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## ORIGINAL ARTICLE

# Common Salivary Protein 1 in Saliva of Diabetes Patients (II)

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**SUMMARY**

***Background:*** **Human common salivary protein 1 (CSP1) is one of a variety of molecules in saliva but its function remains to be determined. The gold standard method for diagnosis of diabetes mellitus (DM) is to check levels of glucose or $HbA_{1C}$ in plasma or serum. The purpose of this study was to examine whether Salivary CSP1 concentration would be useful alternative for DM diagnosis.**

***Methods:*** **The qualities of monoclonal antibodies (mAbs) to recombinant human CSP1 (rhCSP1) were tested by western blotting (WB) and immunohistochemistry. A sandwich ELISA was fabricated with the qualified capture and detector mAbs for measurement of CSP1 level in saliva. CSP1 levels of healthy adults and DM patients were measured by the sandwich ELISA and their results were statistically analyzed by Student's *t*-test. The receiver operating characteristic (ROC) curve was constructed and the area under the curve (AUC) was calculated.**

***Results:*** **The tested mAbs recognized a 27-kDa CSP1 of saliva in WB and stained only a salivary gland in immunohistochemistry. Pearson's correlation coefficient with standard curve between $OD_{450nm}$ value vs. CSP level showed good linearity ($r^2 = 0.995$). The median values (25th to 75th percentiles) of saliva CSP1 in 10 healthy adults and 18 DM patients using the sandwich ELISA were 3.92 µg/mL (3.15 - 4.02) and 4.35 µg/mL (3.94 - 5.11), respectively. Statistically, there was a significant difference of CSP1 level in two groups ($p = 0.026$). The sensitivity value of CSP1 was 64.71 while the specificity value was 88.89 with 0.784 of AUC ($p = 0.003$). These results suggested that the fabricated sandwich ELISA was a good diagnostic test tool for discriminating DM patients from healthy individuals.**

***Conclusions:*** **The present data showed a significant increase of CSP1 levels for DM patients compared with control group, indicating that CSP1 level in saliva could be used as a potential biomarker of detection or screening of DM patients. However, further studies are necessary to provide scientific and clinical validation.**

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## INTRODUCTION

Biomedical studies have demonstrated that the concentrations of biomarker proteins in plasma or serum show good correlations with disease progression and prognosis, and provide valuable tools for monitoring disease states [1-3]. Accordingly, most of diagnostic tests are designed to detect biomolecules' levels in blood plasma or serum [4,5].

Also, human saliva contains a number of distinct salivary proteins, roughly between 100 and 140, including α-amylase, lysozyme, mucins, proline-rich proteins, immunoglobulins, and many others [6,7]. The diagnostic tools targeting salivary molecules have received a great deal of attention due to their simple, inexpensive, and non-invasive procedures. Some recent studies have reported that the salivary proteins can be utilized as biomarkers for early detection of various diseases. For examples, saliva levels of β-amyloid and the enzymatic activity of acetylcholinesterase showed a significant increase for patients with Alzheimer's disease compared with a healthy group [8-10]. Awasthi reported a considerable increase of salivary CYFRA21-1 and lactate dehydrogenase in an oral cancer group compared with controls [11]. Peisker et al. demonstrated a 19.2% significant increase of salivary MMP-9 in patients with oral squamous cell carcinoma compared to controls and suggested that elevation of salivary levels of MMP-9 may be a useful diagnostic tool of oral squamous cell carcinoma [12].

Among the salivary proteins, human common salivary protein 1 (CSP1) was identified as an ortholog of the Demilune cell and parotid protein (Dcpp) of mouse and of CSP1 of rat [13,14]. So far, only a limited number of articles have reported functional aspects of CSP1. An article about pancreatic adenocarcinoma up-regulating factor demonstrated its involvement in the progression of pancreatic cancer [15]. Interestingly enough, its gene turned out to be the same as that of CSP1. Another article reported that CSP1 may play a role in promoting the binding of *Streptococcus mutans* to salivary pellicle, thus influencing the initial colonization of bacterium [16]. Recently, Heo et al. showed that salivary CSP1 levels of periodontal patients were higher than that of a normal group and suggested that CSP1 may be a potential biomarker for screening of periodontitis patients [17].

While trying to find novel biomarkers regarding various diseases from human plasma or serum, we happened to detect and reported that CSP1 level in serum of diabetes patients was higher than diabetes patients [18]. An in-house fabricated sandwich ELISA was used to measure CSP1 level in serum. As a successive experiment to the previous study, the present study pursued development of non-invasive method with saliva and comparison of CSP1 levels between diabetes patients and healthy adults by using the same sandwich ELISA method.

## MATERIALS AND METHODS

### Saliva collection and storage

Saliva samples were obtained from 10 healthy individuals and 18 DM patients who visited the Jeonbuk National University Medical Center in Jeonju, Korea. Informed consent was obtained from volunteers after the nature and possible consequences of the studies had been fully explained. DM diagnosed using the criteria defined at the international workshop for a classification of diabetes and conditions [19]. All female and male participants were between the ages of 32 and 82 years. All participants were advised to checkup for sample collection around at 9:00 morning and not to eat food or drink any beverages after wakeup. After all the saliva samples had been obtained, they were divided into 1.5 mL Eppendorf tubes and centrifuged at 13,000 g at 4°C for 10 minutes. The supernatant was saved, transferred to new 1.5 mL tubes, and the samples were frozen at -70°C until they were ready to be used.

### SDS-PAGE and western blotting

Saliva proteins and GST-tagged recombinant human CSP1 (GST-rhCSP1) were denatured for 2 minutes at 95°C in 2X sodium dodecyl sulfate (SDS) sample buffer and separated on a 10% SDS polyacrylamide gel. Proteins on the gels were either stained with Coomassie blue (CB) or electro-transferred to a PVDF membrane for western blotting (WB) analysis [20]. The PVDF blot was blocked with 5% nonfat powdered milk in PBS and probed with primary mAb-hCSP1#4 for 1 hour at room temperature (RT) or overnight at 4°C. After the blot was rinsed with PBS-0.05% Tween 20 (PBST), interacting mAb was visualized using HRP-conjugated anti-mouse IgG 2nd Ab in PBST and 4-chloro-1-naphtole substrate solution.

### Immunohistochemistry

To locate CSP1 in human tissues, immunohistochemistry was conducted with paraffin-embedded human tissue slides (Super Biochips, South Korea) according to the manufacturer's instructions. Briefly, slides were deparaffinized by treatment with xylenes and rehydrated in a graded alcohol series. Antigen unmasking was carried out by boiling the slides in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) for 10 minutes in a microwave oven. Slides were cooled to RT, rinsed briefly in PBS, and then treated with 0.3% hydrogen peroxide in water for 30 minutes to quench any endogenous peroxidase activity. Slides were blocked for 20 minutes in 3% BSA solution at RT and then probed with biotinylated mAb-hCSP1#4 in blocking solution for overnight at 4°C. The slides were incubated

for 30 minutes at RT with HRP-conjugated avidin and washed 3 times with PBST. The resulting signal was developed with diaminobenzidine tablets (D4293, Sigma, MO, USA) and counterstained with Mayer's hematoxylin (S3309, DAKO, CA, USA). The slides were dehydrated in 80%, 90%, and 100% ethanol, cleaned in xylene for 2 minutes each, mounted with Permount solution, and then covered with coverslip.

**Sandwich ELISA**

Sandwich ELISA was used in this study to measure the concentration of salivary CSP1 in two groups. To each well of the ELIAS plate, 50 µL of capture (mAb-hCSP1#14, 2 µg/mL) was applied 1 hour at 37°C. After three 10 minutes washes with PBST, the plate was blocked with 50 µL of BSA (10 mg/mL) non-specific binding solution. To the wells, 50 µL of the saliva were added, incubated for 1 hour at 37°C, and washed with PBST. To each well, 50 µL of the biotin-conjugated detector (mAb-hCSP1#4, 2 µg/mL) was added and incubated for 1 hour at 37°C prior to being probed with HRP-conjugated avidin (10 mg/mL). Following a final rinse with PBST, the colorimetric detection of the immune reaction was initiated with an addition of 50 µL of methylbenzidine substrate solution (10 mg/mL). The absorbance was measured at 450 nm in an automatic ELISA reader (Bio-Rad model 550, Irvine, CA, USA) after the reaction was stopped by adding 50 µL of 10 µmol/mL $H_2SO_4$.

**Data analysis**

PASW Statistics for Windows version 22.0 (SPSS) and Microsoft version 2016 Excel program were used for analysis and comparison of test results. MedCalc version 19.0 was used for receiver operating characteristic (ROC) curve and the area under the curve (AUC) analysis. p-value < 0.05 and AUC > 0.7 was considered significant. Pearson's correlation coefficient ($r^2$) was used to evaluate correlations between two parameters, CSP1 level and $OD_{450nm}$ value.

## RESULTS

**Quality test of in-house produced mAbs by immunoblot and immunohistochemistry**

GST-rhCSP1 and diabetes patient's saliva was resolved in SDS-PAGE to test the quality of the in-house produced mAbs against rhCSP1. The proteins were electrophoresed in 10% SDS polyacrylamide gel and stained with CB or electro-transferred to PVDF membrane. The PVDF blot was then probed with an in-house produced mAb-hCSP1#4. MAb-hCSP1#4, which was used as a detector for the sandwich ELISA system, recognized a 42-kDa of GST-rhCSP1 and only a single band of approximately 27-kDa in saliva, as shown in lane 1 and 2 of WB corresponding to CB in Figure 1. When the blot was stained with mAb-hCSP1#14, which was used as a capture for the sandwich ELISA system, the result was almost the same as that of mAb-hCSP1#4 (data not shown). In addition, various human tissue section slides were stained with mAb-hCSP1#4 or mAb-hCSP1#14 to check distribution of CSP1. The only stained tissue with mAbs was salivary gland as shown in Figure 2. Two human tissues, pancreas and prostate, were shown as two negative representative tissues among 15 different human tissues. That only saliva gland tissue was stained with mAbs suggested that salivary CSP1 originates exclusively from saliva glands.

**Standard curve and comparison of CSP1 levels in healthy adults and DM patients**

Before measuring CSP1 levels in saliva by a sandwich ELISA system, a standard curve was produced with a series of different concentrations of rhCSP1 (0 - 12 µg/mL). In the standard curve, the rhCSP1 concentration was plotted on the *x*-axis against the $OD_{450\ nm}$ value on the y-axis. mAb-hCSP1#14 and mAb-hCSP1#4 were used as a capture Ab and detector Ab, respectively, to measure concentrations of a standard protein of rhCSP1. The obtained Pearson's correlation coefficient was $r^2$ = 0.995, suggesting that there was good linearity throughout the entire measuring range between the two parameters (Figure 3), and thus the fabricated ELISA system would be reliable for quantification of salivary CSP1. Saliva CSP1 concentrations of 10 healthy adults and 18 DM patients were measured based on the house-fabricated CSP1 sandwich ELISA system and the produced standard curve. The analysis of CSP1 levels from 2 groups was summarized in Table 1. Mean value (± standard deviation) of CSP1 level of healthy adults and 18 DM patients were 3.74 (± 0.66) and 4.65 µg/mL (± 1.05), respectively. Student's *t*-test indicated that there was a statistically significant difference in CSP levels between two groups (p = 0.026). Figure 4 showed the profiles of saliva CSP1 concentration in two groups. The central box and the middle dotted line of each group represented the values from lower to upper quartiles (25th to 75th percentiles) and median, respectively. The median value (25th to 75th percentiles) of the healthy subjects was 3.92 µg/mL (3.15 - 4.02), and that of DM patients was 4.35 µg/mL (3.94 - 5.11). There was one outlier, smaller than 10 or the larger than 90 percentiles, from the healthy adult samples and two outliers from DM patient samples. The profiles of Figure 4 demonstrated that the saliva CSP1 level of DM patients was higher than that of healthy individuals.

**ROC curve for CSP1 sandwich ELISA**

ROC curve was assessed for evaluating the accuracy of the CSP1 ELISA system and AUC was determined to check the ability of the ELISA system to discriminate between the healthy and DM patient groups (Figure 5). The AUC was 0.784 (95% confidence interval; 0.54 to 0.97, p = 0.003) and optimal criterion was > 4.06. The values of sensitivity and specificity were 64.71% and 88.89%, respectively. The obtained ROC curve and corresponding AUC showed salivary CSP1 as a potential

**Table 1. Summary of salivary CSP1 tests in the healthy and DM groups.**

| Characteristics | Healthy | DM patients |
|---|---|---|
| No. of subjects | 10 | 18 |
| Mean (µg/mL) | 3.739 | 4.654 |
| Standard deviation | 0.659 | 1.047 |
| Standard error of the mean | 0.220 | 0.254 |
| Median (µg/mL) | 3.917 | 4.351 |
| 25th to 75th percentiles (µg/mL) | 3.145 - 4.023 | 3.939 - 5.113 |
| Student's *t*-test p-value | p = 0.026 | |

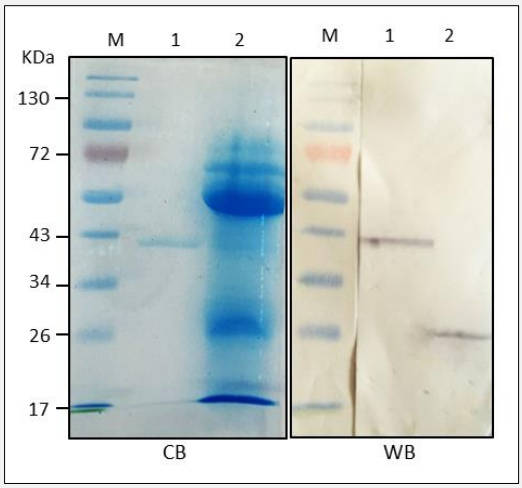


**Figure 1. Detection of CSP1 in diabetes patient's saliva with a house generated mAb to recombinant human CSP1 protein.**

**CB was a Coomassie blue-stained SDS gel resolving molecular size markers (lane M), purified GST-tagged recombinant hCSP1 as a positive control (lane 1) and DM patient's saliva (lane 2). WB was a corresponding immunoblot of CB stained-gel probed with primary mAb-hCSP1#4. The mAb recognized a 42-kDa of GST-tagged rhCSP1 in lane 1 and a single protein band of about 27-kDa in lane 2 of DM patient's saliva.**

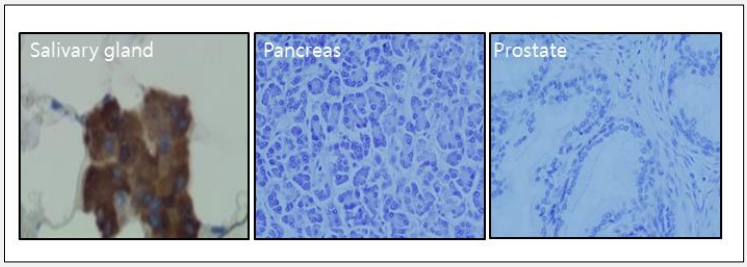


**Figure 2. Immunohistochemical localization of CSP1 in various human tissues.**

**The various human tissues section slides were stained with mAb-hCSP1#4 and localization of CSP1 was examined. The only stained tissue mAb-hCSP1#4 was salivary gland. The pancreas and the prostate tissue stained with mAb-hCSP1#4 were shown as two negative representative tissues among 15 different human tissues.**

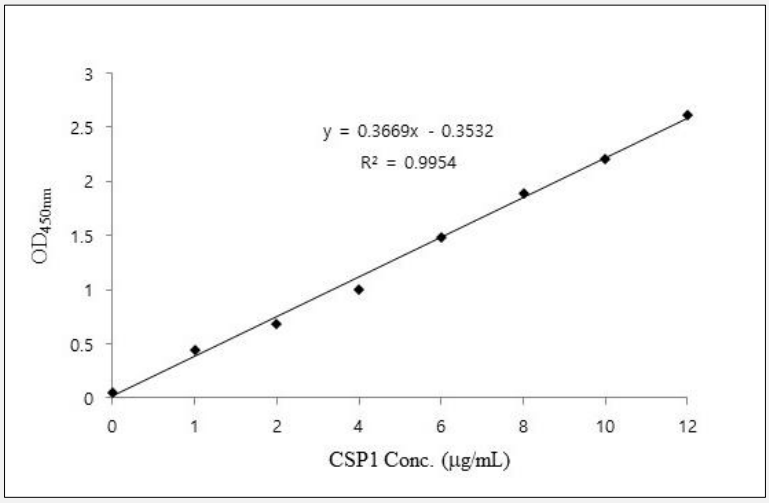


**Figure 3. Standard curve of sandwich ELISA for measurement of CSP1 in saliva.**

**The recombinant human CSP1 (rhCSP1) was used as a standard protein. The in-house generated mAb-hCSP1#14 and mAb-hCSP1#4 were used as a capture and a detector Ab, respectively, for sandwich ELISA. A reliable Pearson's correlation coefficient was obtained between the $OD_{450nm}$ value and rhCSP1 concentration ($r^2$ = 0.995). Good linearity was displayed throughout the entire measuring range (0 - 12 µg/mL) of CSP1.**

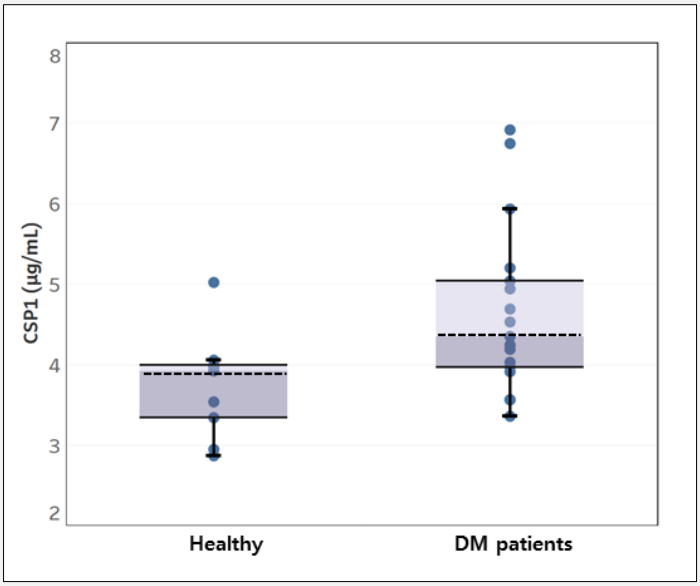


**Figure 4. Profiles of human saliva CSP1 concentrations in the healthy and DM patient groups.**

**The central box and the middle, dotted line of each group represented the values from lower to upper quartiles (25th to 75th percentiles) and median, respectively. The median value (25th to 75th percentiles) of the healthy subjects was 3.917 µg/mL (3.145 - 4.023), and that of DM patients was 4.351 µg/mL (3.939 - 5.113). The horizontal line extended from the minimum to the maximum values, excluding outlier values that were displayed as separate points. Outlier values were defined as those smaller or larger than the 10th or the 90th percentile, respectively.**

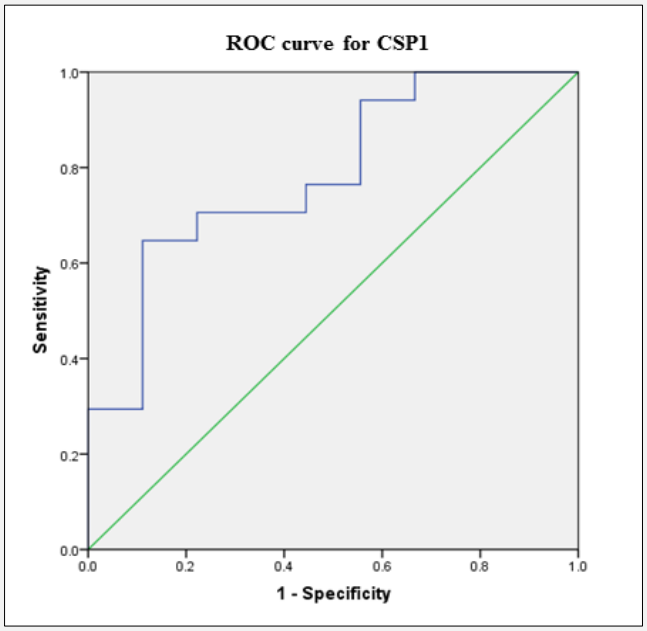


**Figure 5. The ROC curve for CSP1 sandwich ELISA test in healthy adults and DM patients.**

**AUC value was 0.784 (95% confidence interval: 0.54 to 0.97, p < 0.005) and the optimal criterion was > 4.06. Sensitivity and specificity were determined to be 64.71 and 88.89, respectively.**

biomarker had a predictive ability to discriminate DM patients from healthy subjects.

## DISCUSSION

In present study, in order to construct a sandwich ELISA system for measurement of salivary CSP1 level, the in-house generated mAbs were tested for their qualities by WB with human saliva (Figure 1) and immunohistochemistry with human tissue section slides (Figure 2). Furthermore, the standard curve was obtained by plotting a series of different concentrations of rhCSP1 (0 - 12 µg/mL) on the *x*-axis against the $OD_{450nm}$ value on the *y*-axis (Figure 3). The sandwich ELISA system, which was based on the standard curve and capture Ab and detector Ab, was constructed and used for quantification of salivary CSP1 level of normal healthy adults and DM patients. The results of the salivary CSP1 concentrations in the two groups were compared and analyzed (Table 1, Figure 4 and 5).

To measure the salivary CSP1 level accurately, the most important factor is to construct a highly qualified sandwich ELISA system. The highly qualified CSP1 ELISA system should meet the following conditions. First, mAbs should bind to CSP1 protein specifically. Second, the standard curve should show good linearity between CSP1 level and $OD_{450nm}$ value. Thus, we tested the quality of mAb with WB and immunochemistry and checked the linearity of the standard curve with Pearson's correlation coefficient.

When the GST-tagged rhCSP1 protein was subjected to SDS-PAGE and WB, its molecular weight (M. W.) was estimated to be about 42-kDa (lane 1 of CB and WB in Figure 1). Since the M.W. of GST was known to be 28-kDa, it meant that the size of CSP1 was approximately 14-kDa. However, as shown in lane 2 of WB in Figure 1, mAbs stained a single band at 27-kDa from human salivary sample. This discrepancy of M. W. may come from the CSP1 protein existing as a phosphorylated or a glycosylated form in saliva. The fact that the human CSPl sequence contains a potential N-glycosylation site makes the possibility of its existence as a glycosylated form much greater [13]. This postulation would be supported by the three CSPl isoforms in rat that were derived from N-glycosylation of protein but not from different genes [14].

The immunohistochemistry demonstrated that the mAb reacted specifically with salivary glands but not with other human tissues at all (Figure 2). This result was consistent with an article report that the signal for CSP1 mRNA was predominantly great from the sublingual

gland but not from various human organs [13]. The results of WB and immunohistochemistry together indicated that the generated mAbs bind specifically to CSP1 protein.

Next, we set up and obtained a standard curve with a series of different rhCSP1 (0 - 12 µg/mL) and $OD_{450nm}$ value. The sandwiched-complex of mAb-hCSP1#14-rhCSP1-mAb-hCSP1#4 was colorimetrically detected by adding substrate, and the color density of the immune reaction was recorded as absorbance value at $OD_{450nm}$. The obtained Pearson's correlation coefficient between the two parameters was $r^2 = 0.995$, suggesting that the fabricated ELISA system would be reliable for quantification of CSP1 in saliva.

The means (SD) of salivary CSP1 concentrations of 10 normal adults and 18 DM patients were 3.74 µg/mL (0.66) and 4.65 µg/mL (1.05), respectively (Table 1), and the analysis of results showed that the difference in CSP1 level between the two groups was statistically significant ($p < 0.05$). The accuracy of the sandwich CSP1 ELISA method was accessed by AUC to check how well the test separated two different groups (Figure 5). The AUC value was 0.784 (95% confidence interval; 0.54 to 0.97, $p = 0.003$), and optimal criterion was > 4.06. According to traditional academic point system, AUC 0.7 - 0.9 is considered to show that the test system is a good tool for discriminating two groups. Thus, it indicated that salivary CSP1 can be a candidate of potential biomarkers for detection or screening of DM patients.

The current study with saliva corresponded to our previous study with serum [18], such as that the CSP1 level of the DM patient group was higher than that of the healthy group. It showed that the means of CSP1 level in saliva and serum from the healthy group were 3.74 µg/mL (0.66) and 5.67 ng/mL (6.53), and those from DM patients group were 4.65 µg/mL (1.05) and 24.87 ng/mL (11.86), respectively, indicating that saliva contained more than 100 times of CSP1 protein than serum. Our two studies with saliva and serum have a couple of limitations to overcome to say that CSP1 can be useful as a potential biomarker for detection or screening of DM patients. First, the studies were blind tests with respect to information on the participants who were separated into two groups only depending on glucose level on the morning of the checkup day. The factors such as medications and oral diseases were not considered, which could have influenced on CSP1 level. Second, two studies were carried out with the totally independent groups: serum-donated healthy group and saliva-donated healthy group were not same, neither were DM patient groups.

Detection of a biomarker for a specific disease in both the saliva and serum has been reported in many cases. For example, significantly higher concentrations of defensin-1 in oral cell carcinoma patients and of antigen 15-3 in breast cancer patients were detected and compared with healthy controls [22,23]. A highly positive correlation was observed between saliva and serum in both cases.

Therefore, a strictly-controlled and much larger cohort study is required to use CSP1 protein as a biomarker for DM and to overcome the limitations of our previous two studies. Further study needs to investigate whether salivary and serum CSP1 levels have a positive correlation from the same healthy or DM patient groups. If the correlation turns out positive, measurement of salivary CSP1 level would be an easy, simple, and favorable way for diagnosis and screening of DM patients.

## CONCLUSION

The mAbs against rhCSP1 were tested to check whether the mAbs bind specifically to CSP1 protein by WB and immunohistochemistry. Human salivary CSP1 was detected as a glycosylated form of 27-kDa when stained with mAb-hCSP1#4. Salivary gland was the only stained tissue among the various human tissues examined with mAb. The obtained standard curve for a sandwich ELISA system showed good linearity between CSP1 levels and absorbance value of $OD_{450nm}$ ($r^2 = 0.995$). The median values (25th to 75th percentiles) of saliva CSP1 in healthy adults and DM patients were 3.92 µg/mL (3.15 - 4.02) and 4.35 µg/mL (3.94 - 5.11), respectively. Statistically, there was a significant difference of CSP1 level in the two groups ($p = 0.026$). The present data showed a significant increase of CSP1 levels for DM patients compared with the control group, indicating that CSP1 level in saliva could be used as a potential biomarker for detection or screening of DM patients. However, further studies are necessary to provide scientific and clinical validation.

**Declaration of Interest:**
The authors report no competing financial interests.

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