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Dear Editor,

Thank you very much for encouraging us to resubmit our manuscript for publication in Analytica Chimica Acta. Our manuscript entitled "**Proteomic identification of salivary transferrin as a biomarker for early detection of oral cancer**", which has been previously assigned as the Number: ACA-09-2158 and ACA-10-751. Our manuscript has been revised according to reviewer's comments.

Thanks very much again!

Sincerely,

Cheng-Wen Lin, PhD
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The responses to the comments of Reviewer #1 are listed in the following:

(A) Response to the comments on the manuscript of ACA-09-2158

Q1. This manuscript focuses on searching biomarkers for oral cancer. However, no literature was reviewed or discussed regarding the use of proteomics analyses of saliva samples for the discovery of protein markers for oral cancer. It's suggested that the authors discuss relevant literature in the manuscript. For example, in the following paper, Clin Cancer Res 2008;14(19) 6246-6252), it is reported that five protein biomarkers for oral squamous cell carcinoma were found in the saliva. The saliva samples were collected from 64 patients with oral squamous cell carcinoma and 64 healthy patients. Five candidate biomarkers, M2BP, MRP14, CD59, profiling, and catalase, were successfully validated using immunoassays. The presence of these biomarkers confirmed the presence of oral cancer 93 percent of the time.

Ans 1.:

(1) In the Introduction section at Page 6, we have corrected as"Significant increase of salivary soluble CD44 (solCD44) levels has been identified in

head and neck squamous cell carcinoma (HNSCC) patients compared with normal controls [18]. Comprehensive analysis of salivary parameters shows that secretory immunoglobulin A, 8-oxoguanine DNA glycosylase, phosphorylated-Src and mammary serine protease inhibitor (Maspin) are lower, while insulin growth factor I, metalloproteinases MMP-9, carbonyls and Cyclin D1 (CycD1)are higher in OSCC patients [19, 20]. Recent, proteomic analysis of saliva samples from OSCC patients and matched healthy subjects using capillary reversed-phase liquid chromatography with quadruple time-of-flight (LC-Q-TOF) mass spectrometry indicated five candidate OSCC biomarkers (M2BP, MRP14, CD59, profilin, and catalase), being successfully validated using immunoassays on an independent set of OSCC patients and matched healthy subjects [21]. However, these identified candidate OSCC biomarkers have no significant relationship with tumor size, stage and recurrence."

(2) In the Introduction section at Page 20, we have corrected as"the specificity and sensitivity of salivary transferrin-based ELISA was 100% and 100% in T1 group, and 100% and 95% in overall OSCC patients, respectively. By contrast, the sensitivity of salivary solCD44 levels for detection of HNSCC patients ranged from 62% to 70%, and its specificity ranged from 75% to 88% [18]. The sensitivity and specificity values of CycD1 and Maspin as candidate OSCC markers were 100% [20]. In addition, the combination of M2BP, MRP14, CD59, profilin, and catalase as candidate biomarkers yielded a receiver operating characteristic value of 93%, sensitivity of 90%, and specificity of 83% in detecting OSCC [21]. Comparison of salivary transferrin with these reported candidate markers indicated that the receiver operating characteristic value, specificity and sensitivity salivary transferrin were similar to those of reported candidate OSCC markers. Of candidate markers, only salivary transferrin showed the ability for the detection of early-stage oral cancer."

Q2. It's also suggested that the authors compare their finding with those reported in the literature regarding proteomics analysis of saliva for biomarker discovery.

Ans 2.:

In the Introduction section at Page 20, we have corrected as"the specificity and

sensitivity of salivary transferrin-based ELISA was 100% and 100% in T1 group, and 100% and 95% in overall OSCC patients, respectively. By contrast, the sensitivity of salivary solCD44 levels for detection of HNSCC patients ranged from 62% to 70%, and its specificity ranged from 75% to 88% [18]. The sensitivity and specificity values of CycD1 and Maspin as candidate OSCC markers were 100% [20]. In addition, the combination of M2BP, MRP14, CD59, profilin, and catalase as candidate biomarkers yielded a receiver operating characteristic value of 93%, sensitivity of 90%, and specificity of 83% in detecting OSCC [21]. Comparison of salivary transferrin with these reported candidate markers indicated that the receiver operating characteristic value, specificity and sensitivity salivary transferrin were similar to those of reported candidate OSCC markers. Of candidate markers, only salivary transferrin showed the ability for the detection of early-stage oral cancer."

Q3. . Page 2, Abstract, line 9. The term "2D/MALDI-TOF" is not commonly used in the literature and rather confusing. Does it mean "two-dimensional electrophoresis followed by MALDI-TOF-MS analysis"? Or, "two-dimensional MALDI-TOF? If the former is correct, then it is suggest that "2DE followed by MALDI-TOF-MS" be used. If the latter is correct or this term is referred to something else, then the definition of "2D/MALDI-TOF" should be provided.

Ans 3.:

In the Abstract section, we changed two-dimensional electrophoresis followed by MALDI-TOF-MS analysis instead of "2D/MALDI-TOF".

Q4. Page 4, line 4. The parameters used for Mascot search, such as allowed modifications, number of missed cleavages, acceptable peptide score, and number of unique peptides for acceptance, etc., should be reported in the manuscript.

Ans 4.:

In the MATERIALS AND METHODS section at Pages 11 and 12, we have corrected as"Proteins were identified on the MALDI-TOF/TOF-MS spectra by searching against NCBI databases for exact matches using the ProID program (Applied Biosystem/MDS Sciex) and the MASCOT search program

(http://www.matrixscience.com). The derived peak list generated by Mascot.dll v1.6b27 (Applied Biosystems) was searched using a local version of the Mascot (2) search program (v2.2.1; Matrix Science Ltd.) and the Mascot Daemon application (v2.2.0). A human (20,403 sequences) database (Swiss-Prot, release version 57.6, 28-Jul-2009, 495,880 sequences) was used for database searches, and a decoy protein was generated for each of these proteins by sequence reversal to enable estimation of false discovery rates (FDRs). The search parameters are as following: peptide and MS/MS tolerance, ±0.3 Da; trypsin missed cleavages, 1; and variable modifications, carbamidomethylation and Met oxidation; and instrument type, MALDI-TOF-TOF. The interpretation and presentation of MS/MS data were performed according to published guidelines. The MASCOT scores greater than 55 were significant for PMF search (P<0.05). In addition, individual MS/MS spectra for peptides with a Mascot ions score lower than 40 (expect value < 0.015) were inspected manually and included in the statistics only if a series of at least four continuous y or b ions were observed. Protein identification was also based on the assignment of at least two peptides. In all cases, keratins were excluded. Protein expectation value below 0.02 were initially considered true hits with a FDR of <5.0% (estimated by the numbers of reversed matches dividing by the total number of matches).".

In addition, Table 2 at Page 31 had listed the Mascot score, matched peptides and peptide coverage.

Q5.. Page 6, line 23. What was the protein concentration of the saliva samples? Any difference between those from patients and controls?

Ans 5.:

In the MATERIALS AND METHODS section at Page 9 (lanes 11 and 12), we have corrected as"Both pooled OSCC and control salivary samples (100 μg proteins in total each) were analyzed by two-dimensional gel electrophoresis.".

Q6. Page 7, line 6. It is stated here 10% gels were used. However, Figure 1 caption says 12%.

Ans 6.:

In the MATERIALS AND METHODS section at Page 10 (lanes 6 and 7), we

have corrected as“The immobilized pH gradient (IPG) gels were then transferred to the top of 10% polyacrylamide gels (13 × 13 cm) for 2-D electrophoresis.”.

Q7. Page 7, line 12. What was the statistical procedure used for finding spots of two-dimensional gels with altered intensities?

Ans 7.:

In the MATERIALS AND METHODS section at Page 10 (lanes 10 to 12), we have corrected as“Data from three independently stained gels of each sample were used for the correction of spot intensity graphs and statistical analysis with ANOVA analysis.”.

Q8. Page 7, line 25. Is "4-hydroxy cinnamic acid (HCCA)" the correct name of the matrix used for the MALDI experiments? Could "alpha-cyano-4-hydroxy cinnamic acid (CHCA)" be the correct name of the matrix?

Ans 8.:

In the MATERIALS AND METHODS section at Page 11 (lanes 5 to 8), we have corrected as “The peptides were crystallized using the alpha-cyano-4-hydroxycinnamic acid (CHCA) matrix (0.1% TFA, 50% acetone, 1 mg/ml CHCA) and the flight time from the target to the ion detector was calculated.”.

Q9. Page 10, line 1. :Table 2" should be "Table 1".

Ans 9.:

In the Result section at Page 14 (lanes 9 to 11), we have corrected as “A total of 41 OSCC patients were recruited in this study, 39 (95%) males and 2 (5%) females. The age range of OSCC patients was 29 to 79 with an average age of 51.2 years (Table 1).”.

Q10. Page 10, line 9. "using 2D/MS" analysis (Fig. 1)"? There is no MS data here. What is the meaning of "2D"?

Ans 10.:

In the Result section at Page 15 (lanes 4 to 6), we have corrected as “To identify

the potential salivary biomarkers for oral cancer detection, comparison of the salivary protein profiles between OSCC-free control subjects and OSCC patients was performed using 2DE analysis (Fig. 1).”.

Q11. Page 10, line 9. How many gel spots were indentified in those gels? What procedures were used to find out the difference between gels?

Ans 11.:

In the Result section at Page 15 (lanes 8 to 11), we have mentioned that “Silver-stained gels revealed 15 protein spots with at least a 2-fold increase in spot intensity and 31 protein spots with at least a 2-fold decrease in spot intensity in the salivary protein profile of OSCC patients compared with OSCC-free control subjects.”.

Q12. Pag3 10. Why were only four spots selected for further in-gel digestion and mass spectrometry analysis? Were any criteria applied here?

Ans 12.:

In the Result section at Page 15 (lanes 8 to 11), we have mentioned that “Only eleven proteins such as transferrin (Spot ID 1) and transferrin chain A (Spot ID 7) were identified as matching with Mascot score at greater than 55, and MW and pI of indicated proteins in 2DE gel (Figure 2A, Table 2).”

Q13. Table 2. The proteins were identified by Mascot with low protein scores. However, the number of peptides is often greater than 2. It is suggested that the authors double-check the scores and if there were any redundant peptides counted.

Ans 13.:

In the Result section at Page 15 (lanes 15 to 19) and Page 16 (lane 1), we have added that “The amino acid sequence coverage of identified up-regulatory and down-regulatory proteins varied from 20% to 75%. For example, MALDI-TOF MS analysis of transferrin (Spot ID 1) showed a Mascot score of 84, sequence coverage of 56%, and 7 matched peptides (Figure 2A), while S100 calcium-binding protein A8 (Spot ID 9) showed a Mascot score of 94, sequence coverage of 51%, and 23 matched peptides.”.

Q14. Table. It is suggested that theoretical pI and MW values be listed in the table.

Ans 14.:

In the Table 2 at Page 31, we have added theoretical pI and MW values.

Q15. Figure 2. The quality of this product ion scan mass spectral is very poor. Most of the annotated signals corresponding to peptide fragment ions in the figure are in very low S/N ratios. It is required to obtain another MS/MS spectrum with acceptable quality.

Ans 15.:

In Figure 2, we had shown clear data for MALDI-TOF MS analysis and MALDI-TOF-TOF sequencing of transferrin.

Q16. Figure 3. The MW values revealed by Western blot are not consistent with those listed in the Table 2 for either transferrin or transferring chain A.

Ans 16.:

In Figure 3, Western blotting showed that 79 kDa- and 36 kDa-immunoreactive bands for transferrin and transferrin chain A were detected in the saliva of OSCC patients, but not in the saliva of control subjects.”.

Q17. Figure 3. The quality of the Western blots is poor.

Ans 17.:

We had repeated the Western blots and showed a good quality for Figure 3.

Q18. Figure 4. The unit of Y-axis is missing.

Ans 18.:

We had added the unit of Y-axis in Figure 4.

Q19. Figure 4. It is not appropriate to calculate Pearson correlation coefficients since tumor staging is not a continuous variable.

Ans19.:

We had used the ANOVA analysis for correlation between tumor staging and transferrin level.

Q20. Figure 5. The ROC curves presented here are based on the discrimination between normal controls and samples associated with a specific tumor stage. It

is not equivalent to the prediction of oral cancer of different stages. The information presented in this figure is misleading.

Ans 20.:

We grouped T3 and T4 patients into one group. In addition, we had mentioned that "AUROC for predicting OSCC in patients was 0.95 for T1 group (95% CI: 0.48 - 1.05), 0.94 for T2 group (95% CI: 0.51 – 0.88), and 0.91 for T3/T4 group (95% CI: 0.59 - 1.18), respectively." in the RESULT section at Page 17 (lanes 14 to 16).

Q21. Figure 6. It is not appropriate to calculate Pearson correlation coefficients since tumor staging is not a continuous variable.

Ans 21.:

We had used the ANOVA analysis for correlation between tumor staging and transferrin level.

(B) Response to the comments on the manuscript of ACA-10-751

Q1. The cover letter does not address reviewers' comments. It is unclear what has been revised in the main text.

Ans 1.:

Now, we showed the responses to reviewers' comments of ACA-09-2158 above pages in the letter.

Q2. Figure 2B, no need to label all the "b" and "y" ions. It is good enough that the most abundant peaks are matched. Some of the labeled "b" and "y" ions are at noise level.

Ans 2.:

We had removed all labels for "b" and "y" ions in Figure 2B.

The responses to the comments of Reviewer #2 are listed in the following:

(A) Response to the comments on the manuscript of ACA-09-2158

Q1. Figures 2, the MS/MS spectrum is noisy and crowded. I suggest the authors use a better quality spectrum.

Ans 1.:

We have the better MS spectrum (Fig 2A) and MS/MS spectrum (Fig 2B) in

revised manuscript.

Q2. Same issue for Figure 3, the quality of Western blotting data for transferrin is low. Were each band from a single sample or pooled sample?

Ans 2.:

We have used better Western blotting data for analysis of transferrin from pooled samples (Fig 3)

Q3. The accession numbers for proteins should be listed in Table 2. Both transferrin and transferrin chain A are listed in Table 2. One is 16010 Da and the other one is 37241 Da, none of which is matched to the Western blot band (79kDa).

Ans 3.:

We have corrected data in Table 2, which transferrin is 76780 Da. In Figure 3, Western blotting showed that 79 kDa- and 36 kDa-immunoreactive bands for transferrin and transferrin chain A were detected in the saliva of OSCC patients, but not in the saliva of control subjects.”.

Q4. It makes more sense to report box plot graph for the datapoint shown in Figures 4 & 6. The sample size for late stage (T3 & T4) is small. It is hard to tell whether the levels of transferrin in T3 & T4 samples are elevated or not.

Ans 4.:

T3 and T4 were grouped together. T3/T4 group are 9 patients. In addition, we had mentioned that “AUROC for predicting OSCC in patients was 0.95 for T1 group (95% CI: 0.48 - 1.05), 0.94 for T2 group (95% CI: 0.51 – 0.88), and 0.91 for T3/T4 group (95% CI: 0.59 - 1.18), respectively.” in the RESULT section at Page 17 (lanes 14 to 16).

Q5. In the Novelty Statement as well as in the Abstract, the authors stated that "In this study, we identified the elevated levels of salivary transferrin in oral cancer patients as determined by 2D/MALDI-TOF and confirmed by MALDI-TOF/TOF". This is not correct. According to the Materials and Methods, identification of salivary transferrin was based on 2-DE with MALDI-TOF/TOF.

Ans 5.:

We have mentioned “Transferrin levels were elevated in the saliva of OSCC patients as determined using 2DE followed by MALDI-TOF-MS and confirmed by MALDI-TOF/TOF MS, Western blotting and ELISA.” in ABSTRACT section.

Q6. Figure 5, ROC (AUC) values should be reported.

Ans 6.:

We have mentioned “AUROC for predicting OSCC in patients was 0.95 for T1 group (95% CI: 0.48 - 1.05), 0.94 for T2 group (95% CI: 0.51 – 0.88), and 0.91 for T3/T4 group (95% CI: 0.59 - 1.18), respectively.” in RESULT SECTION at Page 17.

Q7. Previous studies on using salivary proteomics for oral cancer biomarker identification were not adequately cited.

Ans 7.:

We have cited several studies about oral cancer biomarker identification using salivary proteomics as the following:

[18] E. J. Franzmann, E. P. Reategui, K. L. Carraway, K. L. Hamilton, D. T. Weed, W. J. Goodwin. Cancer Epidemiol. Biomarkers Prev. 14 (2005) 735 - 739.
[19] T. Shpitzer, G. Bahar, R. Feinmesser, R. M. Nagler. J. Cancer Res. Clin. Oncol. 133 (2007) 613–617.
[20] T. Shpitzer, Y. Hamzany, G. Bahar, R. Feinmesser, D. Savulescu, I. Borovoi, M. Gavish, R. M. Nagler. Br. J. Cancer 101 (2009) 1194–1198.
[21] S. Hu, M. Arellano, P. Boontheung, J. Wang, H. Zhou, J. Jiang, D. Elashoff, R. Wei, J. A. Loo, D. T. Wong. Clin. Cancer Res. 14 (2008) 6246 - 6252.

(B) Response to the comments on the manuscript of ACA-10-751

Q1. The current manuscript is a result of resubmission of a previously reviewed one with revisions. The quality of the manuscript has been much improved. Although several comments made by the reviewers have been addressed as explained in the corresponding author's letter to the journal editor, many concerns raised by the reviewers were neither adequately answered in the letter nor reflected in the revised manuscript. Therefore, it is suggested that the authors should further improve their manuscript before it could be published in the Analytica Chimica Acta.

Ans 1.:

Thanks. We had improved the manuscript again.

Q2. Transferrin is not a novel protein marker for this type of cancer.

Ans 2.:

In the Discussion section at Page 19, we had mentioned that "Recently, parotid acinar cells were reported to synthesize and secrete transferrin into saliva, providing evidence that the detection of transferrin in saliva does not indicate contamination of the sample with blood [24]. In addition, transferrin was found to be significantly increased in the saliva of head and neck squamous cell carcinoma (HNSCC) patients compared with unaffected controls using 2D-DIGE MS proteomic analysis [25]. Therefore, salivary transferrin is a potential candidate as an early detection biomarker and a prognostic marker for oral cancer, allowing for the development of diagnostic assays.".

Q3. It appeared that PMF and MS/MS fragmentation ions were both described in the manuscript. However, it is not clear which method, or both ones, were used to generate the data listed in the Table 2.

Ans 3.:

In the Materials and Methods section at Page 11, we had mentioned that "Proteins were identified on the MALDI-TOF/TOF-MS spectra by searching against NCBI databases for exact matches using the ProID program (Applied Biosystem/MDS Sciex) and the MASCOT search program (http://www.matrixscience.com). The derived peak list generated by Mascot.dll v1.6b27 (Applied Biosystems) was searched using a local version of the Mascot (2) search program (v2.2.1; Matrix Science Ltd.) and the Mascot Daemon application (v2.2.0). A human (20,403 sequences) database (Swiss-Prot, release version 57.6, 28-Jul-2009, 495,880 sequences) was used for database searches, and a decoy protein was generated for each of these proteins by sequence reversal to enable estimation of false discovery rates (FDRs). The search parameters are as following: peptide and MS/MS tolerance, ±0.3 Da; trypsin missed cleavages, 1; and variable modifications, carbamidomethylation and Met oxidation; and instrument type, MALDI-TOF-TOF."

Q4. The current novelty statement looks like an abstract of the manuscript. It needs

to be shortened and focused.

Ans 4.:

We had shortened and focused novelty statement as the following:

The study intends to identify salivary markers for detection of oral cancer. Pooled saliva from oral squamous cell carcinoma (OSCC) patients and OSCC-free controls are further analyzed using two-dimensional gel electrophoresis (2DE), followed by MALDI-TOF MS analyses and MALDI-TOF-TOF MS sequencing. Salivary transferrin elevated in OSCC saliva is identified as a potential marker for detection of early stage OSCC. The increase in salivary transferrin levels in OSCC patients strongly correlates with the size and stage of the tumor. The area under the receiver-operating characteristics curves shows that salivary transferrin-based ELISA is highly specific, sensitive and accurate for the early detection of oral cancer. Salivary transferrin is a potential biomarker for the detection of early-stage oral cancer.

Q5. "PH" is not a correct usage.

Ans 5.:

We had corrected ‘pH’ instead of ‘PH’ in Figure 1.

Q6. The interpretation and presentation of MS/MS data were performed according to published guidelines - which published guidelines? This has to be specified with references cited in the manuscript.

Ans 6.:

We had added the reference as the following:

[24] G. K. Taylor, D. R. Goodlett. Rapid Commun Mass Spectrom 19 (2005) 3420

Q7. The current manuscript claimed that the data in Fig. 2B resulted in a high degree of confidence in the protein identification. However, the quality of MS/MS data presented in the figure was extremely poor.

Ans 7.:

We have the better MS spectrum (Fig 2A) and MS/MS spectrum (Fig 2B) in revised manuscript.

Q8. A p value was reported to be smaller than 0.001 for the data presented in the Fig. 4. However, no statistical model was described anywhere in the current manuscript. Therefore, the p value is not comprehensible to readers.

Ans 8.:

In the Result section at Page 17, we had mentioned that "We then assessed the relationship between the protein level of salivary transferrin and tumor size using ANOVA analysis. Our data showed that a linear increase in salivary transferrin levels strongly correlated with increasing tumor size (P <0.001) (Fig. 4).**".**

Q9. The levels of salivary transferrin of patients were lower than those of healthy controls. In contrast with this trend, the levels of serum transferrin seemed to be higher in the healthy controls. Considering the levels of transferrin in saliva and plasma should be somewhat related, this observation was not a straightforward one. The authors should provide a reasonable explanation for this.

Ans 9.:

In the result section at Page 18 (lanes 6 to 13), we had mentioned that "The protein level of plasma transferrin was lower by 16% in the T1 group, 8% in the T2 group and 21% in the T3/T4 group compared with those in OSCC-free individuals. However, no significant difference of plasma transferrin was found among T1, T2, T3/T4 and OSCC-free groups using two-way ANOVA analysis (P = 0.3) (Fig. 6). The results indicated that the plasma transferrin levels varied in OSCC patients and OSCC-free controls, showing no correlation with the increase of OSCC tumor size.'

Q10. Since many concerns raised by the reviewers were neither adequately answered in the letter nor reflected in this revised manuscript, it is suggested that the authors should further improve their manuscript before it could be published.

Ans 10.:

We carefully corrected the revised manuscript and gave the answers in this letter.

Novelty Statement

The study intends to identify salivary markers for detection of oral cancer. Pooled saliva from oral squamous cell carcinoma (OSCC) patients and OSCC-free controls are further analyzed using two-dimensional gel electrophoresis (2DE), followed by MALDI-TOF MS analyses and MALDI-TOF-TOF MS sequencing. Salivary transferrin elevated in OSCC saliva is identified as a potential marker for detection of early stage OSCC. The increase in salivary transferrin levels in OSCC patients strongly correlates with the size and stage of the tumor. The area under the receiver-operating characteristics curves shows that salivary transferrin-based ELISA is highly specific, sensitive and accurate for the early detection of oral cancer. Salivary transferrin is a potential biomarker for the detection of early-stage oral cancer.

June 25, 2010

Proteomic identification of salivary transferrin as a biomarker for early detection of oral cancer

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Abstract

Oral cancer has a low five-year survival rate. Early detection of oral cancer could reduce the mortality and morbidity associated with this disease. Saliva, which can be sampled non-invasively and is less complex than blood, is a good potential source of oral cancer biomarkers. Proteomic analysis of saliva from oral cancer patients and control subjects was performed to identify salivary biomarkers of early stage oral cancer in humans. The protein profile of pooled salivary samples from patients with oral squamous cell carcinoma (OSCC) or OSCC-free control subjects was analyzed using tow-dimensional gel electrophoresis (2DE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analyses. Potential biomarkers were verified by Western blotting and ELISA assays. Transferrin levels were elevated in the saliva of OSCC patients as determined using 2DE followed by MALDI-TOF-MS and confirmed by MALDI-TOF/TOF MS, Western blotting and ELISA. The increase in salivary transferrin levels in OSCC patients strongly correlated with the size and stage of the tumor. The area under the receiver-operating characteristics curves showed that salivary transferrin-based ELISA was highly specific, sensitive and accurate for the early detection of oral cancer. We have identified salivary transferrin as a biomarker for the detection of early-stage oral cancer. This finding provides a promising basis

for the development of a non-invasive diagnostic test for early-stage oral cancer.

Keywords: Oral squamous cell carcinoma, two-dimensional gel electrophoresis, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, salivary transferrin, receiver-operating characteristics curve, early detection

1. Introduction

Oral cancer accounts for 2–3% of all malignancies [1]. There are more than 300,000 patients newly diagnosed with oral cancer annually worldwide [2]. Squamous cell carcinoma accounts for 90% of these cases [3]. Common risk factors for oral cancer include tobacco, alcohol and chewing betel quid. The risks are synergistic and might result in large areas of mucosal change or stimulate carcinogenesis in the oral cavity [4, 5]. Therefore, oral cancer patients carry a high risk of developing a secondary cancer in the upper aerodigestive tract [6–9]. In accordance with the multi-step theory of carcinogenesis, oral cancer develops from premalignant lesions and causes serial histological and clinical changes [10]. Clinically, premalignant lesions might present as leukoplakia or erythroplakia, but histologically these lesions have various manifestations such as hyperkeratosis, dysplasia, or even carcinoma [10].

The five-year survival rate of patients suffering from oral cancer is as low as 50%, and has not improved significantly in recent decades, despite advances in surgery, radiotherapy and chemotherapy [11-13]. The expected survival rate for patients with advanced staged oral cancer is far lower than that of laryngeal or nasopharyngeal carcinoma, even for those patients achieving complete clinical remission after local therapy [14]. Early detection of oral mucosal lesions followed by

appropriate treatment can increase the recovery rate to 80–90% [12]. Consequently, early diagnosis of malignant or premalignant lesions could reduce the mortality and morbidity associated with oral cancer.

Clinical examination and biopsy are the standard procedures to determine the nature of oral mucosa lesions. Clinical examination includes a thorough head, neck and intraoral visual examination and palpation of the oral cavity. Although the oral cavity is amenable to direct examination, oral cancer is often not detected until a late stage [14, 15]. Biopsy of suspicious lesions remains the gold standard to determine the nature of an oral lesion. The location from which the biopsy sample is taken is crucial in the histopathological verification of oral cancer but selecting this location correctly is often difficult because of the non-uniform appearance of cancerous and precancerous lesions. Biopsies are also associated with patient discomfort. Other clinically available techniques, such as vital tissue staining with toluidine blue and exfoliative cell collection for cytology or molecular analysis, have been developed to aid the early recognition of malignant lesions. These tests have variable false-positive and false-negative rates [3].

There are currently no effective methods to screen for oral cancer. The identification of tumor biomarkers is an essential first step in the development of effective early detection systems for malignant oral lesions, especially in at-risk

populations. Proteomics is a well-established strategy for the discovery of biomarkers for human diseases. In the case of oral cancer, saliva is a good candidate for analysis for the identification of biomarkers [16]. Whole saliva is composed of the secretions of the major and minor salivary glands and gingival crevicular fluid [17]. Compared with blood sampling or biopsy, the use of saliva for oral cancer screening is advantageous because it is easier, less invasive and better tolerated by patients. Significant increase of salivary soluble CD44 (solCD44) levels has been identified in head and neck squamous cell carcinoma (HNSCC) patients compared with normal controls [18]. Comprehensive analysis of salivary parameters shows that secretory immunoglobulin A, 8-oxoguanine DNA glycosylase, phosphorylated-Src and mammary serine protease inhibitor (Maspin) are lower, while insulin growth factor I, metalloproteinases MMP-9, carbonyls and Cyclin D1 (CycD1)are higher in OSCC patients [19, 20]. Recent, proteomic analysis of saliva samples from OSCC patients and matched healthy subjects using capillary reversed-phase liquid chromatography with quadruple time-of-flight (LC-Q-TOF) mass spectrometry indicated five candidate OSCC biomarkers (M2BP, MRP14, CD59, profilin, and catalase), being successfully validated using immunoassays on an independent set of OSCC patients and matched healthy subjects [21]. However, these identified candidate OSCC biomarkers have no significant relationship with tumor size, stage and recurrence.

In this study, we intended to identify salivary biomarkers of early stage oral cancer using two-dimensional gel electrophoresis (2DE) and mass spectrometry (MS). Salivary proteins which showed potential as oral cancer biomarkers were then validated by Western blotting and ELISA. We identified a statistically significant correlation ($P<0.001$) between salivary transferrin levels and the oral cancer tumor grade.

2. Materials and Methods

2.1 Human subjects

Unstimulated whole saliva was collected from patients with oral squamous cell carcinoma (OSCC) and OSCC-free patients at China Medical University Hospital (Taichung, Taiwan) during 2007–2008. Saliva collection protocols were approved by the Institutional Review Board of China Medical University Hospital (permission number DMR96-IRB-80), and informed consent was obtained from each subject. OSCC patients were diagnosed via biopsy. The inclusion criteria for OSCC patients required that the subjects be 20 years or older and immunocompetent, with a primary untreated oral cancer. The exclusion criteria for OSCC patients included previous chemotherapy or radiotherapy, previous oral surgery other than oral biopsy, and the inability to properly consent. The inclusion criteria for

OSCC-free control individuals required that the subjects be 20 years or older and immunocompetent. The exclusion criteria for OSCC-free control individuals included previous chemotherapy or irradiation, systemic conditions associated with immune dysfunction such as diabetes, the presence of any oral mucosa lesions, and pregnancy or lactation. Five categories of subjects were included in the analysis: OSCC-free control subjects and OSCC patients with the tumor size (T) stages 1 to 4 according to the universal TNM staging system of the International Union against Cancer (UICC) [22].

2.2. Collection of whole saliva

The OSCC patients and OSCC-free control individuals enrolled in this study were instructed to abstain from smoking or consuming alcohol for at least 24 hr before the collection of saliva. They were instructed to brush their teeth and rinse their mouths by gargling with clean water before they went to sleep the day before collection. The collection procedure was always performed early in the morning (between approximately 6 and 8 a.m.). Participants were prohibited from any food/water intake or teeth cleaning for one hr before salivary collection. They were instructed to gargle and rinse their mouths with normal saline and to swallow the first bolus of saliva just before starting the expectoration. Whole saliva samples were

collected by subjects spitting into a collecting cup, without mechanical or chemical stimulation. Saliva was collected for 10 min. More than 5 ml of saliva was collected from each subject during this time. After discarding the upper frothy layer, 5 ml of saliva was collected using a syringe and placed in another tube containing 5 μl Complete™ Protease Inhibitor Cocktail, Cat. No. 1697498 (Roche, Mannheim, Germany). The saliva samples were kept in an ice bucket at 0°C, then centrifuged at 12000 rpm (~13400×g) for 10 min at 4°C. The resulting supernatants were collected, then divided in 500 μl aliquots and stored at –80°C.

2.3. Proteomic analysis

Both pooled OSCC and control salivary samples (100 μg proteins in total each) were analyzed by two-dimensional gel electrophoresis. Salivary proteins were precipitated with acetone overnight. The preparation was then resolved in sample rehydration buffer and subjected to first- and secondary-dimension gel electrophoresis on a Multiphor II electrophoresis apparatus (GE Healthcare, Little Chalfont, UK) as described previously [23]. Briefly, saliva samples were prepared in lysis buffer containing 8 M urea and 4% CHAPS. The protein concentration was measured and samples were diluted with 350 μl of rehydration buffer (8 M urea, 4% CHAPS, 18 mM DTT, 0.002% bromophenol blue), and then applied to nonlinear Immobiline

DryStrips (11 cm, pH 3–10; GE Healthcare, Little Chalfont, UK). After 1-D electrophoresis on a Multiphor II system, the gel strips were incubated for 15 min in equilibration solution I (6 M urea, 10% SDS, 50% glycerol, 1% DTT, 0.002% bromophenol blue, 15 M Tris-HCl, pH 8.8), and 15 min in equilibration solution II (6 M urea, 10% SDS, 50% glycerol, 2.5% iodoacetamide, 0.002% bromophenol blue, 15 M Tris-HCl, pH 8.8). The immobilized pH gradient (IPG) gels were then transferred to the top of 10% polyacrylamide gels (13 × 13 cm) for 2-D electrophoresis. Gels were fixed (30% ethanol and 10% glacial acetic acid), and stained with silver nitrate solution, then scanned with a GS-800 imaging densitometer and analyzed with PDQuest software, version 7.1.1 (Bio-Rad, Hercules, CA, USA). Data from three independently stained gels of each sample were used for the correction of spot intensity graphs and statistical analysis with ANOVA analysis.

2.4. In-gel digestion and MALDI-TOF/TOF MS

In-gel digestion was performed according to previously published techniques with slight modifications [23]. Briefly, each spot of interest in the silver-stained gel was excised, placed in a microcentrifuge tube and washed twice with 50% acetonitrile (ACN) in 100 mM ammonium bicarbonate buffer (pH 8.0) for 10 min at room temperature. The excised-gel pieces were then soaked in 100% ACN

for 5 min, dried in a lyophilizer for 15 min, rehydrated in 50 mM ammonium bicarbonate buffer (pH 8.0) containing 20 ng/ml trypsin and incubated at 56°C for 11 hr. After digestion, the peptides were extracted from the supernatant of the gel elution solution (100% ACN in 1% trifluoroacetic acid, TFA), and sonicated for 10 min. The proteins were identified using MALDI-TOF/TOF MS (Bruker). The peptides were crystallized using the alpha-cyano-4-hydroxycinnamic acid (CHCA) matrix (0.1% TFA, 50% acetone, 1 mg/ml CHCA) and the flight time from the target to the ion detector was calculated. Proteins were identified on the MALDI-TOF/TOF-MS spectra by searching against NCBI databases for exact matches using the ProID program (Applied Biosystem/MDS Sciex) and the MASCOT search program (http://www.matrixscience.com). The derived peak list generated by Mascot.dll v1.6b27 (Applied Biosystems) was searched using a local version of the Mascot (2) search program (v2.2.1; Matrix Science Ltd.) and the Mascot Daemon application (v2.2.0). A human (20,403 sequences) database (Swiss-Prot, release version 57.6, 28-Jul-2009, 495,880 sequences) was used for database searches, and a decoy protein was generated for each of these proteins by sequence reversal to enable estimation of false discovery rates (FDRs). The search parameters are as following: peptide and MS/MS tolerance, ±0.3 Da; trypsin missed cleavages, 1; and variable modifications, carbamidomethylation and Met oxidation;

and instrument type, MALDI-TOF-TOF. The interpretation and presentation of MS/MS data were performed according to published guidelines [24]. The MASCOT scores greater than 55 were significant for PMF search (P<0.05). In addition, individual MS/MS spectra for peptides with a Mascot ions score lower than 40 (expect value < 0.015) were inspected manually and included in the statistics only if a series of at least four continuous y or b ions were observed. Protein identification was also based on the assignment of at least two peptides. In all cases, keratins were excluded. Protein expectation value below 0.02 were initially considered true hits with a FDR of <5.0% (estimated by the numbers of reversed matches dividing by the total number of matches).

2.5. Western blotting and ELISA of transferrin in saliva

Salivary proteins were prepared and incubated in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer (0.5 mM Tris-HCl [pH 6.8], 10% SDS, 10% glycerol, 0.5% brilliant blue R) at 95°C for 10 min. The samples were then resolved by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk in Tris buffered saline (TBS) buffer containing 0.1% Tween 20 at

4°C for 2 hr and then incubated overnight with anti-human transferrin antibody (Abcam, Cambridge, UK) at 4°C. The membranes were washed with TBS containing 0.1% Tween 20 and then incubated with HRP-conjugated anti-mouse IgG antibodies (Invitrogen, Carlsbad, CA, USA). The proteins of interest were visualized with the ECL™ Western Blotting Detection Reagents (GE Healthcare) and autoradiography (X-ray films from Kodak, Rochester, NY, USA). In order to validate the transferrin levels in saliva, the wells of a 96-well microtiter plate were coated with 100 µl of 5 µg/ml salivary proteins and incubated at 4°C overnight. Following this incubation (and each subsequent incubation step) the wells were washed three times with TBS containing 0.05% Tween 20 (TBST). The microtiter plate was blocked with 5% skim milk in TBST, and then 2000x dilution of anti-human transferrin antibody was incubated in the salivary protein-coated wells for 2 hr. After incubation with 2000x dilution of goat anti-mouse IgG-HRP conjugate for 2 hr, ABTS/H_2O_2 substrates were added and the optical absorbance of the samples was recorded using an ELISA plate reader (EL X808, BioTek, Winooski, USA).

2.6. Statistical analysis

The relationship between transferrin levels in saliva in OSCC patients compared with OSCC-free control individuals was analyzed by the Chi-squared test

with Yates's correction or by ANOVA analysis using SPSS software (version 10.1, SPSS Inc., Chicago, IL, USA). Differences in mean values between the two groups were compared using the Student's *t*-test. A *P* value of less than 0.05 was considered statistically significant.

3. Results

3.1. Discovery of salivary transferrin as an oral cancer biomarker

A total of 41 OSCC patients were recruited in this study, 39 (95%) males and 2 (5%) females. The age range of OSCC patients was 29 to 79 with an average age of 51.2 years (Table 1). Of 30 OSCC-free control subjects, 23 (76.6%) were male and 7 (23.4%) were female. The age range of OSCC-free control subjects was 29 to 66 with an average age of 44.9 years. Although no significant differences were detected with respect to age or gender distribution, males were more common in the OSCC group.

Details of the patients and their individual histopathological diagnoses are summarized in Table 1. Among these 41 patients, 21 (51.2%) had buccal carcinomas, 19 (46.3%) had tongue carcinomas and one patient had mouth floor cancer. Moderately differentiated keratinizing squamous cell carcinoma was the most common histopathological appearance (28.8%). The 41 patients were classified into

four categories: T1 (41.5%), T2 (36.6%), T3 (9.8%) and T4 (12.1%) according to UICC TNM staging [22].

To identify the potential salivary biomarkers for oral cancer detection, comparison of the salivary protein profiles between OSCC-free control subjects and OSCC patients was performed using 2DE analysis (Fig. 1). The intensity of each protein spot was quantified and analyzed using a GS-800 imaging densitometer and PDQuest software. Silver-stained gels revealed 15 protein spots with at least a 2-fold increase in spot intensity and 31 protein spots with at least a 2-fold decrease in spot intensity in the salivary protein profile of OSCC patients compared with OSCC-free control subjects. These increased and decreased protein spots were selected for in-gel tryptic digestion, and peptide mass fingerprinting by MALDI-TOF MS. Only eleven proteins such as transferrin (Spot ID 1) and transferrin chain A (Spot ID 7) were identified as matching with Mascot score at greater than 55, and MW and pI of indicated proteins in 2DE gel (Table 2). The amino acid sequence coverage of identified up-regulatory and down-regulatory proteins varied from 20% to 75%. For example, MALDI-TOF MS analysis of transferrin (Spot ID 1) showed a Mascot score of 84, sequence coverage of 56%, and 7 matched peptides (Figure 2A), while S100 calcium-binding protein A8 (Spot ID 9) showed a Mascot score of 94, sequence coverage of 51%, and 23 matched peptides. The representative peptide peaks of

transferrin (Spot ID 1) from MALDI-TOF MS analysis were further analyzed using MALDI-TOF-TOF sequencing (Fig. 2B), resulting in a high degree of confidence in the protein identification. To confirm the protein levels of transferrin in saliva, Western blot analysis was performed on saliva from the two groups: OSCC-free control subjects and OSCC patients (Fig. 3). 79 kDa- and 36 kDa-immunoreactive bands for transferrin and transferrin chain A were detected in the saliva of OSCC patients, but not in the saliva of control subjects. The result of Western blotting was consistent with proteomic analyses of silver-stained 2-DE gels as shown in Fig. 1, suggesting that transferrin is a potential salivary biomarker for the diagnosis of oral cancer.

3.2. Correlation between the levels of salivary transferrin and the size of oral tumors

To further investigate the potential of salivary transferrin as a biomarker of oral cancer, ELISA analysis of salivary transferrin protein levels was performed on samples from OSCC patients with stage T1 (n=17), stage T2 (n=15), stage T3 (n=4) or stage T4 (n=5) pathologies and from OSCC-free individuals (n=30). The protein level of salivary transferrin was significantly higher in the saliva of patients suffering from oral cancer than in unaffected individuals (Fig. 4). We then assessed the

relationship between the protein level of salivary transferrin and tumor size using ANOVA analysis. Our data showed that a linear increase in salivary transferrin levels strongly correlated with increasing tumor size (P <0.001) (Fig. 4). These data indicate that salivary transferrin can be used as a biomarker for diagnosis of patients with oral cancer and can also be used to monitor tumor growth.

In addition, receiver-operating characteristics curves (ROC) were used to predict the sensitivity and specificity of transferrin-based ELISA for the detection of each stage of oral cancer (Fig. 5). The threshold optical density value was set at 0.3 and the sensitivity of transferrin-based ELISA for predicting oral cancer was 100% for patients with oral cancer stage T1, 86.6% for stage T2 and 100% for stage T3/T4. The area under the receiver-operating characteristics curves (AUROC) was used to discriminate between OSCC-free subjects and OSCC patients displaying each stage of oral cancer using transferrin-based ELISA (Fig. 5). AUROC for predicting OSCC in patients was 0.95 for T1 group (95% CI: 0.48 - 1.05), 0.94 for T2 group (95% CI: 0.51 – 0.88), and 0.91 for T3/T4 group (95% CI: 0.59 - 1.18), respectively. ELISA assay indicated that transferrin-based ELISA was highly accurate at detecting oral cancer at the T1 stage.

3.3. Correlation between plasma transferrin and oral cancer stages

To further test the correlation between plasma transferrin concentration and different oral cancer stages, plasma samples from OSCC patients at different stages (n=41) and OSCC-free individuals (n=30) were examined by ELISA analysis. The mean plasma transferrin concentration was 256.6 mg/dl in the OSCC-free group, 216.3 mg/dl in the T1 group, 235.0 mg/dl in the T2 group, and 203.6 mg/dl in the T3/T4 group (Fig. 6). The protein level of plasma transferrin was lower by 16% in the T1 group, 8% in the T2 group and 21% in the T3/T4 group compared with those in OSCC-free individuals. However, no significant difference of plasma transferrin was found among T1, T2, T3/T4 and OSCC-free groups using two-way ANOVA analysis (P = 0.3) (Fig. 6). The results indicated that the plasma transferrin levels varied in OSCC patients and OSCC-free controls, showing no correlation with the increase of OSCC tumor size.

4. Discussion

In this study, we compared the proteomic profile of saliva from OSCC patients with an OSCC-free control group. The results indicated that transferrin levels are elevated in the saliva of OSCC patients (Figs. 1–3, Table 2). A significant

increase in the protein level of salivary transferrin correlated with increasing tumor size as represented by the UICC TNM staging system (P<0.001) (Fig. 4). In addition, AUROC of a saliva-based ELISA for the diagnosis of early-stage oral cancer, revealed that salivary transferrin serves as an early-stage biomarker for oral cancer (Fig. 5). Recently, parotid acinar cells were reported to synthesize and secrete transferrin into saliva, providing evidence that the detection of transferrin in saliva does not indicate contamination of the sample with blood [25]. In addition, transferrin was found to be significantly increased in the saliva of head and neck squamous cell carcinoma (HNSCC) patients compared with unaffected controls using 2D-DIGE MS proteomic analysis [26]. Therefore, salivary transferrin is a potential candidate as an early detection biomarker and a prognostic marker for oral cancer, allowing for the development of diagnostic assays.

Transferrin is essential for the growth of rapidly growing cells. It is involved in iron-requiring metabolic processes such as DNA synthesis, electron transport, mitogenic signaling pathways, proliferation and cell survival [27]. Over-expression of the transferrin receptor (CD71), a type II transmembrane homodimer glycoprotein, has been reported in several cancers including lung [26, 28, 29], glioma [30], colon [30, 31], pancreas [32] and breast [33]. Recently, human transferrin was demonstrated to enhance transfection efficiency of cationic

liposome/DNA complexes into oral squamous carcinoma cells [34, 35]. Taken together, these results and the findings from this study support salivary transferrin as a potential diagnostic marker for oral cancer.

Transferrin levels were measured in saliva from 41 OSCC patients displaying various stages of oral cancer and 30 OSCC-free control individuals. ELISA assay showed that the mean level of salivary transferrin in OSCC patients was higher by 91% in T1 group, 88% in T2 group and 84% in T3/T4 group compared with those in control group (Fig. 4). By contrast, the level of plasma transferrin decreased in OSCC patients, but did not significantly associate with OSCC onset or tumor stage (Fig. 6), implying that increase of salivary transferrin levels did not result from the leakage of plasma trasferrin in OSCC patients. Moreover, the specificity and sensitivity of salivary transferrin-based ELISA was 100% and 100% in T1 group, and 100% and 95% in overall OSCC patients, respectively. By contrast, the sensitivity of salivary solCD44 levels for detection of HNSCC patients ranged from 62% to 70%, and its specificity ranged from 75% to 88% [18]. The sensitivity and specificity values of CycD1 and Maspin as candidate OSCC markers were 100% [20]. In addition, the combination of M2BP, MRP14, CD59, profilin, and catalase as candidate biomarkers yielded a receiver operating characteristic value of 93%, sensitivity of 90%, and specificity of 83% in detecting

OSCC [21]. Comparison of salivary transferrin with these reported candidate markers indicated that the receiver operating characteristic value, specificity and sensitivity salivary transferrin were similar to those of reported candidate OSCC markers. Of candidate markers, only salivary transferrin showed the ability for the detection of early-stage oral cancer. In the OSCC-free control group, four samples exhibiting elevated levels of salivary transferrin were from individuals suffering from right parotid mixed tumor, alcoholic liver cirrhosis, nasal polyposis or bronchial asthma. Increased levels of serum transferrin have previously been reported in patients with alcoholic liver disease and asthma [36, 37]. Therefore, the combination of salivary transferrin and other OSCC markers could rule out the false positive and confirm the early diagnosis of OSCC.

Comparison of silver-stained 2DE gels indicated increased levels of zinc finger protein 497, Ig heavy chain variable region and S100 calcium-binding protein A8, and the decreased levels of Kappa B-ras 1, annexin A2, Ig alpha-2 chain, and IgA1-Fc chain A in saliva of OSCC patients (Fig. 1 and Table 2). S100 calcium-binding protein A8 has been reported to up-regulate in prostatic intraepithelial neoplasia and preferentially in high-grade adenocarcinomas, serving as a novel diagnostic marker in human prostate cancer [38]. In addition, S100 calcium-binding proteins S100A12 and S100P were identified as up-regulated

proteins in saliva of OSCC patients using LC-Q-TOF [21]. Therefore, the role of S100 proteins in OSCC diagnosis needs for further investigation.

In conclusion, we performed proteomic analysis of saliva from OSCC patients and OSCC-free control subjects, and identified transferrin as a potential salivary biomarker for the diagnosis of early stage oral cancer in humans. Our findings were confirmed by MALDI-TOF/TOF mass spectra and Western blotting. In addition, salivary transferrin levels strongly correlated with tumor size. ROC demonstrated the high specificity and sensitivity of a transferrin-based ELISA for predicting oral cancer. The AUROC indicated that the transferrin-based ELISA has high accuracy at detecting early stage oral cancer. Future studies will examine the potential application of salivary transferrin as a screening tool for early stage oral cancer.

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Figure and table captions

Fig. 1. Two-dimensional gel electrophoresis of total protein extracts from the pooled saliva from individuals in the OSCC-free control group (A) and from patients in the OSCC group (B). 100 μg of protein sample were diluted with 350 μl of rehydration buffer, and then applied to the nonlinear Immobiline DryStrip. After incubation in the equilibration solutions, the IPG gels were transferred to the top of 10% polyacrylamide gels. 2-D gels were stained with silver nitrate solution. Protein size markers (in kDa) are shown on the left of each gel. The protein spot ID numbers are consistent with those in Table 1.

Fig. 2. MALDI-TOF mass spectrum (A) and MALDI-TOF-TOF mass spectrum (B) for identification of transferrin protein (Spot ID 1). The MALDI-TOF/TOF mass spectrum of the amino acid sequence SVEEYANCHLAR was determined from mass differences in the y and b-fragment ion series and matched residues of transferrin.

Fig. 3. Western blot analysis of salivary transferrin in pooled saliva from OSCC patients and OSCC-free control group. Each pooled salivary sample

was analyzed by 10% SDS-PAGE, and then electrophoretically transferred onto nitrocellulose membrane. The membrane was probed with monoclonal antibodies to human transferrin, and developed with HRP-conjugated secondary antibody and chemiluminescent HRP substrates. Lane 1: OSCC-free control group; lane 2: OSCC group.

Fig. 4. Comparison of salivary transferrin protein levels with tumor size stages in OSCC patients. Each salivary protein sample (5 μg) was coated onto the wells of a 96-well plate, incubated with monoclonal antibodies to human transferrin, and developed with HRP-conjugated secondary antibody and ELISA HRP substrates.

Fig. 5. Receiver-operating characteristic curves of salivary transferrin based on immunoassays for the prediction of oral cancer of different stages: T1 (A), T2 (B), T3 + T4(C).

Fig. 6. Protein levels of plasma transferrin in OSCC patients and OSCC-free control group. Plasma transferrin protein concentrations were determined by

human Transferrin ELISA kit.

Table 1. The characteristics and clinicopathological features of oral cancer patients included in the study.

Table 2. Mass spectrometric identification of proteins found to be increased or decreased in concentration in the saliva of oral cancer patients.

Table 1.

Case	Gender	Age	Clinical diagnosis (type of cancer)	Histology	TNM stage
1	M	63	Buccal	well-diff scc	T1N0M0
2	M	38	Buccal	mod-diff k scc	T1N0M0
3	M	54	Tongue	k scc	T1N0M0
4	M	47	Tongue	k scc	T1N0M0
5	F	47	Mouth floor	k scc	T1N0M0
6	M	52	Buccal	well-diff k scc	T1N0M0
7	M	40	Buccal	residual k scc	T1N0M0
8	M	44	Tongue	k scc	T1N0M0
9	M	47	Buccal	k scc	T1N0M0
10	M	41	Tongue	mod-diff scc	T1N0M0
11	M	62	Buccal	well-diff scc	T1N0M0
12	M	43	Buccal	mod-diff k scc	T1N0M0
13	M	68	Tongue	well-diff k scc	T1N0M0
14	M	29	Tongue	mod-diff k scc	T1N0M0
15	M	65	Tongue	k scc	T1N0M0
16	M	41	Buccal	mod-diff k scc	T1N1M0
17	M	80	Buccal	mod to poor-diff scc	T1N2bM0
18	M	35	Tongue	mod-diff k scc	T2N0M0
19	M	50	Tongue	mod-diff scc	T2N0M0
20	M	41	Tongue	mod-diff k ssc	T2N0M0
21	M	52	Tongue	well to mod-diff k scc	T2N0M0
22	M	70	Buccal	well-diff k scc	T2N0M0
23	M	54	Buccal	mod-diff k scc	T2N1M0
24	M	46	Tongue	mod-diff scc	T2N1M0
25	M	45	Buccal	k scc	T2N1M0
26	M	41	Buccal	mod-diff k scc	T2N2bM0
27	M	37	Tongue	mod-diff k scc	T2N2bM0
28	M	74	Tongue	mod-diff k scc	T2N2bM0
29	M	48	Buccal	mod-diff k scc	T2N2bM0
30	M	61	Buccal	mod to poorly-diff k scc	T2N2M0
31	M	62	Buccal	well-diff k scc	T2N2M0
32	M	64	Tongue	mod-diff scc	T2N1M0
33	M	65	Tongue	k scc	T3N0M0
34	M	58	Buccal	poorly-diff scc	T3N0M0
35	F	57	Tongue	mod-diff k scc	T3N1M0
36	M	56	Tongue	well-diff k scc	T3N1M0
37	M	47	Buccal	well-diff k scc	T4aN0M0
38	M	56	Buccal	well-diff k scc	T4aN0M0
39	M	32	Buccal	well-diff k scc	T4N0M0
40	M	40	Buccal	mod-diff scc	T4N2bM0
41	M	47	Tongue	poorly-diff k scc	T4N2cM1

Mod, moderately; diff, differentiated; k, keratinizing; scc, squamous cell carcinoma.

Table 2.

Spot number	Protein identification	Accession number	Theoretical pI	Theoretical MW (Da)	Mascot score	Matched peptides	Peptide coverage (%)	Fold change
1	Transferrin	gi|248648	8.43	76780	84	7	56%	↑270.23
2	Zinc finger protein 497	gi|38348236	9.27	54780	60	13	37%	↑ 2.02
3	Kappa B-ras 1	gi|9966809	6.00	21120	60	5	42%	↓ >100
4	Annexin A2	gi|73909156	8.41	37290	59	9	26%	↓ >100
5	Ig alpha-2 chain	gi|2135473	5.85	37400	59	8	23%	↓ >100
6	IgA1-Fc chain A	gi|31615935	7.12	23540	73	8	46%	↓ >100
7	Transferrin chain A	gi|7245524	6.49	36190	122	15	43%	↑ 3.19
8	Ig heavy chain variable region	gi|145911751	5.58	10780	67	4	75%	↑ 2.05
9	S100 calcium-binding protein A8	gi|56205193	6.63	10230	94	23	51%	↑ 5.24
10	Chain A, Crig Bound To C3c	gi|119390092	6.82	70620	226	20	45%	↑2.1
11	hypothetical protein	gi|34364649	6.15	55660	63	7	20%	↓>100

Figure

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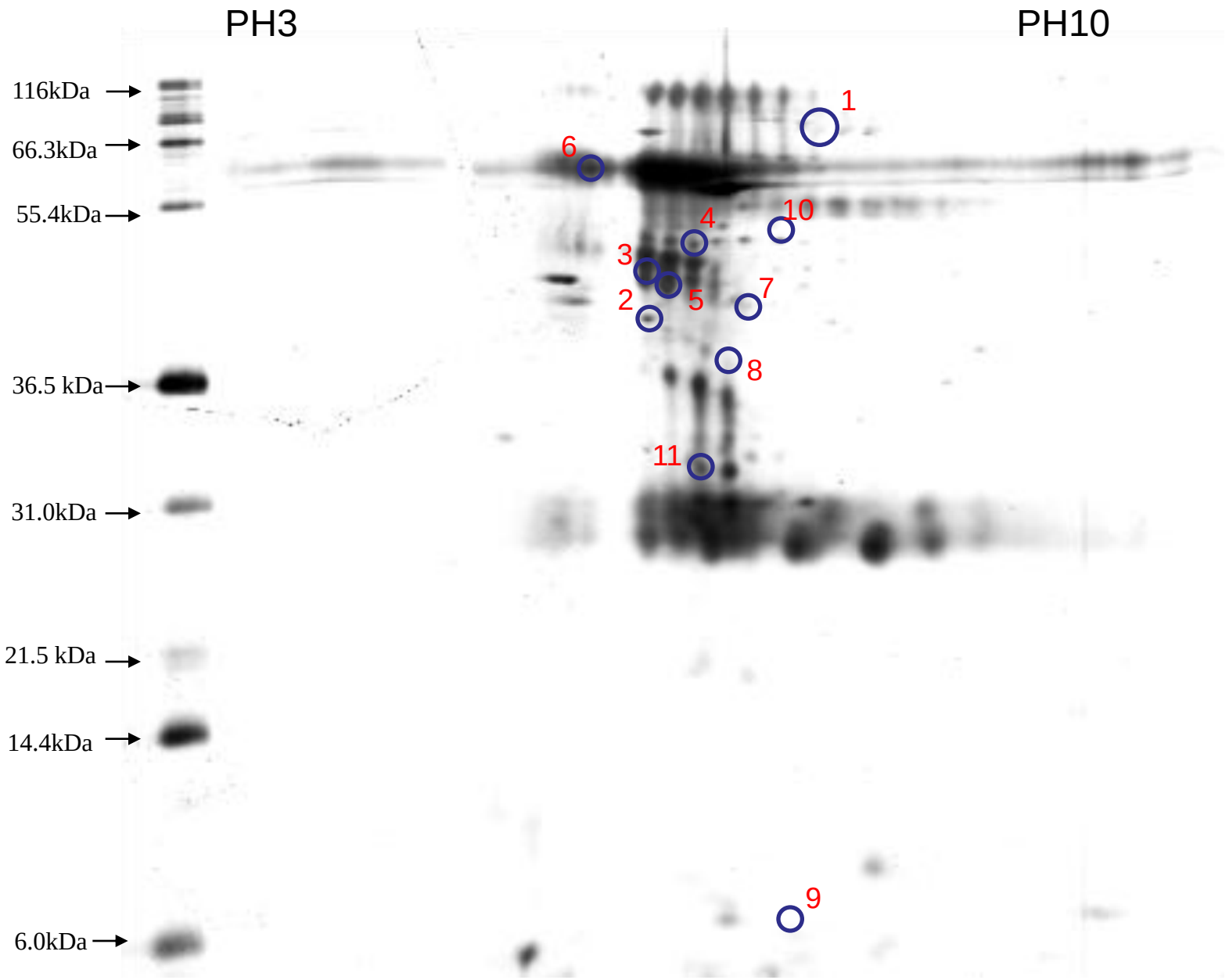

Figure
Fig. 1B
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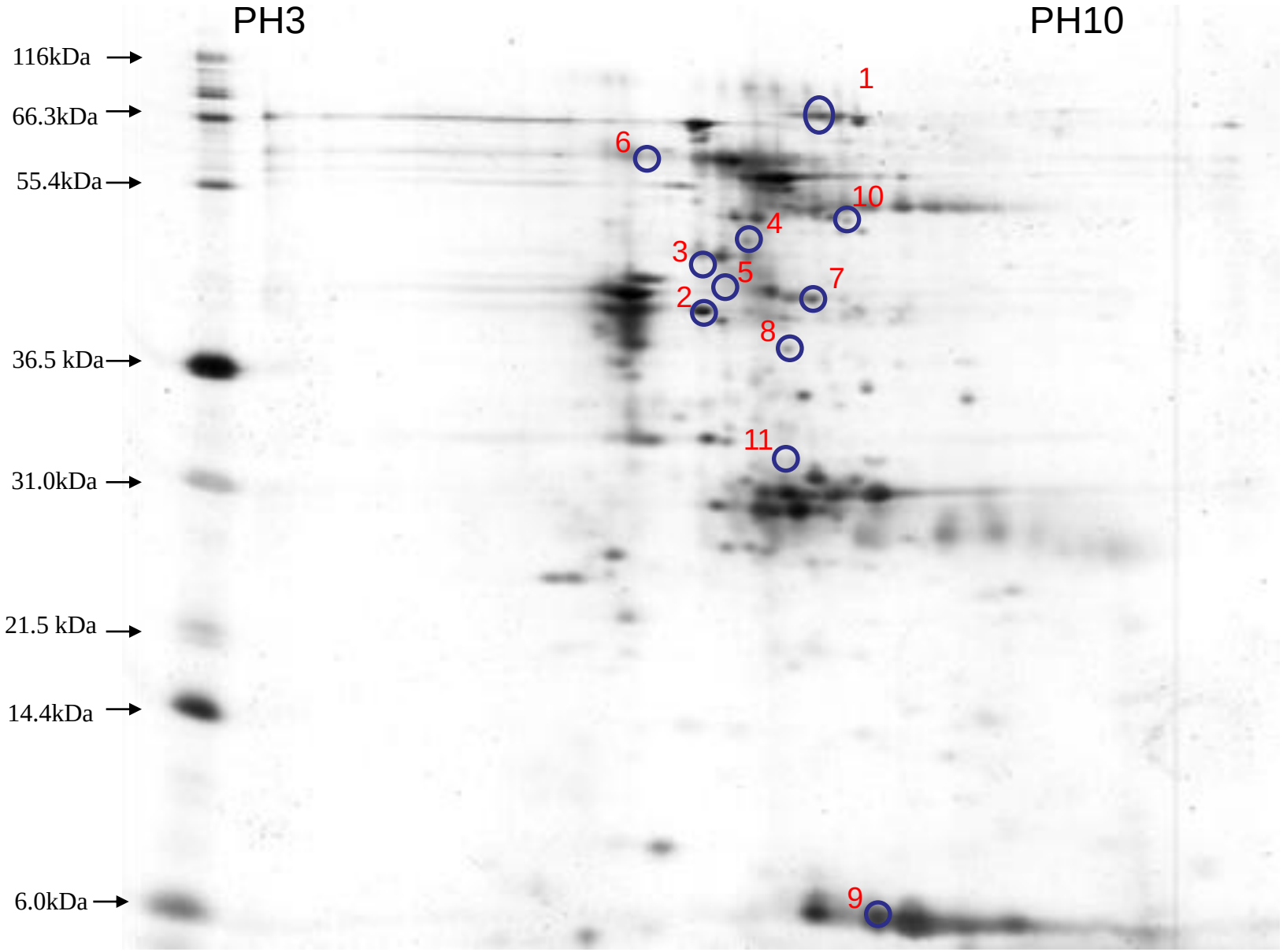

Fig. 2A

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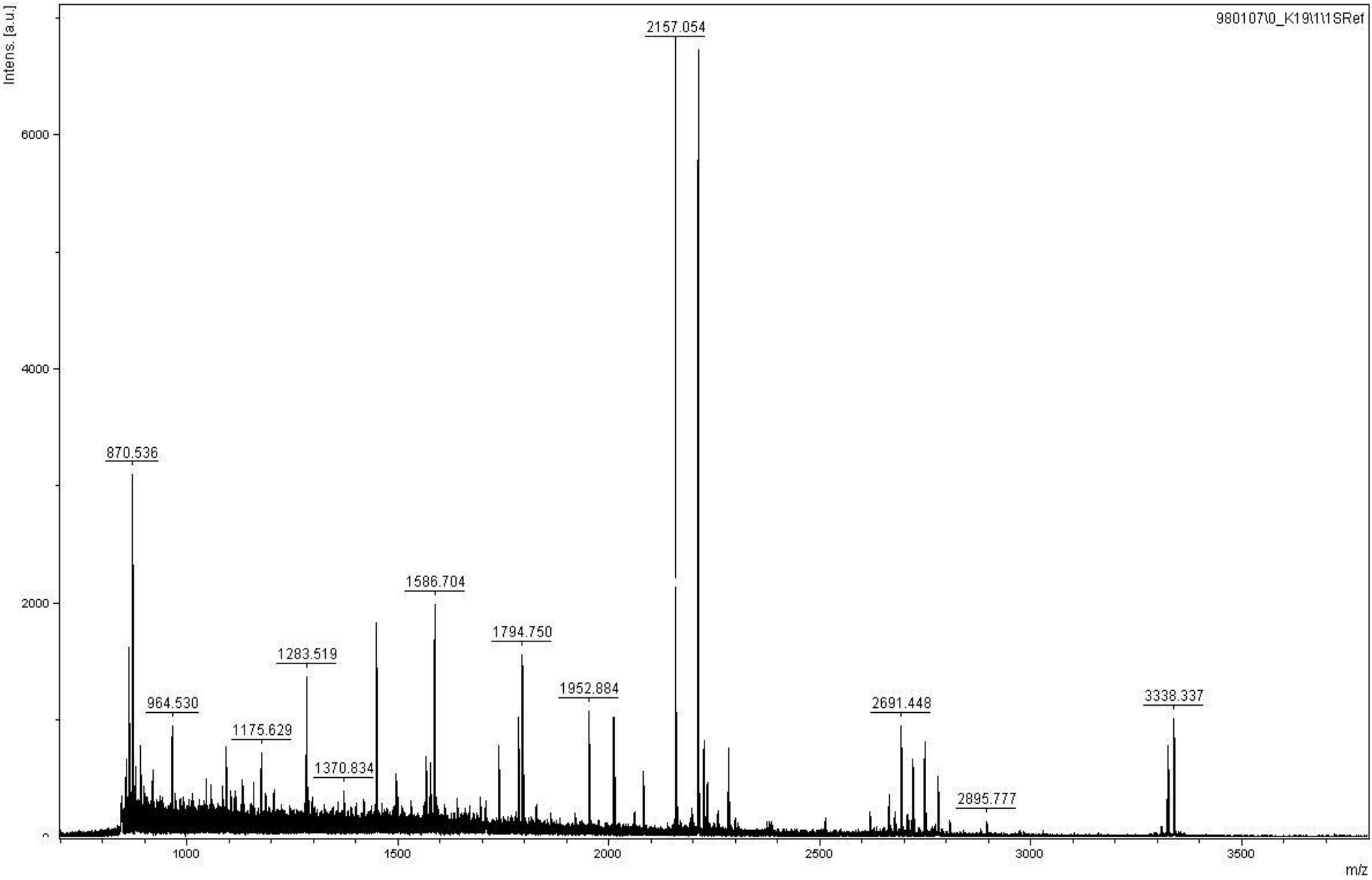

Fig. 2

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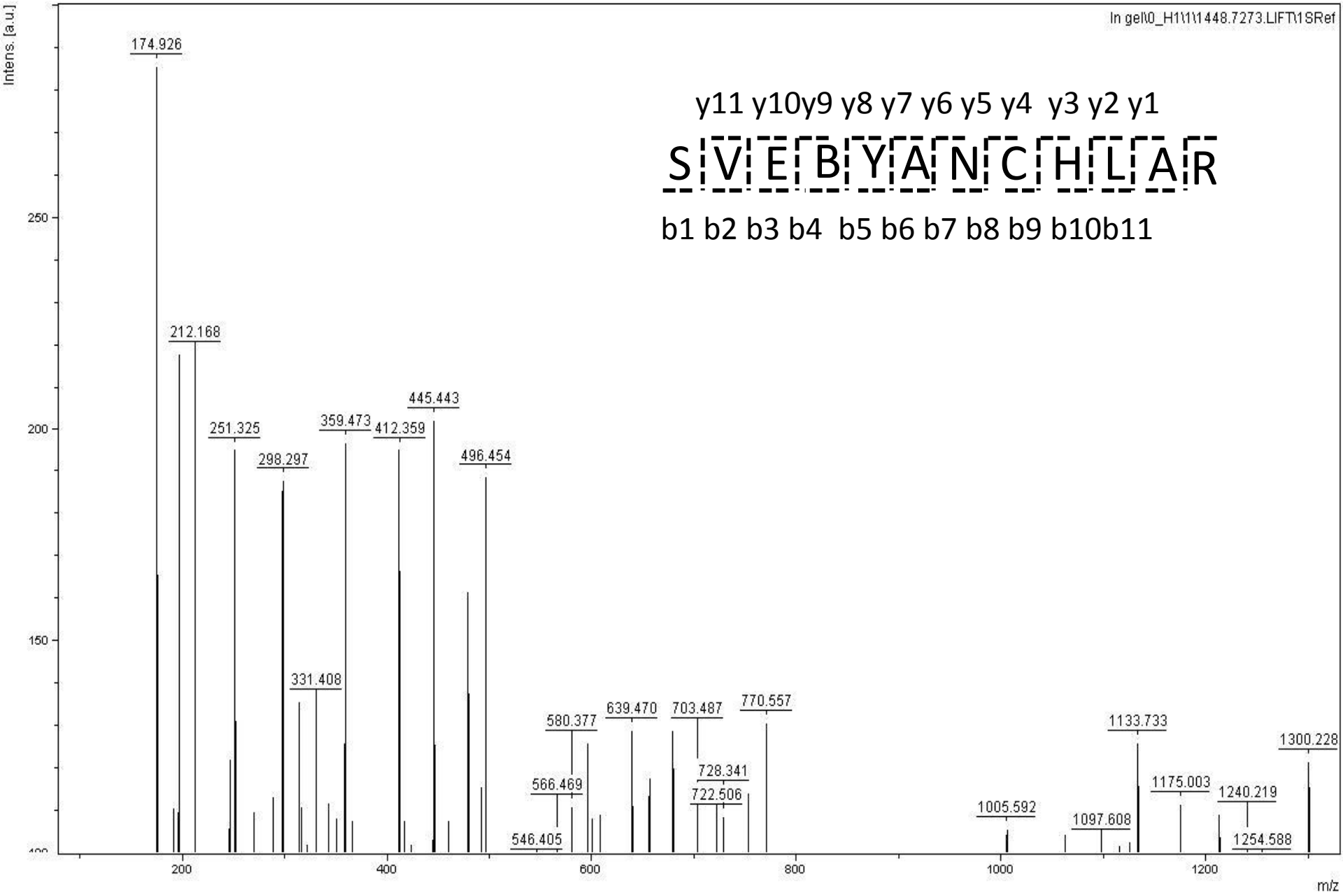

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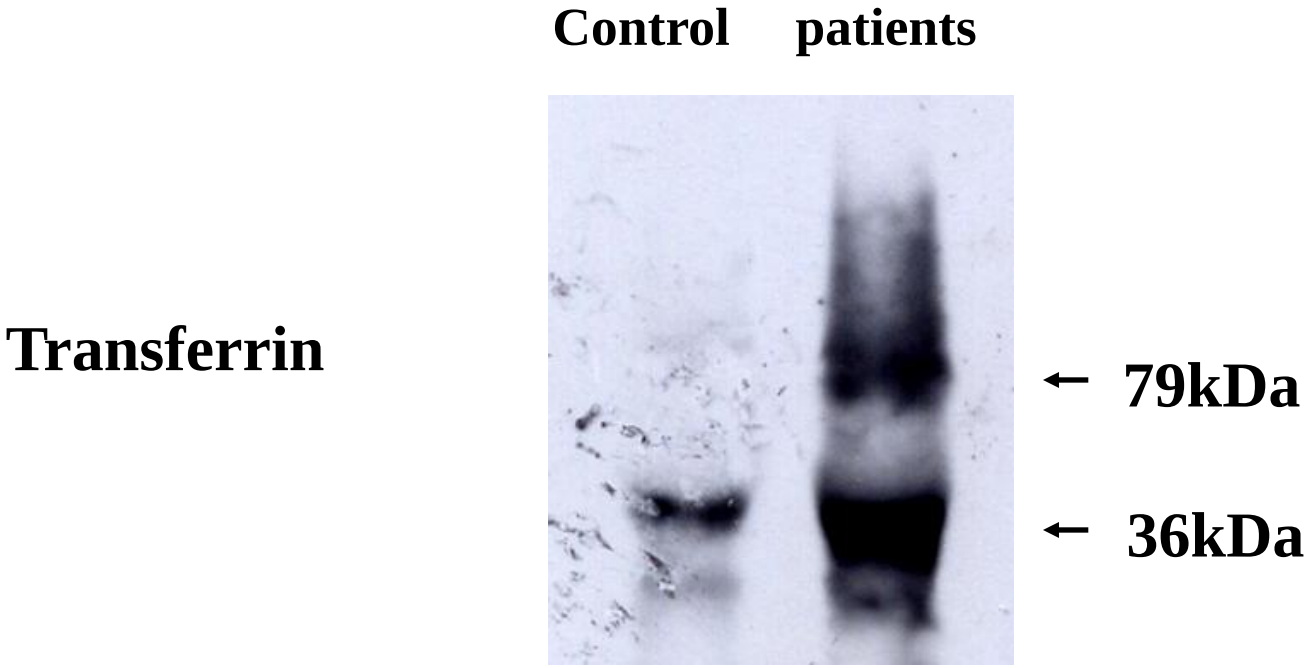

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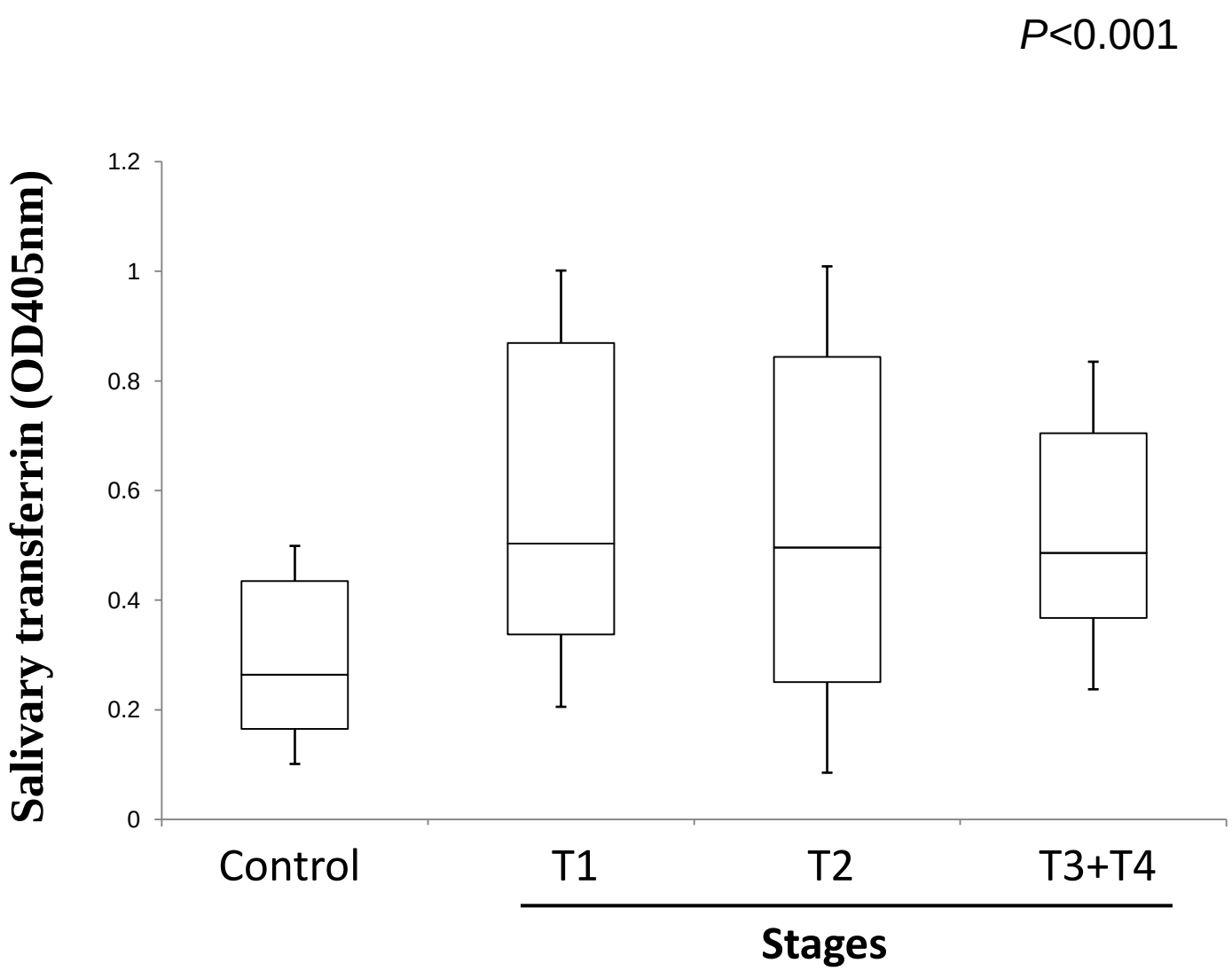

Figure
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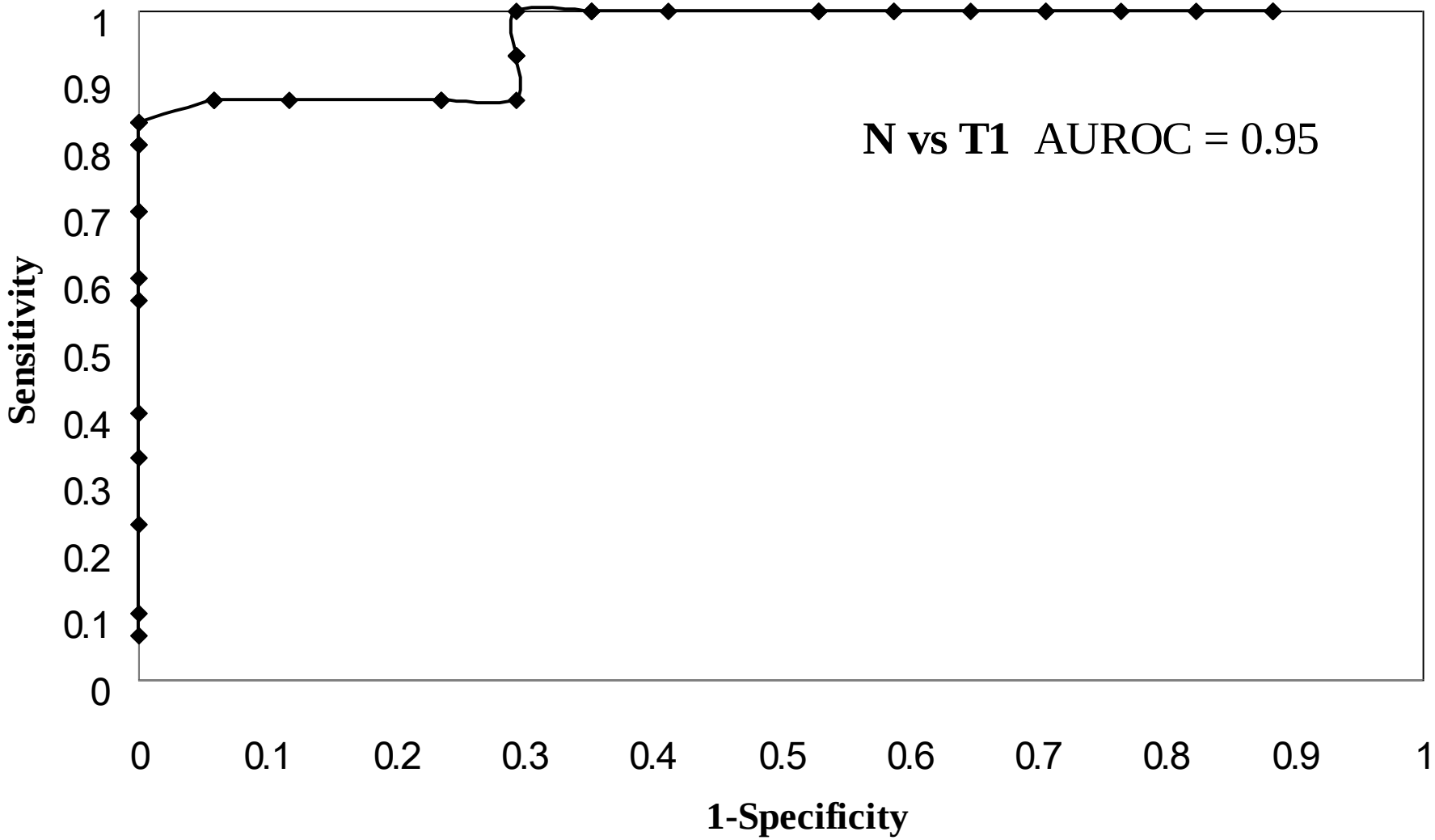

Figure
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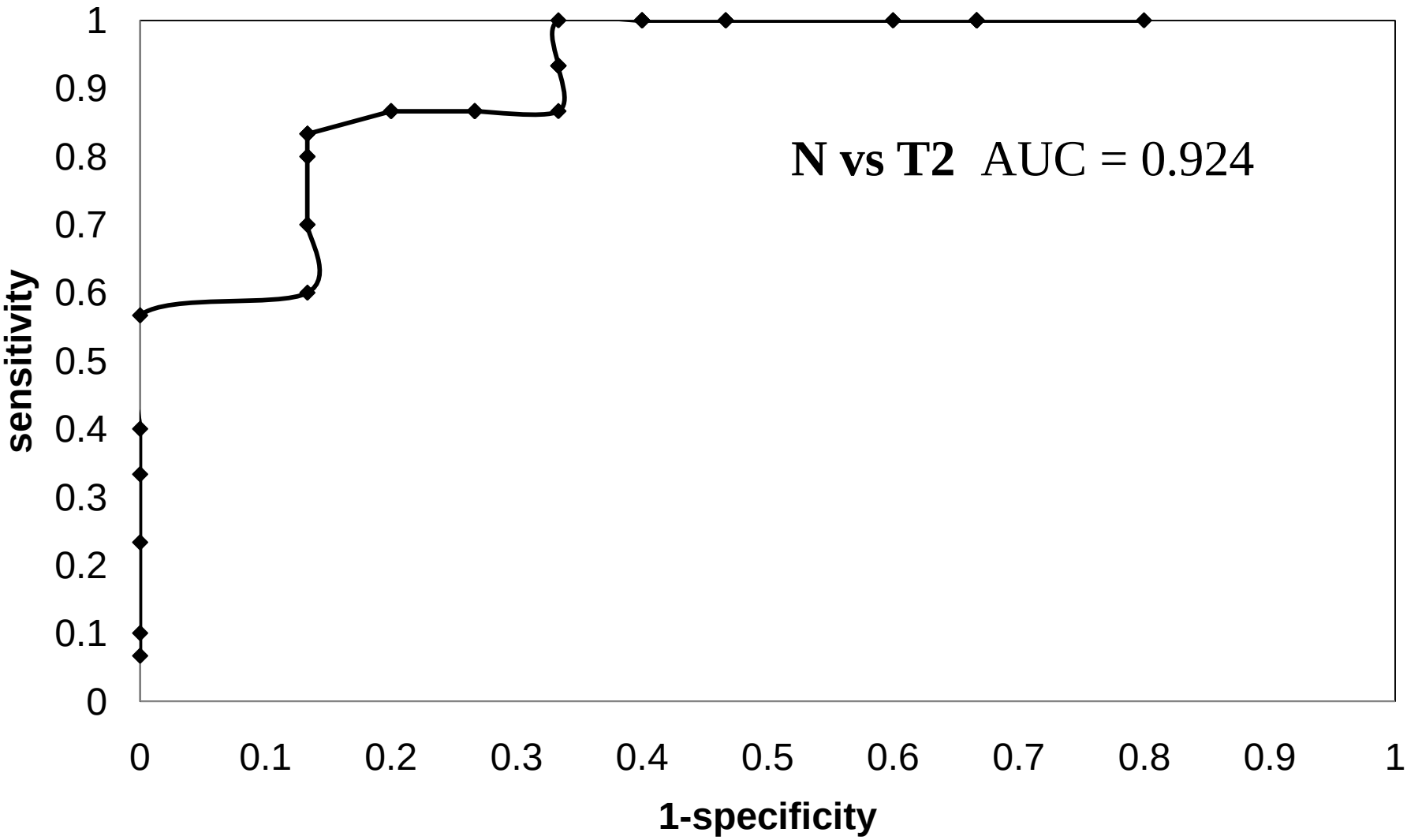

Figure
Fig. 5C
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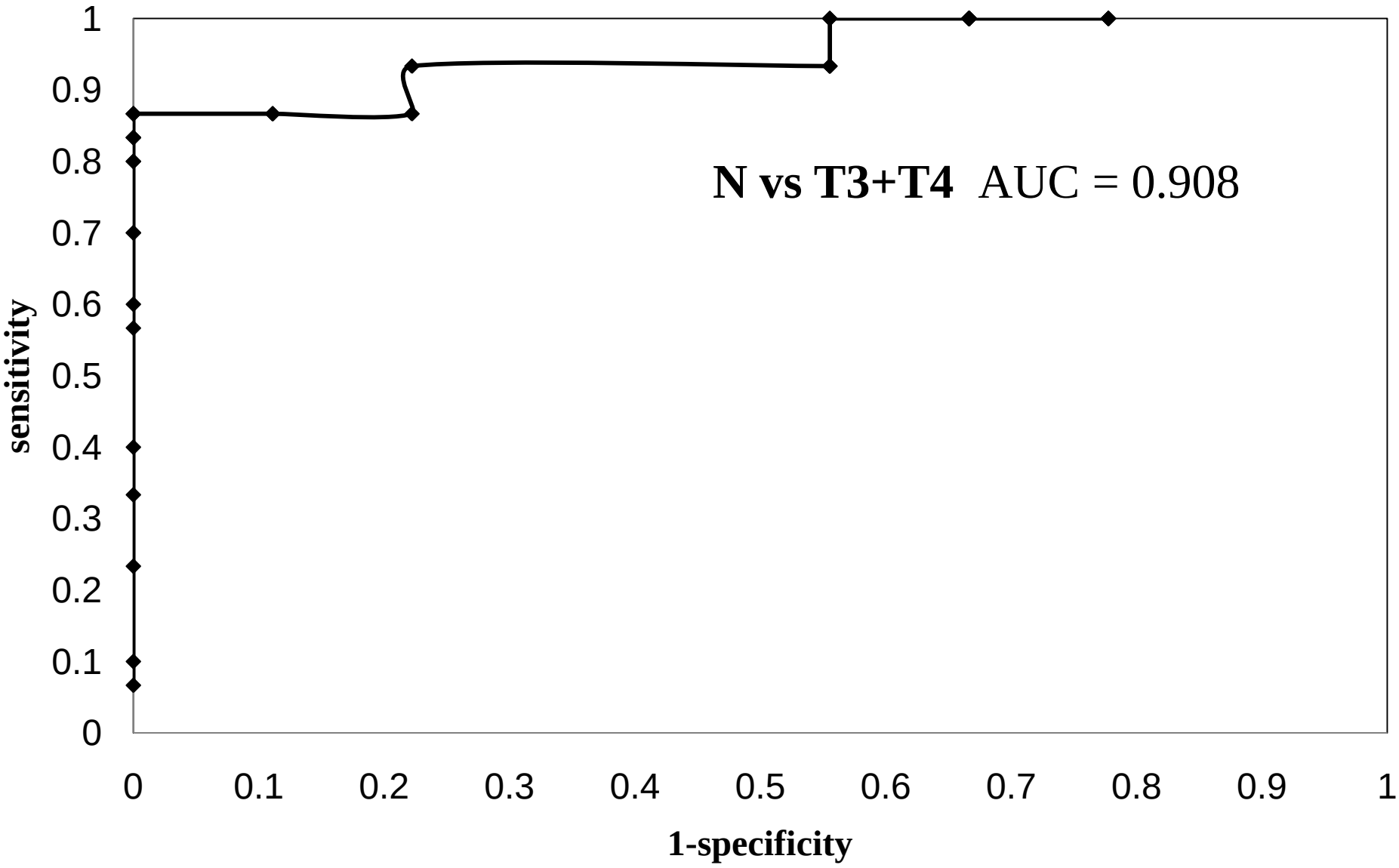

Figure
Fig. 6
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