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Assessment of candidate biomarkers in paired saliva and plasma samples from oral cancer patients by targeted mass spectrometry

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ABSTRACT

For oral cancer, numerous saliva- and plasma-derived protein biomarker candidates have been discovered and/or verified; however, it is unclear about the behavior of these candidates as saliva or plasma biomarkers. In this study, we developed two targeted assays, MRM and SISCAPA-MRM, to quantify 30 potential biomarkers in both plasma and saliva samples collected from 30 healthy controls and 30 oral cancer patients. Single point measurements were used for target quantification while response curves for assay metric determination. In comparison with MRM assay, SISCAPA-MRM effectively improved (> 1.5 fold) the detection sensitivity of 11 and 21 targets in measurement of saliva and plasma samples, respectively. The integrated results revealed that the salivary levels of these 30 selected biomarkers weakly correlated ($r < 0.2$) to their plasma levels. Five candidate biomarkers (MMP1, PADI1, TNC, CSTA and MMP3) exhibited significant alterations and disease-discriminating powers (AUC = 0.914, 0.827, 0.813, 0.77, and 0.753) in saliva sample; nevertheless, no such targets could be found in plasma samples. Our data support the notion that saliva may be more suitable for the protein biomarker-based detection of oral cancer, and the newly developed SISCAPA-MRM assay could be applied to verify multiple oral cancer biomarker candidates in saliva samples.

Significance: In this work we systematically determined the abundance of 30 selected targets in the paired saliva and plasma samples to evaluate the utility of saliva and plasma samples for protein biomarker-based detection of oral cancer. Our study provides significant evidence to support the use of saliva, but not blood samples, offer more opportunity to achieve the success of protein biomarker discovery for oral cancer detection.

1. Introduction

Oral cavity cancer is a major worldwide health problem with high prevalence and mortality. Oral squamous cell carcinoma (OSCC) accounts for > 90% of oral cavity cancer cases. Although conventional survey policy and therapy regimens of surgery and anticancer drug treatment have improved, the survival outcome of OSCC patients has not significantly improved in recent decades. The main reasons for poor outcome are delay diagnosis of lesions in advanced stages, secondary cancer occurrence, local recurrence, and metastasis [1–3].

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Epidemiological studies have shown that the 5-year survival rates of OSCC patients in stages I, II, III and IV are 72–90%, 39–85%, 27–70% and 12–50%, respectively [1,3,4]. Therefore, better strategies for detecting early-stage OSCC and/or predicting the aggressiveness of tumor lesions are urgently needed to improve the diagnosis and treatment of OSCC.

Body fluid-accessible biomarkers hold potential for detecting early-stage cancers, which are often manageable and curable. Serum and plasma are the major sample types in routine clinical practice for disease screening, diagnosis, and monitoring. They are different blood matrices imposing different considerations (regarding pre-analytical and analytical methods) and detection tendencies [5]. For instance, a team effort made by the HUPO Plasma Proteome Project (HUPO PPP) had found the dramatic differences in peptide contents between serum and plasma samples, presumably due to various ex vivo processes occur during processing of venous blood into serum, leading to neogeneration of many peptides [6]. Based on this finding, the Specimens Committee of HUPO PPP concluded that plasma is preferable to serum, due to less degradation ex vivo, for certain peptidomic studies [7]. For oral cavity disease, saliva is the biofluid most proximal to oral lesions, and can be obtained easily and non-invasively [8,9]. By a meta-analysis, the usefulness of the salivary biomarkers for the OSCC detection has been suggested [10]. During the past two decades, numerous candidate biomarkers for OSCC have been discovered by different laboratories using serum, plasma and/or saliva specimens, including proteins, RNAs, DNAs, metablolites and exosomes [11–16]. However, their clinical utilities for OSCC detection and prognostic prediction remain to be further validated. For example, Wang et al. explored and showed the potential of tumor-specific DNA (somatic mutations or human papillomavirus genes) in the saliva and plasma as a valuable biomarker for detection of head and neck squamous cell carcinomas (93 cases in this study) [17]. In addition, Gleber-Netto et al. evaluated the discriminatory power of salivary transcriptomic (7 mRNAs) and proteomic (2 proteins) biomarkers in differentiating OSCC cases from controls and potentially malignant oral disorders (180 cases in this study) and concluded the combination of mRNA and protein markers is of great value for OSCC detection [18].

Recently, we have reported the verification of 49 candidate OSCC biomarkers in 460 saliva samples using multiple reaction monitoring-mass spectrometry (MRM-MS) assays [19], as well as the development of a 24-plex SISCAPA-MRM (stable isotope standards and capture by anti-peptide antibodies coupled with MRM-MS) assay for verifying OSCC candidate biomarkers in saliva samples [20]. For those 49 candidate biomarkers selected for our previous verification study, which were prioritized from 302 proteins examined in 277 papers with small-size clinical specimens, our literature mining revealed that 23 targets had been quantified in plasma/serum specimens but only 6 targets were measured in saliva samples (see Table S2 in Ref. [20]). This observation indicates the previous preference of using blood samples for measuring OSCC biomarkers. Although studies from our and other groups have shown the high potential of specific salivary proteins for OSCC detection, to our knowledge, only a few studies explored the behavior of biomarker candidates as saliva or plasma biomarkers in the same cohort. A recent study verified the serum and salivary levels of chemerin and MMP-9 in OSCC and showed that the elevated levels of MMP-9 (in saliva) and chemerin (in both saliva and serum) might be considered as diagnostic biomarkers [21]. In contrast to consistent changes of chemerin in serum and saliva samples, the inverse changes of serum and salivary EGFR were observed in OSCC patients compared with health subjects [22]. Moreover, Lee et al. measured 14 cytokines in both plasma and saliva samples from the same individuals using Luminex Bead-based multiplex assay and found that the correlation between saliva and plasma biomarkers in OSCC was weak [23].

Targeted mass spectrometry has recently been demonstrated as a robust technology platform for identifying and quantifying multiple proteins in complex biological samples for disease biomarker discovery and verification. The MRM-MS assay is a targeted acquisition technique capable of verifying the presence and abundance of > 100 candidates of interest in a single analysis [24–27]. Employing spiked-in, stable heavy isotope-labeled peptides with known amounts as a standard, liquid chromatography (LC)-MRM-MS measurements enable sensitive, reproducible and specific quantification of 100 or more peptide features in complex biological matrices [28,29]. Further coupling peptide immunoaffinity enrichment with LC-MRM-MS analyses, known as SISCAPA-MRM [30] or immuno-MRM [31,32], can improve the sensitivity and specificity of MRM-MS assays in quantitatively measuring disease biomarkers and cellular signaling proteins.

Although a few studies have explored the behavior of biomarker candidates as saliva or plasma biomarkers in the same cohort, most of them are cytokines [23], and only three proteins (MMP-9, chemerin and EGFR) other than cytokines were investigated [21,22]. Since many cytokines are known to be also activated in non-cancer, inflammatory conditions, a more comprehensive blood/saliva comparative study of biomarker candidates other than cytokines should benefit future development of clinically useful OSCC biomarkers. In the present study, we established two types of targeted MS assays for 30 potential OSCC protein biomarkers selected from the literature and our in-house studies [19,20], and then applied these assays to determine the abundance of selected targets in paired saliva and plasma samples from 30 controls and 30 OSCC cases. Because various ex vivo degradation processes are highly enhanced in serum compared to plasma samples, which might have a strong effect on the peptide-based targeted MS assays [7], we thus chose the paired plasma/saliva, but not serum/saliva samples for this comparative study. Our data demonstrated the superiority of SISCAPA-MRM versus MRM assay for quantifying larger numbers of selected targets in both saliva and plasma samples, and showed that both methods could reliably quantify more targets in saliva than plasma samples. More importantly, we found that many candidate biomarkers were significantly elevated in saliva, but not paired plasma samples, from OSCC patients.

2. Materials and methods

2.1. Study subjects and sample collection

Thirty individuals with no signs of cancer (healthy controls) and 30 OSCC patients were enrolled at Chang Gung Memorial Hospital (Linkou, Taiwan) with Institutional Review Board approval (IRB No. 102-5685A3). All subjects signed an informed consent form to participate and permit the use of plasma/saliva samples collected before treatment. The demographic characteristics of these subjects are summarized in Supplemental Table S1. All experiments were performed in accordance with the approved guidelines. The plasma and saliva samples from the donors were collected simultaneously in the morning or afternoon. One hour before collection at least, the donors avoided eating, drinking, smoking, and using oral hygiene products for collecting saliva samples. Before saliva collection, donors were asked to rinse the mouth with clean water. The passive drooled saliva samples from the donors were collected using 15-ml sterile centrifuge tubes, and the obtained saliva samples were centrifuged at 3000 $\times g$ for 15 min at 4 °C. The resulting supernatant was collected, treated with a protease inhibitor cocktail (v/v 1:50, Sigma, St. Louis, MO, US), and stored in aliquots at −80 °C. Whole blood sample were collected into commercially available EDTA-containing tube, and then centrifuged at 2000 $\times g$ for 15 min at 4 °C. The resulting supernatant was collected, treated with a protease inhibitor cocktail (v/v 1:50, Sigma, St. Louis, MO, US), and stored in aliquots at −80 °C. The time from collection to centrifugation for all samples were within 90 min. Protein concentrations in saliva and plasma samples were determined using a BCA assay kit (Pierce Chemical, Rockford, IL, US) according to the manufacturer's protocol.

2.2. Selection of protein targets and surrogate peptides

Protein targets were selected from literature survey and prioritized according to their clinical and scientific relevance as OSCC-related biomarker candidates, including 1) the number of articles; 2) the consistency in the differential expression under two different conditions (cancer versus healthy control) among multiple studies; and 3) detection in multiple sample types (tissue and body fluids) of OSCC. Detailed descriptions of the 30 selected targets are summarized in Supplemental Table S2. Thirty surrogate tryptic peptides representing these 30 proteins (one peptide for each target protein) were selected as previously described [19]. Briefly, the list of tryptic peptides was generated in silico by the MRMPilot software (version 2.1; AB-Sciex, Forster, CA, US) and further selected according to the following criteria: (a) peptides without terminal RP and KP sequences that could potentially lead to missed cleavages; (b) unique peptides containing 8 to 23 residues without any known post-translational modification sites; (c) peptides without chemically reactive amino acids (such as Cys, Met) and unstable sequences (such as NG, DG, QG, and N-ter Q). (d) peptides containing more hydrophilic, charged, or branched amino acids and/or proline residue in consideration of antigenicity for anti-peptide antibody production. However, for which no suitable peptide fully fitted in the above criteria, we reluctantly allowed the presence of unstable sequences (NG, DG, QG or N-ter Q). The uniqueness of these selected peptides was further checked via a BLAST search with human protein database of UniProt. The sequence and mass values of the surrogate peptides and their heavy version are summarized in Supplemental Table S3.

2.3. Peptide synthesis

Synthetic (light) peptides were purchased from Kelowna International Scientific (Taipei, Taiwan). The heavy version of [$^{13}C_6{}^{15}N_2$]Lys- and [$^{13}C_6{}^{15}N_4$]Arg-coded proteotypic peptides, used as stable isotope standard (SIS) peptides, were synthesized and purified at the UVic-Genome BC Proteomics Centre (Victoria, BC, Canada). The purity of synthetic stable isotope-labeled peptides was at least 90%, and in most cases was > 95%. The powder of each synthetic peptide was resolved in 0.1% FA and divided into aliquots of 1 nmol. The aliquots were then dried by Speed-Vac and stored at −80 °C.

2.4. Production and characterization of mouse monoclonal anti-peptide antibodies

The synthetic peptides with a C-terminal cysteine or GSGC linker (purchased from Kelowna International Scientific) were chemically conjugated to a carrier protein and used as the antigens. Mouse monoclonal anti-peptide antibodies against 30 target proteins were produced and characterized according to previously described procedures [20], including selection of the signature peptide for each target protein, preparation of peptide-carrier protein conjugates, mouse immunization, enzyme-linked immunosorbent assay (ELISA)-based monoclonal antibody screening, measurement of antibody-peptide antigen binding kinetics using surface plasmon resonance, and screening of the peptide-capture ability of produced antibodies by immuno-affinity enrichment-conjugated qTOF MS analysis. The characteristics of these 30 mouse monoclonal anti-peptide antibodies are described in Supplemental Table S4.

2.5. Sample preparation

Based on the BCA assay, the total protein concentration of each sample was defined (Supplemental Table S5). Triplicate aliquots of each sample containing 30 μg (saliva) or 120 μg (plasma) proteins was diluted with an appropriate amount (as indicated in Supplemental Table S5) of 100 mM Tris-HCl buffer at pH 8.5 (Sigma) to achieve the final volume of 60 μL and then mixed with 20 μL of 20% sodium deoxycholate (DOC; Sigma). The samples were heated at 100 °C for 5 mins, and the denatured proteins were then reduced with 5 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP; Sigma) at 60 °C for 30 min and alkylated with 10 mM iodoacetamide (Sigma) at 37 °C for 30 min in the dark. The samples were further diluted with 100 mM Tris-HCl buffer to achieve 1% DOC before adding trypsin (Agilent, Santa Clara, CA, US) to the substrate protein solution. At an enzyme to substrate ratio (w/w) of 1:25, the digestion was performed at 37 °C overnight, and then stopped by boiling for 15 min. To remove DOC, the samples were adjusted to 0.1% trifluoroacetic acid (TFA; Alfa Aesar, Haverhill, MA, US) and 0.4% formic acid (FA; J.T. Baker, Phillipsburg, NJ, US) and then centrifuged at 15,000 × *g* for 10 min at room temperature. Afterwards, the supernatants were mixed with a set of SIS peptides containing 90 fmol (for saliva) or 360 fmol (for plasma) of each target peptide and then desalted by solid-phase extraction (SPE) equipment using Waters Oasis HLB 96-well plates (10 mg) (Waters, Milford, MA, US), as described previously [20]. Finally, the aliquots of saliva (25 μg) and plasma (100 μg) were taken for target peptide enrichment using anti-peptide antibodies coupled with LC-MRM-MS measurement (SISCAPA-MRM). The residual samples were lyophilized and stored at −20 °C until the analysis of LC-MRM-MS (MRM).

2.6. Automated and multiplexed SISCAPA assay

A KingFisher magnetic particle processor was used for automated handling of the multiplexed SISCAPA assay against 30 target peptides, as described previously [20]. Briefly, the slurry of 200 μL protein G magnetic beads (GE Healthcare, Little Chalfont, UK) was washed and suspended in phosphate buffered saline (PBS) with 0.03% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (USB Corp., Cleveland, OH, US). The pre-washed beads were incubated with 1 μg of specific anti-peptide antibodies for each of 30 analytes (Supplemental Table S4) for 1 h under a medium mixing condition, and then transferred to next plate which contained trypin-digested saliva or plasma samples for a 2-h incubation. The beads were washed twice with 200 μLof PBS (plates 4 and 5) and once with 1/10× PBS. The captured peptides were then eluted in plate 7 which contained 50 μL of 5% acetic acid (J.T. Baker) and 70% ACN (the elution buffer). The eluted samples were lyophilized and stored at −20 °C until further analysis by LC-MRM-MS.

2.7. LC-MRM-MS analysis

A nanoACQUITY HPLC system equipped with a nanoACQUITY UPLC C18 column (100 μm × 100 mm, 1.7-μm particle size; Waters) was coupled with a triple quadrupole mass spectrometer (QTRAP 5500; AB Sciex, Redwood, CA, US). The lyophilized samples were rehydrated with appropriate amount of 0.1% formic acid to achieve the concentration of 0.25 μg/μL, and 4 μL of each eluate was injected onto the LC-MRM-MS system. After 20-min injection of sample with 3% buffer B, samples were separated at a flow rate of 0.4 μL/min with a 38-minute linear gradient from 3% to 22% of buffer B, a 5-minute linear gradient from 22% to 30% of buffer B, a 5-minute flat of 30% buffer B, and a final 2-minute linear gradient from 30% to 95% of buffer B, followed by a re-generation with a 2-minute linear gradient from 95% to 5% of buffer B and a 5-minute flat of 5% 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). The acquisition parameters (collision energy, CE; declustering potential, DP; entrance potential, EP; collision entrance potential, CEP; and collision exit potential, CXP) for each target peptides were experimentally determined as described in our previous studies [19,20]. The instrument setting of acquisition methods: ion spray voltage,

2300 V; curtain gas setting, 20 psi (UHP nitrogen); interface heater temperature, 150 °C; auto-sampler temperature, 4 °C; MS operating pressure, 1.1×10^{-5} Torr; Q1 and Q3 were set to unit resolution (0.6–0.8 Da full width at half height). A scheduled MRM option using three MRM ion pairs per peptide (Supplemental Table S3) was used for all data acquisition with a target cycle time of 1.5 s and a 6-min scheduled MRM detection window. As the overlapping number of transitions ≤72 transitions, the 1.5-seconds cycle time would offer a dwell time ≥ 20 msec per transition in our experiments. The triplicate samples prepared from each clinical specimen were continuously subjected to LC-MRM analysis, and each sample run was separated by two wash runs with a short gradient (from 3% to 95% ACN within 30 mins).

2.8. Generation of response curves

Bulk saliva or plasma protein digests were prepared as background matrices. As shown in Supplemental Table S6, the digests were mixed with a constant amount of a set of light peptides and a variable amount of heavy peptides for the generation of reverse response curves. The samples with > 2000-fold range of levels from 0.024 to 50 fmol/μg (MRM in plasma), from 0.01 to 40 fmol/μg (MRM in saliva), from 0.0061 to 12.5 fmol/μg (SISCAPA-MRM in plasma), and from 0.00976 to 20 fmol/μg (SISCAPA-MRM in saliva) along with a zero sample were subjected to MRM and SISCAPA-MRM analyses, respectively. The quintuplet samples of each level were prepared, and the data acquisition was sequentially performed from zero sample to the highest concentration sample followed by a blank sample (0.4% FA) for one replicate. Each sample run was separated by two wash runs with a short gradient (from 3% to 95% ACN within 30 mins). According to the linear regression of log-to-log scales, the data point nearest to the turning point of the slope (i.e. the point at which the slope begins to flatten out) was determined as the low end of curve. The quantifier ion (a single transition per peptide) was selected according to the following criteria: (1) lower value of LOD and/or CV% obtained in the response curve, and (2) lower interference status in clinical samples.

2.9. Bead-based suspension immunoassay for the detection of MMP1, MMP3, LGALS3BP and TNC

Protein concentration of MMP-1, MMP-3, LGALS3BP, and tenascin (TNC) in saliva and plasma specimens were determined by Luminex multiplex assay kit (R&D Systems, Minneapolis, MN, US) according to the manufacture's protocol. By using filter-bottom 96-well microplates (Millipore, Burlington, MA, US) 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, US). The detection range was 36.95–26,939 pg/mL for MMP-1, 27.6–60,364 pg/mL for MMP-3, 249.64–545,970 pg/mL for LGALS3BP, and 33.67–24,545 pg/mL for TNC. Saliva and plasma specimens were respectively analyzed in 5-fold and 20- fold dilution, and the detected protein concentration lower than the detection limit was considered as zero.

2.10. Data analysis and statistical methods

All MRM data were processed using Skyline software based on the defined precursor (Q1) and fragment (Q3) mass list of the target peptides [33]. All spectra were manually double-checked for confident quantitative results, and the unqualified signals were recorded as zero concentration for the quantification of clinical samples. The single point measurements were used for the quantification of targets in clinical samples. The levels (fmol/μg) of the target peptides in each sample were determined as the ratio of the peak area to that of the heavy-labeled peptides, and multiplied by the known input of each heavy peptide. The concentrations (ng/mL) of target proteins were determined as the observed levels (i.e. fmol/μg) multiplied by the molecular weight of each target protein and multiplied by the total protein concentration of each sample. The reverse response curves were generated for assay metric determination. The limit of detection (LOD) and lower limit of quantification (LLOQ) of each target were determined based on response curves generated using the MRM statistical software, QuaSAR, as previously reported [34]. The LLOQ was calculated as the LOD value multiplied by 3. The differences in biomarker levels between two groups were analyzed using the Mann Whitney test. The area under the receiver operating characteristic (ROC) curve (AUC) was analyzed using SPSS statistical software. Consistency between quantifications by MRM and SISCAPA-MRM assay were analyzed using linear regression. Correlations between plasma levels and saliva levels of candidate proteins were analyzed by Pearson's correlation. The statistical significance of each target in multiple comparison assay was further adjusted by Benjamini–Hochberg procedure with the false discovery rate defined as 5%.

3. Results

3.1. Establishment of 30-plex MRM and SISCAPA-MRM assays for target protein quantification in saliva and plasma samples

Based on our previous study of OSCC-related biomarker in saliva samples [19], we attempted to verify the potential of applying these biomarkers in plasma samples and compare the levels of biomarkers in both plasma and saliva samples collected from the same individuals. In this study, we adapted two approaches, MRM and SISCAPA-MRM, to quantify the protein biomarkers in both plasma and saliva samples. The MRM and SISCAPA-MRM assay methods used in this study were primarily established in our previous studies [19,20] against 24 targets, and the additional 6 targets (ENO1, MYO5A, OASL, PADI1, S100A2, and SERPINE1) with newly in-house produced anti-peptide antibodies were included for establishing the 30-plex MRM and SISCAPA-MRM assays. The sequence and mass values of the surrogate peptides and their heavy version are summarized in Supplemental Table S3. A representative run shows the intensity and liquid chromatography distribution of the 30 peptides analyzed by scheduled MRM-MS (Supplemental Fig. S1). The single chromatograms of light and heavy XICs for the 30 target peptides are shown in Supplemental Fig. S2. The characteristics of these 30 mouse monoclonal anti-peptide antibodies are described in Supplemental Table S4. After optimization of instrument parameters for peptide detection and protocols for sample preparation and immuno-enrichment, we constructed reverse response curves for the 30 target peptides using a serial dilution of heavy peptides (13 designed data points) plus a constant amount of light peptides of each target, which was mixed with saliva or plasma digest as background matrix. Limit of detection (LOD) and lower limit of quantification (LLOQ) values for 30-plex MRM and SISCAPA-MRM assays in saliva and plasma samples were determined by reference to reverse response curves. The different set points of the dynamic range and input amount of saliva or plasma digest in MRM and SISCAPA-MRM assays are detailed in Supplemental Table S6.

In consideration of the application in clinical use, we intended to use 1 μg saliva or plasma digest for direct MRM assay. For SISCAPA-MRM measurements, 25-fold or 100-fold more digests (25 μg saliva or 100 μg plasma protein digests, finally) than the one used in MRM assay were tested to enhance the signals of selected targets. Therefore, we used the determined amount of digest as background matrices for MRM and SISCAPA-MRM assay response curves, respectively. The detection sensitivity (LOD and LLOQ) and linearity (slope and r^2) of assays as applied to saliva and plasma matrices are shown in Supplemental Table S7 (for MRM assay) and Supplemental Table S8 (for SISCAPA-MRM assay). The performance of the MRM and SISCAPA-MRM assays using

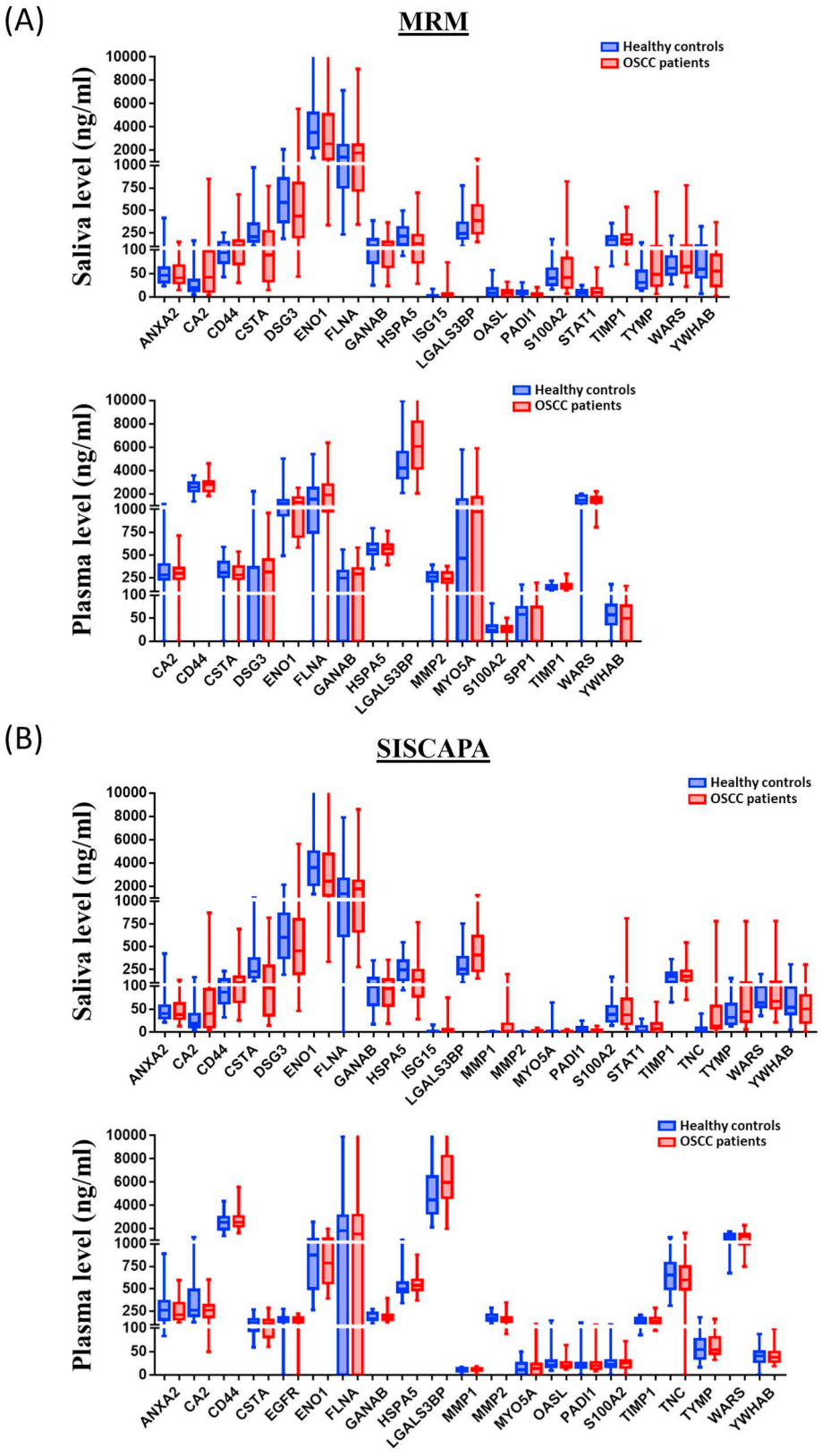

Fig. 1. Abundance of target proteins in saliva and plasma samples, determined by MRM and SISCAPA-MRM assays. (A) For MRM assay, shown here are the quantification results of 19 and 16 targets which could be measured in > 30 saliva and plasma samples among total 60 samples, respectively. (B) For SISCAPA-MRM assay, shown here are the quantification results of 22 and 21 targets which could be measured in > 30 saliva and plasma samples among total 60 samples, respectively. Box-whisker plot showing the levels of the proteins quantified in Hc (blue) and Oc group (red), presented as the upper and lower quartiles and range (box), the median value (horizontal line), and the middle 90% distribution (whisker line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

saliva were much better than that using plasma. As expected, SISCAPA-MRM assay showed improvement of LLOQ to its corresponding MRM assay. Applying an additional immuno-enrichment step using the anti-peptide antibodies effectively improved (> 1.5 fold) the detection sensitivity of 11 targets in saliva samples and 21 targets in plasma samples (Supplemental Table S8).

3.2. Abundance of the 30 biomarker candidates in paired saliva and plasma samples from healthy subjects and OSCC patients, determined by MRM and SISCAPA-MRM analyses

We applied the developed 30-plex MRM and SISCAPA-MRM assays to determine the levels of 30 selected candidates using single point

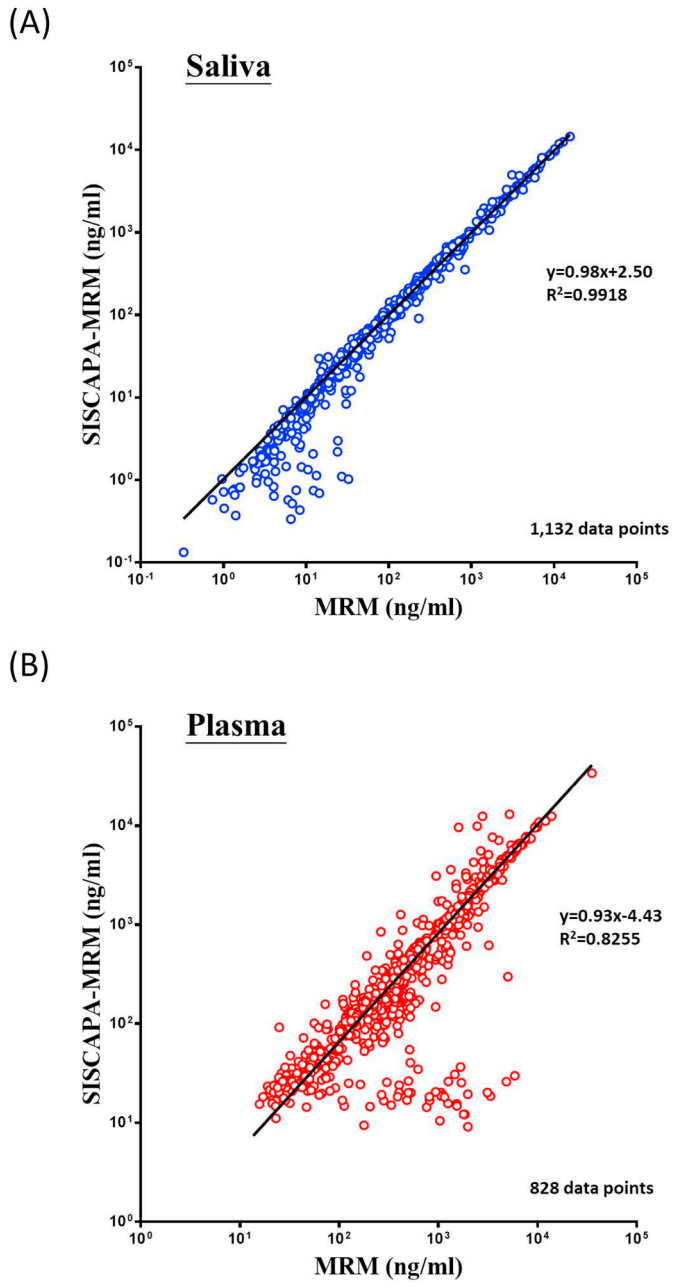

Fig. 2. Consistency analysis of target protein abundance determined by MRM versus SISCAPA-MRM assay in saliva and plasma samples. A total of 1800 data points (30 targets × 60 samples) were obtained using MRM (or SISCAPA-MRM) assay in saliva (or plasma) samples. (A) In saliva samples, 1132 data points for target proteins that could be quantified (i.e., target protein concentration > 0) by both MRM and SISCAPA-MRM assays were used in a linear regression analysis to evaluate the consistency between measurements obtained by MRM and SISCAPA-MRM assays. The results are shown in a log-scale plot. (B) Similar analysis is shown for plasma samples, in which 828 data points were used for the analysis.

measurements in both saliva and plasma samples from each of the 30 healthy subjects and 30 OSCC patients. Of the 30 candidate biomarkers measured directly by MRM, 19 and 16 targets could be measured in > 30 saliva and plasma samples among total 60 samples, respectively (Fig. 1A). We further applied anti-peptide antibodies to capture 30 targets from larger amounts of samples (saliva samples, 25 μg; plasma samples, 100 μg) to improve the detection power of the established MRM assay. Consequently, 22 and 21 detectable targets could be measured in ≧30 saliva and plasma samples, respectively, using this newly developed 30-plex SISCAPA-MRM assay (Fig. 1B). Quantification data for these 30 targets in paired saliva and plasma samples from the 60 individuals are detailed in Supplemental Tables S9. The measured abundance of these proteins ranged from 0.16 ng/mL (IL6) to 4175.59 ng/mL (ENO1) in saliva and from 0.57 ng/mL (ISG15) to 7475.53 ng/mL (LGALS3BP) in plasma. The dynamic range of detection of MRM and SISCAPA-MRM assays covered five orders of magnitude. In addition, an analysis of the consistency between the two MS-based quantification assays revealed a good linear correlation (R^2 = 0.9918) and good accuracy (slope = 0.98) in saliva samples (Fig. 2A) as well as in plasma samples (R^2 = 0.8255; slope = 0.93; Fig. 2B). Collectively, these results indicate that inclusion of an immuno-enrichment process prior to LC-MRM-MS analysis improves detection sensitivity while retaining detection accuracy in both saliva and plasma samples.

3.3. Relative abundance of each target in saliva and plasma samples from 60 individuals

To evaluate the relative abundance of each target in saliva and plasma samples from the 60 individuals, the $\log_2$ ratio of the determined concentration in each sample to the mean concentration of the healthy group was calculated and displayed in Fig. 3. Among total 1800 quantitative data events (30 targets × 60 samples) in saliva or plasma samples, the SISCAPA-MRM results showed that the levels of many candidate biomarkers, including CA2, EGFR, ISG15, MMP1, MMP2, MMP3, OASL, SERPINE1, TNC and TYMP, were elevated in saliva, but not plasma samples, from OSCC patients (Fig. 3). Similar results could be obtained from the MRM analysis, which are shown in Supplemental Fig. S3. We further analyzed the correlation between saliva levels and plasma levels of these 10 biomarkers except two targets (MMP3 and SERPINE1) which didn't have pairwise data in both saliva and plasma samples. All the eight targets, except ISG15, showed poor correlation between their saliva and plasma levels ($r < 0.2$); for ISG15, its levels in saliva and plasma exhibited positive correlation (Pearson's $r = 0.688$, $p = .0192$) although there are only 11 pairwise data points (Supplemental Fig. S4). Moreover, for all target proteins simultaneously measurable by MRM or SISCAPA-MRM in paired saliva and plasma samples, we obtained correlation r values of 0.0736 (for MRM) and 0.1358 (for SISCAPA-MRM) between saliva and plasma (Supplemental Fig. S5A and B), indicating that the determined concentrations of multiple candidate biomarkers in these paired saliva and plasma samples were very poorly correlated ($r < 0.2$). These results reveal that the plasma concentration of proteins was not reflective of saliva concentrations, and suggest that the concentration of these candidate protein biomarkers in saliva is more meaningful for oral cancer detection.

3.4. Evaluation of the discrimination power of candidate biomarkers quantified using SISCAPA-MRM

Since the SISCAPA-MRM assay was capable of greater detection sensitivity and measured more data points, we further investigated the quantitative results of SISCAPA-MRM analysis to address clinical utility of each target in use of the two biofluid sample types (saliva and plasma). Ten targets—CSTA, EGFR, HSPA5, ISG15, LGASL3BP, MMP1, MMP3, PADI1, SERPINE1, TNC—showed significant ($p < .05$) changes in saliva levels in the OSCC group compared with the healthy group (Fig. 4A & Supplemental Table S10); however, only one target (LGALS3BP) displayed a significant relative increase in levels (Oc/Hc = 1.5) in plasma (Fig. 4B & Supplemental Table S11). Concerning the multiple comparisons could affect the statistical finding, we further adjusted the statistical significance by Benjamini–Hochberg procedure and found that seven targets (CSTA, ISG15, MMP1, MMP3, PADI1, SERPINE1, and TNC) still remained significant change in saliva samples but no such targets could be found in plasma samples (Supplemental Table S12). Consequently, seven candidates elevated in saliva samples

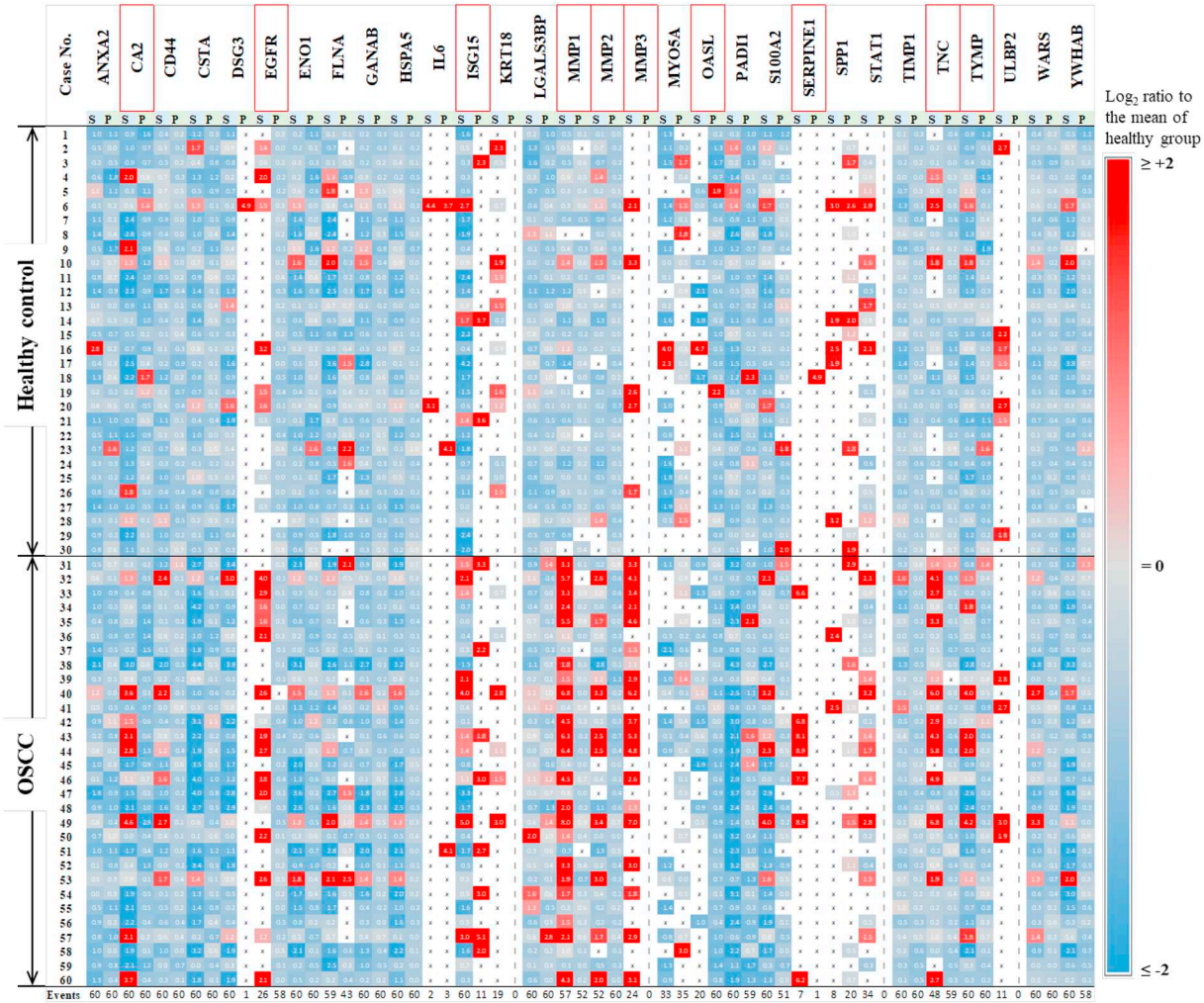

Fig. 3. Heat maps of the relative abundance of each target in saliva and plasma samples from 60 individuals. The levels of each target protein in paired saliva (S) and plasma (P) samples from each of the 30 healthy subjects and 30 OSCC patients were quantified by SISCAPA-MRM assay. For each target protein, the $\log_2$ ratio of the determined concentration to the mean concentration of the healthy group (case numbers 1–30) was first calculated in the 60 saliva or plasma samples, and data from the 30 target proteins were then combined to generate the heat map, which illustrates significant changes in candidate proteins in OSCC patients. "×" indicates that the protein in question was not detectable in the corresponding sample, and "|" denotes the protein in question was not detectable in any sample.

of OSCC groups were selected, and their assay results are detailed in Table 1. In addition to fold-change and *p*-value, the ROC curve analysis further showed that five of the seven targets (MMP1, PADI1, TNC, CSTA and MMP3) showed AUC values being 0.914, 0.827, 0.813, 0.77 and 0.753, suggesting their good potential for further clinical validation. To further confirm the results observed by using MS-based quantification, we performed multiplexed bead-based immunoassays to quantify the protein levels of four selected targets (MMP1, MMP3, TNC and LGALS3BP) in both saliva and plasma samples. As shown in Fig. 5, the results are, in principle, consistent with those obtained using the MS-based assay, which also indicated the elevated saliva levels of three targets (MMP1, MMP3 and TNC) in OSCC cases versus healthy controls while only one target (LGALS3BP) elevated in plasma samples from OSCC cases.

4. Discussion

Detection of disease biomarkers in body fluids has great impact for disease diagnostics and treatment. Both saliva and blood samples have been widely used for oral cancer biomarker discovery, but there is limited information about the systematic comparison of disease-discriminating power of candidate protein biomarkers measured in these two biofluids. The study by Lee et al. [23] represents one such effort to address this issue, which measured 14 cytokines and found that the levels of several cytokines, such as IL1β, IL-6, IL-8 and TNF-α, were significantly increased in saliva from OSCC patients but showed no obvious difference in plasma samples between patient (n = 41) and control (n = 24) groups. Our current study addressed the same issue but focusing on quantification of candidate biomarkers other than cytokines (which were prioritized from a review of literatures) using in-house developed, MS-based multiplex assays. Our data showed that five candidates (MMP1, PADI1, TNC, CSTA and MMP3) with significantly altered levels and displaying high disease-discriminating power (AUC = 0.914, 0.827, 0.813, 0.77 and 0.753) were only observed in saliva, but not plasma, samples from OSCC patients (Table 1). Taken together with the findings by Lee et al. [23], these results indicate that, for OSCC, disease-caused alterations in protein biomarkers in the local microenvironment of the oral cavity may poorly be reflected in the systemic circulation. Our study highlights the importance to validate the oral cancer biomarkers in saliva, which is less invasive and close to tumor nest than blood samples.

While serum/plasma is generally regarded as one of standard specimens for evaluation of systemic alternations including malignant disease, saliva is a local infiltrate of oral cavity, respiratory tract and digestive tract. Approximate one third of the whole-saliva proteins are found in plasma, and nearly 40% of biomarker candidates discovered in

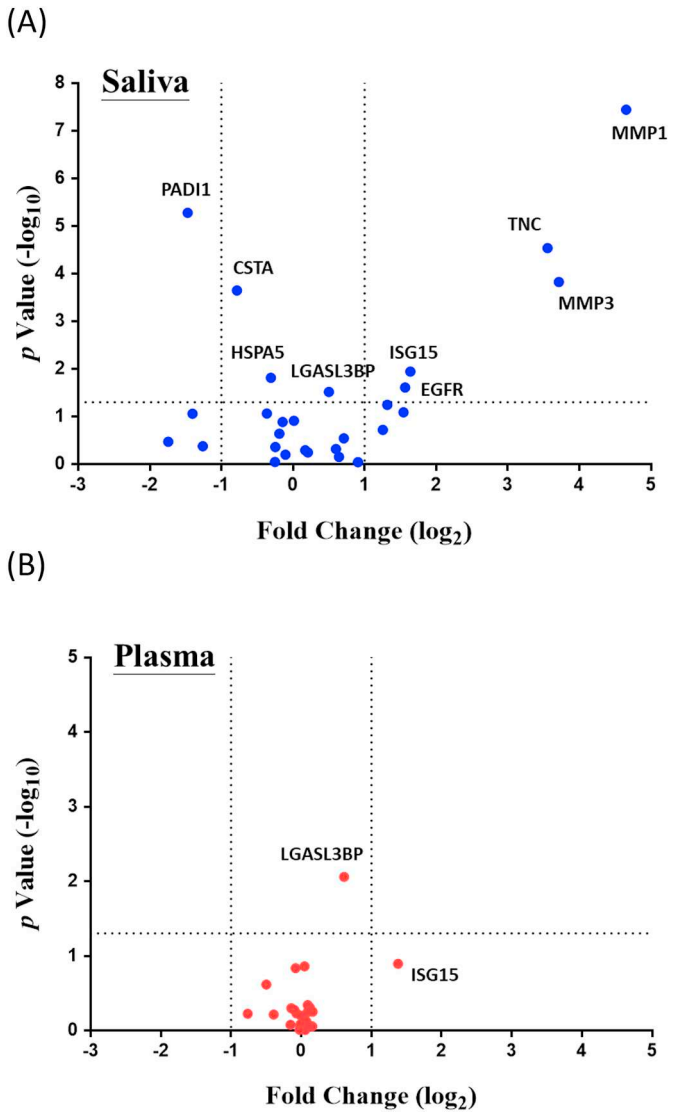

Fig. 4. Evaluation of the performance of SISCAPA-MRM–quantified candidate biomarkers in distinguishing oral cancer patients from healthy controls using saliva and plasma samples. A volcano plot (fold change vs. *p*-value) of 29 targets quantified in saliva (A) and 26 targets quantified in plasma (B) showing significant ($p < .05$) changes in nine targets in saliva and one target in plasma. The horizontal dotted line indicates $p = .05$, and the vertical dotted line denotes fold-change = 2. It is noted that the saliva level of SERPINE1 was significantly elevated in OSCC group ($p = .006$), but its fold change between healthy and OSCC group couldn't be estimated (the average concentration of SERPINE1 in healthy group was zero). Therefore, SERPINE1 is not shown in this figure.

plasma also existed in saliva [26,35,36]. However, the correlation of protein abundances between saliva and plasma was not largely explored. A previous study compared the expression levels of multiple biomarkers (27 cytokines) in plasma and saliva samples from 50 healthy adults, and only two cytokines (IL-6 and MIP-1β) showed week correlation ($r = 0.31$ and 0.34) between saliva and plasma [37]. We preformed correlation analysis between saliva and plasma samples in the abundance levels (Supplemental Fig. S5) and relative abundance levels (Supplemental Fig. S4) and obtained similar results to support the week correlation of protein level between saliva and plasma. However, some factors such as the variation of concentration of salivary substances affected by the collection time and dietary habits of donors have to be considered and further evaluated. In the concern of this issue, we have reported that the average variation of 90 proteins in the intraday- and interday-collected saliva samples from the same subjects was 38% and 43%, respectively [38], suggesting that the variation of concentration of proteins in saliva samples collected via a well-controlled protocol might be small.

For biomarker discovery, the reproducibility and sensitivity of the applied assays would profoundly affect the reliability and validity of the results. The extreme dynamic range of protein concentration in plasma appears to be the major factor affecting the sensitivity of MRM assays. As expected, the detection sensitivity of many targets in both plasma and saliva was enhanced using the SISCAPA-MRM assay (Supplemental Table S8); specifically, up to 21 targets in plasma showed improvement (2.4- to 22.9-fold) in LLOQ values compared with those determined using the MRM assay. In our experimental design, 100 µg of plasma protein (equivalent to 1–2 µL of plasma) was used for the SISCAPA-MRM assay. Improving the LLOQ value to near sub-nanogram per milliliter levels could require ~1-mL plasma samples [32]. On the other hand, LLOQ values determined by SISCAPA-MRM assay were unsatisfactory in 12 targets in saliva and 2 targets in plasma (SISCAPA-MRM/MRM < 0.66; Supplemental Table S8). Lower-affinity of antigen-antibody interactions might account for this observation. Therefore, antibodies with higher capture efficiencies are recommended to achieve higher quality and sensitivity of SISCAPA-MRM assays.

In the present study, single point measurements based on the internal standards were used for the quantification of targets in clinical samples while the reverse response curves were generated for the determination of signal linearity and LOD/LLOQ corresponding to the selected peptides. The concentration ranges of 8 proteins (EGFR, IL6, KRT18, MMP2, MYO5A, SERPINE1, SPP1 and ULBP2) below the respective assay LLOQs will challenge the accuracy of the quantification results (Supplemental Tables S7, S8, S10 and S11). However, if both stable-isotope-labeled internal standards and endogenous peptides could be detected with confident peak sharp and co-elution feature, the determined values were included for quantification analysis. Among the 30 targets, 12 have been detected in saliva and/or plasma samples by immuno-based assays in previous studies (Supplemental Table S2). In comparison of our quantitative results (Supplemental Tables S10 and S11) with the concentration ranges reported (Supplemental Table S2), the levels measured by MS-based assay were similar to those by immuno-based assays for IL6 and CD44 (saliva sample), and TNC, LGALS3BP, MMP2, and SPP1 (plasma sample). However, it is quite different between MS-based and immuno-based quantifications for some proteins, such as LGALS3BP, MMP1, MMP3 (saliva sample) and CD44 (plasma sample). Percy et al. reported the concentration ranges of 158 proteins in healthy donor's saliva samples by LC-MRM assay [26]. Among the 6 target proteins measured in both our and Percy's studies, four targets (CD44, HSPA5, LGALS3BP, and TIMP1) showed similar concentrations but two targets (CSTA and ENO1) displayed different levels. The differences in method and target peptide selected for quantification, as well as the race of study subjects might contribute to the observed discrepancies between studies.

By statistical analysis and AUC value evaluation of the SISCAPA-MRM assay results, our study revealed significant increase of three proteins (MMP1, MMP3 and TNC) and decrease of two proteins (CSTA and PADI1) in saliva samples from OSCC patients (Table 1). Although sample cohort of this study is small, the salivary levels of MMP1, MMP3, and CSTA have been verified in a large cohort consisting of 460 individuals enrolled in an oral cancer screening program in our previous study, showing that MMP1 is the most highly increased protein in the OSCC group with a disease-discriminating power value (AUC) being 0.871 [19]. It has been shown that metalloproteinases, a family of multifunctional proteins, are up-regulated in various types of cancer, including oral cavity cancers [39–41]. Transcriptional assays and multiplex sandwich-ELISAs have shown that the RNA and protein levels of both MMP1 and MMP3 are highly elevated in tumor tissues and saliva from OSCC patients and display an increasing trend with higher disease stage [39]. Increases in tenascin-C (TNC) in oral cancer are

Table 1
Concentrations of seven candidate biomarkers in saliva and plasma samples, determined by SISCAPA-MRM assay.

Protein	Hc (n = 30)		Oc (n = 30)		Oc vs. Hc				
	Concentration[a] (ng/mL)	Detectable	Concentration (ng/mL)	Detectable	Fold-change[b]	*p*	Rank	(i/m)Q[c]	AUC
Saliva sample									
MMP1	0.784 ± 0.501	27	19.728 ± 39.983	30	25.18	< 0.0001	1	0.002	0.914
PADI1	8.591 ± 6.440	30	3.099 ± 2.902	30	0.36	< 0.0001	2	0.003	0.827
TNC	7.145 ± 8.797	20	83.967 ± 170.794	28	11.75	< 0.0001	3	0.005	0.813
MMP3	0.829 ± 2.029	5	10.865 ± 21.537	19	13.11	< 0.0001	4	0.007	0.753
CSTA	314.332 ± 223.102	30	182.679 ± 212.763	30	0.58	< 0.0001	5	0.008	0.770
SERPINE1	0.000 ± 0.000	0	5.685 ± 13.035	7	–	0.006	6	0.010	0.617
ISG15	2.368 ± 3.083	30	7.381 ± 14.804	30	3.12	0.011	7	0.012	0.689
Plasma sample									
MMP1	9.838 ± 5.023	25	11.008 ± 4.902	27	1.12	0.564	12	0.020	0.544
PADI1	24.761 ± 20.545	29	26.095 ± 21.305	30	1.05	0.764	18	0.030	0.523
TNC	669.708 ± 216.236	30	668.396 ± 310.371	29	1.00	0.633	17	0.028	0.537
MMP3	0.000 ± 0.000	0	0.000 ± 0.000	0	–	–	–	–	–
CSTA	132.910 ± 52.051	30	124.247 ± 52.917	30	0.93	0.532	11	0.018	0.548
SERPINE1	12.325 ± 67.505	1	0.000 ± 0.000	0	–	0.334	7	0.012	0.483
ISG15	0.572 ± 1.866	3	1.489 ± 3.741	8	2.60	0.128	2	0.003	0.578

[a] Mean ± SD.
[b] Fold change of protein levels in OSCC group over healthy control group.
[c] (i/m)Q, where i is the rank, m is the total number of tests, and Q is the false discovery rate defined as 5%; significance adjusted according to p < (i/m)Q.

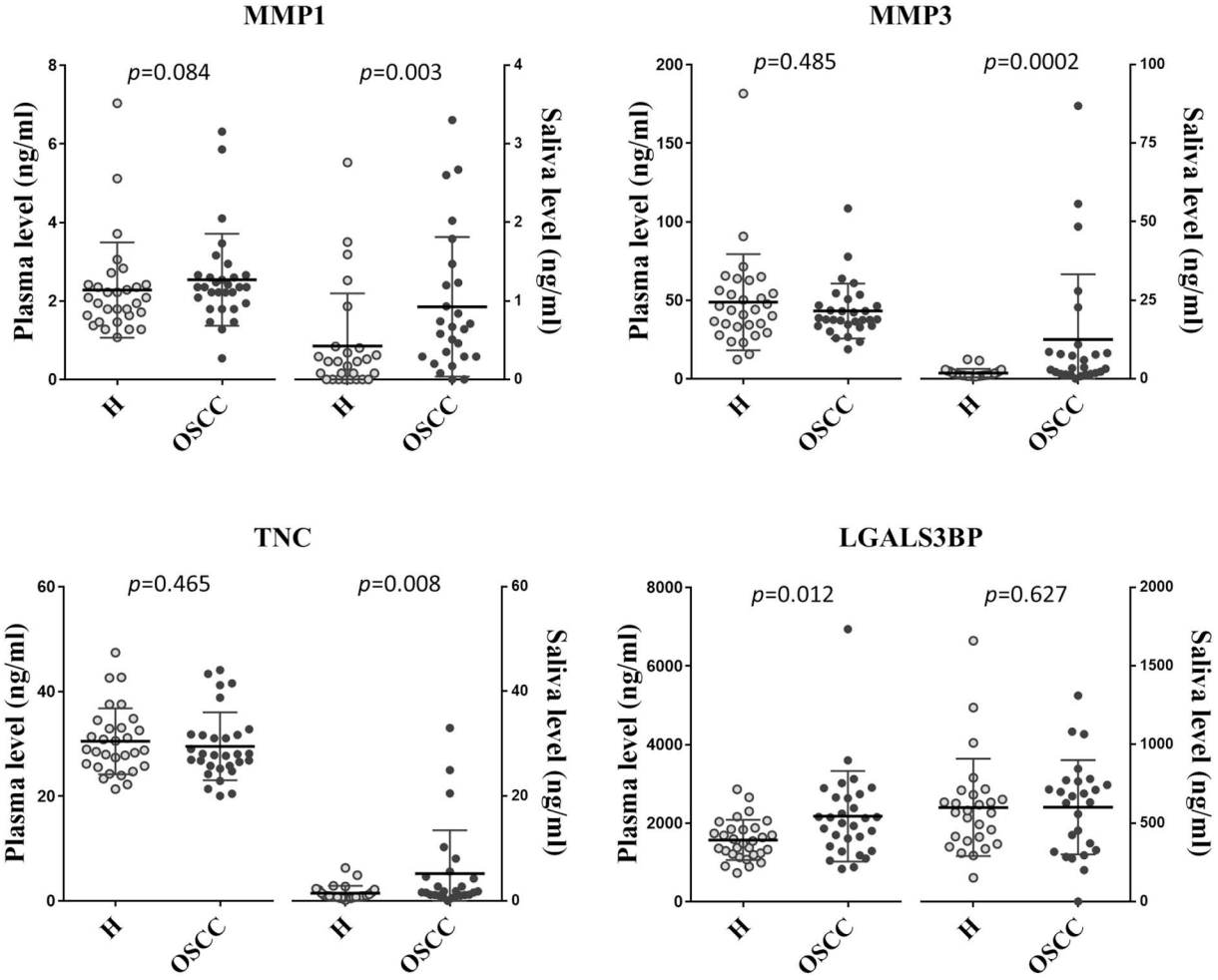

Fig. 5. Quantification of four targets in plasma and saliva samples using the multiplexed bead-based immunoassay. For plasma sample, 60 samples (30 H and 30 OSCC) were diluted 20-fold with sample diluent and then subjected to the multiplexed bead-based immunoassay (R&D luminax) according to the manufacture's protocol. For saliva samples, 53 samples (27 H and 26 OSCC among 30 cases each group) remained enough amount for this analysis. The saliva samples were diluted 5-fold with sample diluent and then analyzed.

positively associated with tumor metastasis, implicating TNC in modulation of the extracellular matrix at the invasive tumor front [42]. However, no previous studies have investigated its salivary levels in OSCC patients. Cysteine protease inhibitor cystatin A (CSTA), belonging to type 1 cystatin super-family, acts as a tumor suppressor in many cancers, including esophageal, skin and Lung cancers. In head and neck cancer, overexpression of CSTA was found in tumor tissues as compared with the non-tumor part, but the risk of tumor recurrence was high in patients with low CSTA level [43,44] PADI1, encoding peptidyl arginine deiminase type-1, was found to be down-regulated in oral cancer tissue specimens using Affymetrix cDNA arrays. PADI1 can catalyze the post-translational deamination of proteins such as filaggrin and keratins by converting arginine residues into citrulines in the last steps of epidermal differentiation, a process that may support the growth and movement of tumor cells [45,46]. Due to the small sample size tested in this study, the saliva biomarkers found in this study have to be further verified in a large cohort to clarify their clinical relevance and significance.

5. Conclusions

We have developed 30-plex MRM and SISCAPA-MRM assays for candidate OSCC biomarkers and applied these assays to compare the disease-discriminating power of selected biomarkers in paired saliva and plasma samples from 60 subjects. SISCAPA-MRM assay has higher detection sensitivity than MRM assay and can quantify more targets in both saliva and plasma samples. For most quantified targets, a very poor correlation was observed between their saliva and plasma levels. Five targets (MMP1, PADI1, TNC, CSTA and MMP3) displayed significantly altered salivary levels in OSCC patients as compared to healthy subjects with AUC = 0.914, 0.827, 0.813, 0.77 and 0.753, but none of these alterations could be observed in the plasma samples from the same subjects. Our data support the notion that saliva may be more suitable for protein biomarker-based detection of oral cancer. Moreover, the newly developed 30-plex SISCAPA-MRM assay could be used to verify the clinical utility of multiple oral cancer biomarker candidates in a large cohort of saliva samples in future studies.

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Availability of data and material

All data generated or analyzed during this study are included in this article and its additional files. The raw files of mass spectrometry-based quantitative data have been deposited to the Peptide Atlas with the link of https://db.systemsbiology.net/sbeams/cgi/PeptideAtlas/PASS_View. Dataset title: Assessment of candidate biomarkers in paired saliva and plasma samples from oral cancer patients by targeted mass spectrometry; Dataset identifier: PASS01299; Password: HN9894s. The raw files include the four sets of response curve (MRM and SISCAP-MRM assay in each of saliva or plasma), applications of two assays on individual samples.

Authors' contributions

LMC, YCH, and JSY contributed to analysis and interpretation of data and drafting of the manuscript. KYC contributed to acquisition of data and technical support. SFC, YNC, SYL, and WSW contributed to acquisition of data and processing of raw data. IYC, CY, and LJC contributed to statistical analysis of data. WFC, CYC, and KPC contributed to collection of clinical sample and providing clinical information. YSC, KPC and JSY contributed to study concept and design.

Ethics approval and consent to participate

This study was approved by the Institutional Review Board of Chang Gung Memorial Hospital, and all subjects signed an informed consent form to participate and permit the use of plasma/saliva samples collected before treatment.

Consent for publication

Not applicable.

Declaration of Competing Interest

The authors declare that they have no competing interests and no potential conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2019.103571.

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