration of MMP-1, MMP-3, and tenascin (TNC) in saliva specimens were determined by Luminex Bead-based Suspension Immunoassay (Bead-based Suspension Immunoassay for the Detection of MMP1, MMP3, and TNC).
multiplex assay kit (R&D Systems, Minneapolis, MN) according to the manufacture’s protocol as previously described (23, 56). By using filter-bottom 96-well microplates (Millipore) and vacuum manifold, the recommend protocols were performed automatically. At the end of all reactions, the beads were suspended in assay buffer and analyzed using the Bio-Plex 200 system (Bio-Rad Laboratories, Hercules, CA). The detection range was 23.18–11011 – 16,900 pg/ml for MMP-1, 41.5–11011 – 30250 pg/ml for MMP-3, and 11.22–11011 – 24545 pg/ml for tenascin. All saliva specimens were analyzed in 5-fold dilution, and the detected protein concentration lower than the detection limit was considered as zero.

Statistical Analysis—Consistency of quantification between two ELISA kits for measuring IgG concentrations and between measured concentration and theoretical concentration (response curve for MRM-MS analysis) were evaluated by linear regression with R2 and p value. Correlations between quantifications by SISCAPA-MRM and Bio-plex assay were analyzed using Spearman’s correlation.

RESULTS

Experimental Workflow for Development of a Multiplexed SISCAPA-MRM Assay Targeting OSCC Biomarker Candidates—The strategy used here is schematically depicted in Fig. 1. We first retrieved ~300 OSCC candidate biomarkers from published reports with positive verification data for small-volume clinical samples and our in-house dataset, and then narrowed this list to 49 targets, with a focus on body fluid-accessible proteins that could be predicted by the secretory pathway or the secretome database, or which could be detected directly by saliva/plasma proteome profiling. Signature peptides of prioritized targets were then selected for mouse mAb production and synthesis of stable isotope-labeled (SIS) peptides, and the sequence of the synthetic peptides were confirmed by MALDI-TOF/TOF MS analysis. Using both SPR and SISCAPA-MS assays, we then further analyzed culture supernatants of hybridomas that passed the first ELISA screening to obtain peptide-binding affinity and peptide-capture ability data for each mAb. Twenty-four mAbs that showed high peptide-binding affinity, acceptable peptide-capture ability, and reasonable production yield were selected for development of the multiplexed SISCAPA-MRM assay. Experimental conditions, reagents and parameters for this assay were extensively examined, optimized and characterized, and finally applied to quantify more than

Fig. 1. Study design and experimental workflow for the development of a multiplexed SISCAPA-MRM assay targeting OSCC biomarker candidates. See text for details.
Selection of Proteotypic Peptides of the 49 Candidate Proteins and Production of Antipeptide mAbs—We applied the criteria described in Materials and Methods to select a representative, MS-friendly tryptic peptide for each of the 49 candidate proteins as a surrogate for quantification by MRM, as well as for use as an antigen for the production of mAbs. The 49 candidate proteins and their signature peptides are listed in supplemental Table S1. Synthetic peptides containing an extended C-terminal linker (GSGC or C) were chemically conjugated to the large carrier protein, BSA, and used as antigens for immunization in mice. After immunization for 1 month, a serum sample was taken from each immunized mouse to test the titer of antibodies by ELISA using free peptides or peptide-ovalbumin conjugates as screening antigens. Forty-two peptide antigens elicited acceptable to strong immune responses in mice, and these mice were further processed to hybridoma fusion and selection. This process was generally successful, yielding 363 IgG clones against 36 targets; no positive clones were obtained from mice immunized with the other five target peptides (GDF15, HMGCS1, TGFB1, HGF and PLAU) (supplemental Table S1). The numbers and isotypes (IgG1, IgG2a and IgG2b) of the positive clones pro-
The majority (~78%) of the 36 targets produced mAbs with two or three IgG isotypes; the exceptions were CYR61, DSG3, FLNA, GANAB, GBP1, IGFBP3, MMP1, and SOD2 (Fig. 2A). The overall proportions of the three isotypes in these 363 clones were 38.6% (140/363) for IgG1, 22.8% (83/363) for IgG2a, and 38.6% (140/363) for IgG2b (Fig. 2B). To further screen these mAbs by SPR and SISCAPA-MS assays, we first determined the concentration of IgG in the culture supernatant of each hybridoma using sandwich ELISAs with anti-Fab and anti-Fc antibodies. IgG concentrations in supernatants of these 363 hybridoma clones ranged from 0.5 to ~400 µg/ml, and the average concentration of IgG1, IgG2a, and IgG2b was 22.3, 22.4, and 103.4 µg/ml, respectively (Fig. 2C). Notably, the concentrations of IgG2b were generally much higher than those of the other two isotypes (IgG1 and IgG2a), an observation that was further confirmed using a different ELISA against IgGs (supplemental Fig. S1).

Screening of mAbs Using a Biacore 3000 Optical Biosensor and SISCAPA-MS Assay—To assay the performance of each mAb as a catcher for peptide antigen, we used the SPR-based Biacore 3000 optical biosensor to screen high-affinity mAbs, and then evaluated the immuno-capture recovery for target peptides using a quantitative, MS-based SISCAPA assay. Representative results of SISCAPA-MS and SPR assays using four mAb clones against MMP3 are presented in

Fig. 3. Screening of hybridoma supernatants by SISCAPA-MS and SPR, and comparative analysis of binding affinity and peptide-capture ability of the 363 mAbs. A, SISCAPA-MS and SPR analyses of a representative set of mouse mAbs (clone no. 1, 4, 10, and 13) against the MMP3 signature peptide (QIAEDFFGIDSK). The extracted-ion chromatogram (left panel) and line-spectrum profile (middle panel) of antibody-captured light peptide (m/z (H2+ ) = 664.33) and spiked heavy peptide (m/z (H2+ ) = 664.33) were used to calculate the peptide-capture ability of individual antibodies. Right panel: The binding kinetic curve and equilibrium dissociation constant (K_D) of each antibody toward the peptide, coated on the SPR sensor chip. B, Correlation analysis of the peptide-capture ability and K_D value of the 363 mAbs. C, The distribution of mAbs with different K_D values. The number of mAb clones with different K_D values is indicated above the columns. The peptide-capture recovery rates (<1%, 1–5%, or >5%) of these mAbs are denoted by columns with different gray scales. D, The K_D value distribution of mAbs with different peptide-capture recovery rates (<1%, 1–5%, and >5%). The mean ± S.D. value for each group is indicated by a black solid line.
Fig. 3A, which showed the difference in signals of captured light peptide measured by SISCAPA-MS (left and middle panels) for antibodies with poor and good affinities (KD value) measured using the SPR platform (right panel). These results indicate the feasibility of our strategy using two assay platforms to screen the peptide-capture ability of each mAb.

In this screening procedure, different clones of mAbs produced against specific peptide antigens were first analyzed on a peptide-immobilized chip, which is reusable for multiple clones against the same peptide antigen, to obtain a reliable comparison between different mAbs. Considering the bivalent effect contributed by the two flexible antigen-binding arms of IgGs, we first optimized our SPR assay condition using a model mAb, the SPP1 clone 5E7. As shown in supplemental Fig. S2, these tests indicated that high peptide density (RU > 200) on a chip or low IgG concentration (< 2 µg/ml) caused a more apparent bivalent effect in binding curve analyses, as evidenced by very slow disassociation between antibody and peptide antigen. Accordingly, we immobilized each peptide antigen on a chip at a density of ~130 RU and applied the IgG solution at a single concentration, in the range of 3–5 µg/ml, for each of the 36 targets. The equilibrium dissociation constant $K_D$, which represents the peptide-binding response of each mAb, is calculated from association (ka) and dissociation (kd) rate constants. To further confirm the stability and reproducibility of the measured $K_D$ values between days for different clones of a particular target, we continuously monitored the response and baseline signals of a total of 28 clones against CA2 peptide on two different days. As shown in supplemental Fig. S3A, baseline signals were stable in the range of ±15 RU, and antibody-binding responses to the immobilized peptide were much higher than baseline signals. Interday experiments also indicated good reproducibility of the $K_D$ values determined for these mAbs (supplemental Fig. S3B). We applied the same assay conditions to the remaining mAb clones, and determined the $K_D$ values of all 363 clones, which were found to range from 1.18 × 10^{-10} to 7.04 × 10^{-7} m (supplemental Fig. S4A). The frequency distribution of clone $K_D$ values was bell-shaped, and most clones (81.3%, 295/363) exhibited $K_D$ values in the range of 1 × 10^{-8} to 1 × 10^{-10}.

In addition to binding kinetics analysis, we conducted a KingFisher magnetic bead processor-assisted SISCAPA-MS assay to determine the peptide-capture ability of each mAb. This assay was performed by adding 1 µg IgG (from the hybridoma culture supernatant) into binding reaction mixtures containing 1000 fmol of synthetic $^{12}$C-light peptide. After capture, wash and elution steps in different plates, eluents were spiked with 20 fmol of $^{13}$C-heavy peptide and analyzed by LC-MS using a qTOF-MS. Using the post-spiked $^{13}$C-heavy peptide as an internal standard, we could determine the recovery rate of light peptide captured by each mAb (see Materials and Methods for details). The peptide-capture recovery rates of these 363 clones were determined to range from 0% to 53%, with 93 clones showing recovery rates >5% (supplemental Fig. S4B). The details of binding kinetic constants (ka, kd, and $K_D$) and peptide-capture recovery rate for each of the 363 mAbs are shown in supplemental Table S4.

**Correlation Between Binding Affinity and Peptide-capture Ability of the 363 mAbs**—Because there is very limited information relating to the correlation between binding affinity and the peptide-capture ability of antipeptide mAbs in a large-scale setting, we addressed this issue using data generated for the 363 mAbs (9 without SISCAPA-MS analysis). As expected, the 2-D scatter plot showed the tendency of negative association between the peptide-capture abilities (linear scale in the y axis) and their $K_D$ values (reverse and logarithmic scale in the x axis) of these anti-peptide mAbs, and the Spearman’s correlation analysis revealed a moderate correlation ($r = 0.579, p < 0.0001$) between the two variables (Fig. 3B). The percentage of clones with peptide-capture recovery rates >5% (black brick) increased proportionally (6.25%, 40.5%, 100%, and 100%) as their $K_D$ values decreased ($< 1 × 10^{-8}$, $<1 × 10^{-9}$, $<1 × 10^{-10}$, and $<1 × 10^{-12}$ m) (Fig. 3C); additionally, the majority of mAbs with $K_D >1 × 10^{-9}$ (75.4%, 159/211) exhibited peptide-capture recovery rates <1% (light gray brick, Fig. 3C). The average $K_D$ of clones with recovery rates >5% was 3.82 × 10^{-10} m, which is much lower than that of the other two populations with recovery rates <1% (1.32 × 10^{-8} m) and 1–5% (7.64 × 10^{-9} m) (Fig. 3D).

Because a lower $K_D$ value reflects a faster association rate (larger ka) and/or a slower dissociation rate (smaller kd) in antibody-antigen interactions, we also analyzed the relationship between kinetic constants (ka, kd, and $K_D$) and peptide-capture recovery rates. As shown in Fig. 4A, mAbs with peptide-capture recovery rates >5% congregated predominantly in the upper-right corner of the plot, corresponding to faster association rates (larger ka) and slower dissociation rates (smaller kd). Applying a ROC analysis to evaluate the power of ka, kd and $K_D$ to predict mAbs with higher peptide-capture ability (recovery rate >5%), we found that the area under the curve (AUC) values of $K_D$, ka and kd were 0.91, 0.86, and 0.68, respectively (Fig. 4B), indicating that $K_D$ and, to a lesser extent, kd, were good predictors for selecting mAbs suitable for SISCAPA assay development. A tentative cut-off value of $K_D <2.82 × 10^{-9} m$ would select most mAbs (92/93, 98.9%) with a recovery rate >5% and provide filter-out rate (89/195, 45.6%) for clones with a recovery rate <1%, which might be useful for establishing a filter-out rule for quick screening of mAbs that could be applied to the SISCAPA assay.

**Optimization of a 24-plex SISCAPA-MRM Assay for OSCC Candidate Biomarkers**—Recent reports demonstrated the feasibility of assembling a multiplexed SISCAPA-MRM assay for measuring more than 50 target peptides in a single assay in matrices such as plasma and tissue extracts (40, 57). In the current study, we attempted to assemble a multiplexed
SISCAPA-MRM assay for quantification of more than 20 OSCC candidate biomarkers in saliva samples. Among the 36 candidate biomarkers for which antipeptide mAbs were successfully produced and characterized, we sieved out the high-quality antipeptide mAbs against 24 targets (one antibody per target) for further development of the 24-plex SISCAPA-MRM assay (supplemental Table S5), and 16 of which exhibited a peptide-capture recovery rate >5%.

To capture the 24 mAbs used in this assay, we first chose to use magnetic protein G beads (DynaBeads). However, some polymer-like interference was detected during LC-MS analysis using DynaBeads (supplemental Fig. S5, bottom); a series of single-charged compounds (428–780 \( m/z \)) with a mass increase of 44Da was observed at ~20 to 35 min during LC-qTOF MS analysis (supplemental Fig. S5, lower part of left panel). These polymer-like signals were not detected in the elution buffer (directly analyzed by LC-MS), in mock tests of elution that applied SISCAPA reactions without using any beads, or with a different protein G magnetic bead preparation from GE Healthcare (supplemental Fig. S5). Supplemental Fig. S6A shows a comparison of the properties of these two different magnetic protein G bead preparations. We further evaluated the performance of these two preparations in terms of antibody-capture ability, loss of beads during KingFisher processing, and peptide-capture recovery rates for the 24-plex SISCAPA-MRM assay. We found that the antibody-capture capacity of DynaBeads (240 \( \mu l \)) was better than that of GE beads (120 \( \mu l \)) (supplemental Fig. S6B), and both bead preparations exhibited almost equal loss (~10%) during well transfer in the KingFisher magnetic particle processor (supplemental Fig. S6C). In terms of the recovery of specific peptide targets in the 24-plex SISCAPA-MRM assay, GE beads showed higher recovery efficiency for nine targets, DynaBeads showed higher recovery for four targets, and both beads provided similar peptide recovery rates for the other 11 targets (supplemental Fig. S6D).

“Passengers” during target peptide elution, such as hydrophobic polymer-like molecules and acid-released antibodies, could reduce LC-MS performance in the SISCAPA-MRM assay. Therefore, we examined the stability of binding between antibody and beads, as well as the efficiency of peptide elution from the peptide-antibody-bead complex. The extent of antibody release from both types of beads was significantly reduced by increasing ACN concentration (from 0% to 70%) in the elution solution (5% acetic acid) (supplemental Fig. S7A), without compromising the efficiency of peptide elution from either type of bead (supplemental Fig. S7B). In fact, inclusion of 70% ACN in the elution solution enhanced the signals of several target peptides, especially in the assay using GE beads (supplemental Fig. S7B, lower panel). Based on these results, we adopted GE beads as the capture material and 70% ACN in 5% acetic acid as the elution solution in our optimized SISCAPA-MRM assay.

Performance Characteristics of the 24-plex SISCAPA-MRM Assay—To further characterize the performance of this newly developed SISCAPA-MRM assay for candidate biomarker verification in saliva samples, we constructed reverse response curves for the 24 target peptides using scheduled MRM in Qtrap5500. The response of a serially diluted heavy peptide (0.24–1000 fmol, 13 data points) mixed with a constant amount of light peptide (100 fmol) for each target was analyzed in a background of saliva digest (25 \( \mu g \) protein). Using MMP2 as an example, we observed the expected linear increase in heavy peptide signals and a constant light peptide signal (Fig. 5A). The measured peptide concentrations (fmol/25 \( \mu g \) saliva) of three optimized Q1/Q3 transitions showed a linear response to their theoretical concentrations (Fig. 5B); the Q1/Q3 transition 2. y8.1, which displayed a
much better response, was defined as the quantifier ion for the determination of response curves. The response curves of all 24 target peptides are shown in supplemental Fig. S8. The linearity of the response curves, and values for LOD, LLOQ, coefficient of variation (CV), and accuracy for all 24 targets are detailed in Table I. Most of these 24 targets were detected in more than 10 points on the 13-point response curve (blank plus 13 points of 2-fold serial dilutions) with a good linear

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**Fig. 5.** Response curve for quantification of MMP2 using the assembled 24-plex SISCAPA-MRM assay and the overall performance characteristics of 24 targets in a saliva background matrix. Serially diluted heavy peptide mixtures containing different amounts (0, 0.24, 0.49, 0.98, 1.95, 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250, 500, and 1000 fmol) of each of the 24 signature peptides were mixed with a constant amount (100 fmol) of light peptide mixtures for each of the 24 signature peptides. A 14-sample set for generation of a response curve for each target was prepared by adding the resulting mixture of heavy and light peptides to a 25-μg saliva digest. The ion signals of both heavy and light peptides for each of the 24 signature peptides in these 14 samples were detected using the SISCAPA-MRM assay. A, Ion signals (peak areas) of heavy and light signature peptides for MMP2 (IDAVYEAPQEEK) measured simultaneously in these 14 samples. B, Response curve of the MMP2 signature peptide, showing the calculated heavy peptide concentrations versus the expected heavy peptide concentrations, according to the observed heavy/light peak area ratio of three selected Q1/Q3 transitions. C, D, All 14 samples were analyzed using the same 24-plex SISCAPA-MRM assay in sextuplicate. The overall coefficients of variation (C) and accuracy (D) of the 24 targets at each point of the response curve are shown in the range from 0.24 to 1000 fmol with a boxplot, presented as the upper and lower quartile and range (box), the median value (horizontal line), and the middle 90% distribution (extended line).
response ($R^2 > 0.95$); the exceptions were FLNA, which was detected in only 9 points, and CD44, which exhibited a relatively poor linear response ($R^2 = 0.62$). Twenty targets showed LLOQ values for peptides less than 0.1 fmol/μg, whereas four targets (CD44, EGFR, LGALS3BP, and FLNA) had higher LLOQ values ranging from 0.126 to 1.044 fmol/μg. The LLOQ value for each target is also expressed as protein concentration (in ng/ml), which represents the amount of protein corresponding to the determined level of proteotypic peptide. The background saliva digest. Fourteen targets (ANXA2, CA2, CSTA, HPSA5, IL6, ISG15, KRT18, MMP1, MMP2, MMP3, TIMP1, TNC, TNP, and YWHAB) had LLOQ values less than 10 ng/ml protein. Of the remaining targets, seven (CD44, DSG3, GANAB, SPP1, STA1, TNC, and WARS) had LLOQ values between 10 and 50 ng/ml, and three (EGFR, FLNA, and LGALSB3BP) had LLOQ values greater than 50 ng/ml. The median value of CV, calculated in the linear range of the response curve for each target, was less than 25% for most targets (22/24, 92%), indicating high stability in the assay (Table I). Overall, this 24-plex SISCAPA-MRM assay showed low CV values for sextuplicate data at each data point on the response curve (Fig. 5C) and high accuracy of quantification over more than 5 orders of magnitude range in concentration (0.1 to 1000 fmol), based on the ratio of the measured concentration to the theoretical concentration, expressed as a percentage (Fig. 5D). Taken together, these data indicate that this newly developed multiplexed assay shows good accuracy and target-dependent LLOQ in saliva samples containing 25 μg protein, with a majority of targets exhibiting LLOQ values less than 50 ng/ml.

**Multiplexed Quantification of Target Proteins in Saliva Samples From Healthy Donors and OSCC Patients—**To evaluate the applicability of this newly developed 24-plex SISCAPA-MRM assay for quantifying multiple OSCC candidate biomarkers, we first used it to quantify these 24 targets in two model saliva samples pooled from 20 healthy donors and 20 OSCC patients. From 25 μg of saliva protein digests (equivalent to 10–30 μl of saliva sample), this assay was able to measure the endogenous peptide signal of 19 targets at levels higher than or close to their LLOQ values (red line) in a saliva matrix background (Fig. 6). Among these 19 measurable targets, nine targets were detected at an abundance less than 1 fmol/μg (Fig. 6A), whereas the other 10 targets were quantifiable between 1 and 25 fmol/μg (Fig. 6B).

In addition, we prepared another pooled saliva sample (from seven OSCC patients) to evaluate the intra-/inter-day reproducibility of the 24-plex SISCAPA-MRM assay as well as the stability of the targets in unprocessed or trypsin-digested saliva sample during storage at different conditions (–80, 4, and 25 °C) for various time periods (1–7 or 1–14 days) (see supplemental Fig. S9 and Experimental Procedures for the experimental design and detailed protocol). Results from these analyses revealed that 18 of the 24 targets could be detected/quantified in this pooled saliva sample processed at different conditions, and the majority of the quantified targets exhibited low CV values (< 20%) for the intra- and inter-day
assays, which indicates a good reproducibility of our 24-plex SISCAPA-MRM assay (Fig. 7A and supplemental Table S6). In addition, we found that the protein levels of most targets determined in the unprocessed saliva sample stored at −80 °C or 4 °C for 1–7 days fall within the 80–120% range of those determined at day 0 (Fig. 7B), indicating that most of the protein targets in the unprocessed saliva sample remain quite stable when stored at −80 °C or 4 °C for up to 1 week. When the un-processed saliva sample was kept at 25 °C for more than 1 day, the measurable levels of several targets (including DSG3, GANAB, ISG15, LGALS3BP, MMP1, MMP3, TYMP, WARS, and YWHAB) decreased drastically (Fig. 7B). We then performed similar evaluation using the trypsin-digested saliva sample, and the results showed that storage of saliva sample in the trypsin-digested format could significantly improve the measurable levels of those “labile” targets kept at 25 °C for 3–14 days (supplemental Table S7).

On the basis of these encouraging results, we further applied this assay to measure these targets in individual saliva samples from another independent sample set consisting of 20 healthy controls and 21 OSCC patients; demographic data for controls and patients are shown in Table II. As summarized in Table III, this 24-plex SISCAPA-MRM assay was capable of quantifying 17 targets in more than 75% (31 cases) of the 41 saliva samples, whereas the remaining seven targets were measurable only in 2–20 cases. The median value of CV, calculated in triplicate quantification of each target in the 41 saliva samples, was less than 25% for most targets (18/19, 95%), showing the precision of this assay applied to clinical saliva specimens (supplemental Fig. S10). The quantification range of this 24-plex SISCAPA-MRM assay was from 0.21 ng/ml (for IL-6) to 2349.42 ng/ml (for FLNA). This analysis showed significantly increased levels (1.3- to 23.4-fold) of four proteins (LGALS3BP, MMP1, MMP3 and TNC), and decreased levels (0.5- to 0.7-fold) of two proteins (CSTA and HSPA5) in the OSCC group compared with the control group (p < 0.05) (Fig. 8). Notably, MMP1 displayed the highest fold-change in salivary level (23.4-fold) between OSCC patients and healthy controls (Table III). The AUC value of six significantly changed proteins were calculated to be 0.933 (MMP1), 0.793 (CSTA), 0.767 (TNC), 0.708 (MMP3), 0.690 (HSPA5), and 0.686 (LGALS3BP), indicating that MMP1 has the best power among the selected targets to distinguish OSCC patients from healthy controls.

To examine if the changes of potential biomarkers measured on the peptide level reflects the changes in protein concentration in saliva, we performed a multiplexed bead-based immunoassay to quantify the protein levels of three selected targets (MMP1, MMP3, and TNC) in saliva samples because of their highly elevated saliva levels in some OSCC patients and the availability of commercial assay reagents for these targets. Among those 41 saliva samples used for the 24-plex SISCAPA-MRM assay, we could retrieve 24 samples (7 healthy controls and 17 OSCC) for this immunoassay whereas other samples were not available. As shown in supplemental Fig. S11, the quantification results are consistent with those from the MS-based quantification assay, which also indicated the elevated protein levels of the three targets in OSCC cases versus healthy controls. In addition, we also compared the protein concentrations of each target measured respectively by SISCAPA-MRM and bead-based immunoassay in these saliva samples. This analysis revealed a rather good correlation (r > 0.75, Spearman’s correlation) between the protein concentrations measured by the two

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**Fig. 6.** Detection of target proteins in pooled saliva samples using the developed 24-plex SISCAPA-MRM assay. The assembled 24-plex SISCAPA-MRM assay was used to quantify target proteins in two saliva samples pooled, respectively, from healthy controls and OSCC patients (20 cases for each group). For each pooled sample, 25 μg of saliva protein was digested with trypsin, and the saliva digest was spiked with the 13C-heavy peptide mixture (100 fmol for each peptide) for subsequent multiplexed SISCAPA-MRM-MS analysis. The experiment, from protein digestion to MS analysis, was performed in triplicate. The salivary levels determined for individual targets were categorized into two groups based on concentrations less than (A) and greater than (B) 1 fmol/μg in the OSCC saliva sample. The bar chart shows the results of quantification of each target in the healthy control (white bar) and OSCC (gray bar) groups, and the red line indicates the LLOQ of each target. Targets showing >2-fold increased/decreased levels in the OSCC group relative to the control group are denoted by asterisks.
methods for these three targets (supplemental Fig. S12). Collectively, the results demonstrated that the measured peptide concentrations can indeed reflect the change in protein concentration in saliva.

**DISCUSSION**

Verifying the utility of candidate biomarkers discovered in independent studies by simultaneously comparing their levels in clinical samples using precise, multiplexed assays is a critical step toward identifying truly valuable candidate biomarkers that warrant further development for clinical use (20, 21, 58). SISCAPA-MRM represents one of the most attractive technologies for this purpose (36–40, 59, 60). The aim of this study was to develop a multiplexed SISCAPA-MRM assay for quantifying more than 20 candidate OSCC biomarkers retrieved and prioritized from the published literature and our own studies. These targets were independently discovered and verified in small-volume clinical samples, either tissue specimens or body fluids (serum, plasma and/or saliva). Although the same concept has been illustrated by Whiteaker et al. (60), who employed an engineered mouse model to verify potential breast cancer biomarkers using a biomarker panel obtained from a proteomics-based study, our current study is nonetheless significant in three respects. First, this is the first
SISCAPA-MRM Assay for Oral Cancer Biomarker Verification

TABLE III
Concentrations of the 24 protein biomarkers in control and OSCC groups

| No. | Protein | Control (n = 20) | OSCC (n = 21) | Control vs. OSCC |
|-----|---------|-----------------|--------------|-----------------|
|     |         | Concentrationa | Detectable  | Concentration    | Detectable | Fold-changec | p     | AUC  |
|     |         | (ng/ml)         |             | (ng/ml)         |             |             |       |      |
| 1   | ANXA2   | 45.89 ± 18.93   | 20/20       | 47.15 ± 23.73   | 21/21       | 1.0         | 0.9688 | 0.495|
| 2   | CA2     | 38.39 ± 41.09   | 20/20       | 96.84 ± 190.37  | 21/21       | 2.5         | 0.1967 | 0.619|
| 3   | CD44    | 109.81 ± 59.24  | 20/20       | 156.52 ± 153.55 | 21/21       | 1.4         | 0.4263 | 0.574|
| 4   | CSTA    | 376.84 ± 256.35 | 20/20       | 183.57 ± 216.27 | 21/21       | 0.5         | 0.0014 | 0.793|
| 5   | DSC3    | 732.91 ± 490.13 | 20/20       | 632.74 ± 492.79 | 21/21       | 0.9         | 0.3823 | 0.581|
| 6   | EGFR    | 4.19 ± 6.34     | 3/20        | 6.80 ± 9.45     | 8/21        | 1.6         | 0.4292 | 0.569|
| 7   | FLNA    | 1883.74 ± 1945.21 | 17/20    | 2349.42 ± 2361.27 | 18/21    | 1.2         | 0.3821 | 0.581|
| 8   | GANAB   | 116.55 ± 81.03  | 20/20       | 113.70 ± 78.51  | 21/21       | 1.0         | 0.9688 | 0.495|
| 9   | HPSE    | 264.97 ± 141.20 | 20/20       | 197.28 ± 174.73 | 21/21       | 0.7         | 0.0381 | 0.690|
| 10  | IL6     | 0.21 ± 0.67     | 2/20        | 0.00 ± 0.00     | 0/21        | N/A         | N/A   | N/A  |
| 11  | ISG15   | 2.83 ± 3.38     | 20/20       | 7.23 ± 15.39    | 21/21       | 2.6         | 0.1709 | 0.626|
| 12  | KRT18   | 3.86 ± 2.58     | 20/20       | 3.44 ± 3.36     | 21/21       | 0.9         | 0.3153 | 0.593|
| 13  | LGAL3BP | 317.54 ± 203.45 | 20/20       | 421.57 ± 176.10 | 21/21       | 1.3         | 0.0432 | 0.686|
| 14  | MMP1    | 0.90 ± 0.61     | 20/20       | 20.97 ± 45.84   | 21/21       | 23.4        | <0.0001| 0.933|
| 15  | MMP2    | 0.96 ± 0.79     | 16/20       | 2.15 ± 2.62     | 15/21       | 2.2         | 0.2554 | 0.605|
| 16  | MMP3    | 0.66 ± 1.49     | 1/20        | 10.03 ± 22.23   | 11/21       | 15.3        | 0.0112 | 0.708|
| 17  | SPPl    | 0.61 ± 1.17     | 3/20        | 0.20 ± 0.57     | 0/21        | 0.3         | 0.3178 | 0.564|
| 18  | STAT1   | 7.08 ± 7.47     | 7/21        | 9.94 ± 10.41    | 12/21       | 1.4         | 0.4661 | 0.567|
| 19  | TIMP1   | 175.95 ± 78.73  | 20/20       | 200.33 ± 93.04  | 21/21       | 1.1         | 0.5750 | 0.552|
| 20  | TNC     | 8.60 ± 10.07    | 11/20       | 88.40 ± 185.81  | 19/21       | 10.3        | 0.0036 | 0.767|
| 21  | TYMP    | 45.20 ± 38.11   | 20/20       | 98.91 ± 168.48  | 21/21       | 2.2         | 0.1478 | 0.633|
| 22  | ULPBP   | 2.21 ± 3.84     | 3/20        | 1.76 ± 4.43     | 3/21        | 0.8         | 0.3585 | 0.562|
| 23  | WARS    | 70.74 ± 37.64   | 20/20       | 116.93 ± 178.68 | 21/21       | 1.7         | 0.6689 | 0.540|
| 24  | YWHAB   | 80.69 ± 75.85   | 20/20       | 67.47 ± 66.59   | 20/21       | 0.8         | 0.5060 | 0.562|

aMean ± S.D.
bDetectable (concentration > 0) case number/total case number.
cFold change of protein levels in OSCC group over healthy control group.
automatically screen high-affinity mouse antipeptide mAbs against two tryptic peptides of two targets (ADAM17 and CRP). Similarly, Razavie et al. (64) employed KingFisher SISCAPA assays combined with MALDI-TOF-MS analysis (MiSCREEN) to screen anti-peptide rabbit or mouse mAbs against 15 tryptic peptides of nine targets (six for rabbit mAbs and three for mouse mAbs). They also applied SPR-based kinetic analysis, showing that the selected mAbs bound with high affinity and had low dissociation constants (kd). Our current study exploited the essential features of these three studies, namely SPR and KingFisher SISCAPA assays coupled with LC-SRM-MS analysis, and hence provided the opportunity to systematically evaluate the relationship between binding kinetic parameters and peptide-capture ability for each peptide-mAb pair based on data obtained from the 363 mAbs analyzed. Our analyses confirmed the negative association between peptide-capture ability and $K_D$ value (Fig. 3B) and further highlighted $K_D$ and, to a lesser extent, kd as good predictors of mAbs suitable for SISCAPA assay development (Fig. 4B). This observation is consistent with the findings of Razavie et al. (64), who showed that mAbs with low kd values (i.e. lower off rates), which are preferentially selected by the MiSCREEN procedure, tend to be suitable for use in immuno-MS assays. The tentative cut-off $K_D$ value capable of retaining most mAbs with higher peptide-capture ability, determined here to be $2.82 \times 10^{-9}$ M, could provide a useful reference for scientists or companies desiring to select mouse mAbs for further testing in SISCAPA-MS assays.

We have observed a moderate correlation between binding affinity and the peptide-capture ability of anti-peptide mAbs in a large-scale setting (Fig. 3B). However, this analysis also showed that several antibodies possess very high affinity but low or zero activity in the SISCAPA-MS assay. The difference in the principles used for the Biacore 3000 optical biosensor...
and SISCAPA-MS assay might partly explain the discrepancy. Basically, the SPR-based measurement of binding affinity was performed using a peptide-coated chip to capture the antibodies in liquid flow; this design can faithfully reflect the affinity between target peptide and the peptide (antigen)-binding domain of antibody, which locates on the Fab region. However, the activity of antibodies in SISCAPA-MS assay is dependent not only on their binding affinity to cognate peptide antigen but also other factors, such as the integrity of heavy chain and light chain structure and the ability of Fc region to bind to protein G magnetic beads. Certain kinds of structure defects introduced into the Fc region during the antibody production process might result in the generation of some antibodies that have high affinity but shows zero activity in SISCAPA-MS assay. Another possibility is that the binding affinity of these antibodies to their cognate peptide antigens might be too strong to be disrupted by the elution buffer applied (5% acetic acid in 70% ACN).

Sensitivity, stability, and reproducibility represent critical factors for application of multiplexed SISCAPA-MRM assays to verification of candidate biomarkers in clinical samples. Although the performance of this 24-plex assay varied among individual targets, especially with respect to LLOQ values (Table I), its ability to detect individual targets reflected both the abundance of these targets in clinical samples and their LLOQ values, rather than LLOQ alone. For example, FLNA exhibited the highest LLOQ (1.044 fmol/µg saliva protein) in the 24-plex SISCAPA-MRM assay among the 24 targets assessed, but nonetheless showed quantifiable levels above its LLOQ in the two pooled saliva samples and in most (35/41) of the saliva samples tested in this study. However, five targets (EGFR, IL6, SPP1, STAT1, and ULBP2) with lower LLOQ values (0.206, 0.032, 0.076, 0.030, and 0.051 fmol/µg saliva protein, respectively) could not be well quantified against a background of 25 µg of pooled or individual saliva samples (Fig. 6, Tables I and III), indicating the low abundance of these five targets in saliva collected from healthy controls and OSCC patients. Increasing the input of saliva sample may improve the detection of these targets using the newly developed SISCAPA-MRM assay.

High precision and reproducibility are essential in protein biomarker research, particularly where translation to clinic is sought; meanwhile, the issue of preanalytical variability in saliva collection and analysis should be carefully considered. The controls of standard operating procedures handling serum/plasma sample have been widely discussed, including sample selection, collection, stability and storage (65). In contrast to blood sample, saliva is a non-sterile body fluid comprising exocrine contributions from salivary glands and non-exocrine components, such as micro-organisms, desquamated oral epithelial cells, leukocytes, and a serum-like transudate (66). For saliva collection, the donors were asked to refrain from eating, drinking, smoking, and using oral hygiene products for at least one hour and rinse the mouth with drinking water twice before specimen collection by followed the protocol as described previously (52). This would prevent the saliva from contamination with consumptions taken orally; however, it is likely insufficient to wash out the effects of their behavior and personal life style. Exposure to chemical consumption, such as nicotine, arecoline, etc., might affect the turnover of proteins or transiently induce the expression of some proteins. In addition, previous studies have reported the high susceptibility of salivary proteins to proteolytic degradation caused by endogenous proteases in whole saliva and suggested whole saliva collection, short-term storage of samples on ice and whole saliva centrifugation to separate the cells from the protein-containing supernatant as the safest, most practical, and most suitable handling protocol (66, 67). This protocol has been adopted by the present study with a minor modification by adding proteinase inhibitor mixture immediately to the protein-containing inhibitor mixture collected after whole saliva centrifugation at 4 °C. The whole saliva supernatants prepared were then stored at −80 °C for further study, and our data showed that most of the targets selected for the present study are quite stable in the whole saliva supernatant stored at −80 °C (Fig. 7B). Another concern is the volume of saliva collected from each testee, which could be varied significantly between persons. As the idea of salivary proteins being biomarkers for oral cancer is based on the concept that the candidate biomarkers can be directly secreted/released from oral tumors or tumor-like lesions into the saliva, the high inter-person variation of saliva volume can have a profound effect on the measurement of “concentration” of a putative biomarker (e.g. ng protein biomarker per ml of saliva). Moreover, the intra-day and inter-day variations of saliva proteins in the same person have not been thoroughly investigated. Although the above-mentioned issues remain to be further studied, saliva, the most proximal biofluid to oral lesions, is still an ideal specimen for oral cancer biomarker discovery.

Saliva is an ideal specimen for OSCC biomarker discovery and verification, as it is protein-rich, easily accessible and, more importantly, the biofluid most proximal to oral lesions (68). A considerable number of salivary proteins have been profiled using proteomics approaches (69–72), and some studies have explored differentially expressed proteins in saliva samples obtained from OSCC and healthy controls (73, 74). In the current study, we applied the developed 24-plex SISCAPA-MRM assay to quantify and compare these targets in 41 saliva samples from healthy controls and OSCC patients. Although the size of saliva samples used here was small, we were encouraged by the ability of our assay to identify significantly increased or decreased levels of six targets in OSCC patients compared with healthy controls (Fig. 7). This observation supports the feasibility of our overall strategy, which encompasses careful selection of targets and signature peptides, production/characterization of mouse mAb, assembly of the multiplexed SISCAPA-MRM assay for quantification of
multiple targets in clinical saliva specimens, and ultimately the development of a fit-for-purpose multiplexed assay for OSCC biomarker verification.

Among the six proteins whose levels were significantly different between OSCC and healthy control saliva samples, five (MMP1, TNC, LGALS3BP, CSTA, and HSPA5) were previously identified by us, including in our previous OSCC cell secretome study (47), and two (TNC and HSPA5) are also highly overexpressed in OSCC tissues (49). Additionally, LGALS3BP, CSTA, and HSPA5 have been identified in the OSCC-related saliva proteome (73, 74). Notably, it has also been reported that serum levels of TNC are increased in patients suffering from head-and-neck squamous cell carcinomas, especially late-stage or recurrent patients (75). Furthermore, it has been reported that the levels of MMP1, which showed the most drastic elevation in OSCC saliva in our current study, are elevated in both OSCC tissues (76–78) and saliva (79). Although we have shown a good correlation between the target protein concentrations measured in saliva samples by SISCAPA-MRM and bead-based immunoassay, the absolute values of protein concentration for specific target determined by the two methods can be different (supplemental Fig. S12). A similar observation has been reported by Lin et al. (80), in which protein immunoprecipitation-MRM and ELISA were used to quantify the same biomarker candidates in plasma samples. In our case, this phenomenon could be because of the fact that the capture antibodies and protein/peptide standard used in the two assays are different. Other factors involved in this phenomenon might include the loss of target peptide during sample preparation in MS-based assay, such as digestion efficiency or hydrophobic absorption to plastic materials used in entire procedure of the assay, and uncertain interferences in immuno-based assay (24–27). Although only one tryptic peptide per target protein was applied to quantify target protein in the present study, we demonstrated that the measured peptide concentrations reflect similar changes obtained in protein quantification by multiplexed and immuno-based assay (Fig. 8 and supplemental Fig. S11).

In summary, we describe the first attempt to develop a mouse mAb-based, multiplexed SISCAPA-MRM assay targeting more than 20 potential body fluid-accessible OSCC biomarker candidates, prioritized from a literature search and our own studies. Both SPR and SISCAPA-MS assays were applied to characterize and select mouse anti-peptide mAbs suitable for assembling this multiplexed SISCAPA-MRM assay. By demonstrating the ability of this multiplexed assay to quantify more than 20 targets in clinical saliva specimens, we have established the feasibility of using multiplexed SISCAPA-MRM assays in future to compare the clinical utility of dozens of prioritized OSCC biomarker candidates in a large cohort of saliva samples.

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DATA AVAILABILITY

The chromatograms used in quantification of analyses and the mass spectrometry-based proteomics data (raw files and transition list) have been deposited to the Peptide Atlas with the link of https://db.systemsbiology.net/sbeams/cgi/PeptideAtlas/PASS_View. Dataset title: SISCAPA-MRM assay for oral cancer biomarker verification; Dataset identifier: PASS00910; Password: QD2653hv. The files contain the raw data for analyzing (1) samples for generating response curves (84 raw files), (2) two model saliva samples (pooled from 20 healthy donors and 20 OSCC patients, respectively) (6 raw files), (3) 41 individual samples (20 healthy donors and 21 OSCC patients) (123 raw files), and (4) one model saliva sample (pooled from seven OSCC patients) (65 raw files).

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[5] This article contains supplemental material.

[6] These authors contribute equally to this work.

[7] To whom correspondence should be addressed: Department of Cell and Molecular Biology, College of Medicine, Chang Gung University, Taoyuan, Taiwan. Tel.: 886-3-2118800 ext. 5171; Fax: 886-3-2118891; E-mail: yusong@mail.cgu.edu.tw.

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