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# Proteomic analysis of secretion from human transplanted submandibular gland replacing lacrimal gland with severe keratoconjunctivitis sicca

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ABSTRACT

*Purpose*: Proteomic analysis of secretions from transplanted or non-transplanted submandibular glands in patients with severe keratoconjunctivitis sicca and tears from normal eyes. *Experimental design*: Secretions from submandibular glands transplanted to replace lacrimal glands and non-transplanted submandibular glands were collected at 1 year from 5 patients with severe keratoconjunctivitis sicca undergoing transplantation, and tears were collected from 3 normal subjects. 2-D electrophoresis (2-DE), then mass spectrometry was used to identify proteins. Western blot analysis was used to confirm protein expression. *Results*: We identified 34 and 11 distinct proteins in the saliva from transplanted submandibular glands and tears, respectively. The saliva from transplanted submandibular glands contained almost all the proteins abundant in tear fluid. The functions of identified proteins in the saliva from transplanted submandibular gland were mainly immune response and anti-bacterial. In total, 7 proteins showed differential expression between the saliva of transplanted and non-transplanted submandibular glands. The upregulation of short palate, lung and nasal epithelium carcinoma-associated protein 2 and carbonic anhydrase VI was confirmed by Western blot analysis. *Conclusions*: Identified proteins in saliva from transplanted submandibular glands may protect ocular structures. These findings can help in understanding the functional status of transplanted submandibular glands.



## 1. Introduction

Keratoconjunctivitis sicca (KCS), also known as dry eye syndrome, is a common ophthalmological condition. The incidence is between 15% and 28% in people older than 60 years [1,2]. In China, about 30 million people have the disorder [3]. Treatments include the use of tear substitutes and surgical corrections such as obliteration of the lacrimal drainage pathways, which are effective only for mild cases but not for severe cases [3–5].

In 1986, Murube-del-Castillo first described microvascular autologous submandibular gland transplantation for surgical correcting severe keratoconjunctivitis sicca. The whole submandibular gland is transferred to the temporal region of the skull. The related blood vessels are anastomosed to the superficial temporal artery and vein. Wharton's duct is transplanted to the upper lateral conjunctiva fornix without connecting to the duct of the lacrimal gland. The gland is left denervated, and the damaged lacrimal gland is left *in situ* [4]. The procedure is now in use in several medical institutions in Australia, Germany, China, United Kingdom, and the United States [3,5–8]. Long-term follow-up revealed that secretion from the transplanted gland significantly improves dry eyes in both subjective and objective parameters [8].

However, whether the protein composition of the secretion from the transplanted submandibular gland differs from that of eye fluid or saliva of normal submandibular gland remains unknown. Previous studies showed that newly created tear film of KCS patients undergoing transplantation of submandibular gland remains mainly salivary, with lower osmolality relative to tears [8,9]. Therefore, proteomic examination of the composition of secretions from transplanted submandibular glands is important to fully understand the status of transplanted submandibular glands and further improves transplantation therapy for severe KCS.

Proteomics is a fast-growing discipline that has recently been used for analysis of whole saliva and parotid and submandibular-sublingual

*Abbreviations:* KCS, Keratoconjunctivitis sicca; 2-D, Two dimensional; SPLUNC2, Short palate, lung and nasal epithelium carcinoma-associated protein 2; CA VI, Carbonic anhydrase VI; CBB, Coomassie brilliant blue; WS, whole saliva; MS, Mass spectrometry; FA, formic acid

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glandular secretions, and proved to be a valuable approach in the field of saliva research [10–12].

In the present study, we performed proteomic analysis of secretions from transplanted or non-transplanted submandibular glands of patients with severe KCS and normal tears.

## 2. Materials and methods

### 2.1. Human subjects

The project was approved by the Scientific and Ethics Committee of Peking University. We included 5 patients with severe KCS (3 males, mean age 49.4 ± 12.5 years) with autologous submandibular gland transplantation on one side in the Department of Oral and Maxillofacial Surgery, Peking University School of Stomatology. We transplanted the submandibular gland as described [3] with modification, and the 5 patients underwent partial glandular transplantation (about 2/3 submandibular gland transplanted) because the secretion of 1/2 normal submandibular gland is enough to lubricate the ocular surface. In addition, all patients underwent surgery to reduce the size of the transplanted gland 1 year after transplantation because of epiphora or persisting excessive secretion. The transplanted submandibular gland was left denervated. All patients underwent complete systemic examination before transplantation. None had Sjögren's syndrome, symptoms of xerostomia or history of smoking. The underlying causes of the severe KCS were idiopathic (4 patients) and chronic keratoconjunctivitis (1 patient). Schirmer test results were <1 mm for all 5 patients, and scientigraphy with $^{99m}$Tc pertechnetate revealed no hypofunction of submandibular glands before transplantation.

Three normal subjects (2 males, mean age 25.3 ± 1.9 years) were recruited for tear analysis. All subjects did not wear contact lens and had normal ocular and general health. None was taking any medication. All subjects gave their signed informed consent to be in the study.

### 2.2. Chemicals and reagents

Iodoacetamide (IAA), DL-dithiothreitol (DTT), urea, thiourea, glycerol, acetonitrile (ACN), trifluoroacetic acid (TFA) and 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonate (CHAPS) were from Sigma-Aldrich (St. Louis, MO, USA). Polyclonal antibody targeting the C-terminal peptide of human SPLUNC2 (VDNPQHKTQLQTLI) was raised from rabbits by Cowin Biotech Co. (Beijing, China). Anti-CA VI goat monoclonal antibody (targeting human CA VI) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

### 2.3. Sample collection and processing

#### 2.3.1. Secretion samples

Unstimulated submandibular saliva was collected 12 months after the transplantation from the submandibular gland on the contralateral side to the transplantation side using the special collecting system as described by Wolff et al. [13]. The unstimulated secretion from the transplanted submandibular gland was collected 12 months after the transplantation from the inferior marginal tear strip of the impaired eye with a calibrated capillary tube. We collected at least 1 mL saliva from the non-transplanted or transplanted submandibular gland from each patient. Total collection time ranged from 25 to 35 min.

Tear fluid from normal subjects was stimulated by the yawn reflex elicited voluntarily by the subjects. The subject's lower eyelid was gently pulled down and the tip of a 20-μL sterilized glass microcapillary tube (Microcaps, Drummond Scientific, USA) was placed in contact with the tear meniscus near the lateral canthus. We collected about 500 μL tears from one eye of each subject. Total collection time ranged from 25 to 40 min.

Immediately after collection, 1/10 volume protease cocktail inhibitor (Sigma, USA) was added to the secretion samples to prevent proteolytic degradation. To remove debris, samples were centrifuged at 14,000 *g* for 15 min at 4 °C. Proteins in the samples were precipitated by use of the Liquor Protein Extraction Kit (Applygen Technologies, Inc., China). After centrifugation at 14,000 *g* for 15 min at 4 °C, the protein pellets were washed 3 times with ethanol, air dried, and solubilized in the buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, and 0.2% bio-lytes ampholyte pH 3–10 (rehydration solution). Protein concentration was measured by the Bradford assay (Bio Rad). Protein solutions were stored at −80 °C until use.

#### 2.3.2. Tissue samples

Since all five patients underwent two operations, the first was partial glandular transplantation of submandibular gland, and the second was reducing the size of the transplanted gland 1 year after transplantation, tissues of normal submandibular glands were collected at the first operation while tissues from transplanted submandibular glands were collected at the second surgery. All the tissue specimens were snap frozen in liquid nitrogen for further procedure.

Tissue samples (0.1 g) were cut into pieces of about $2\times2\times2$ mm$^3$ and homogenized by use of homogenizer (Ultra-Turrax T10, IKA Lab Technology, Staufen, Germany) in an ice-cold denaturing lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin), then sonicated by use of a Sonifer II 450 (Branson, Danbury, CT) in an ice bath for 8 cycles, each consisting of 5-s sonication followed by a 10-s break, then held for 30 min on ice with periodic vortexing. The lysates were centrifuged at 14,000 *g* for 30 min at 4 °C. The protein concentration in the supernatants was determined by use of a Bio-Rad protein quantitation kit. Protein samples were aliquoted and stored at −80 °C for Western blot analysis.

### 2.4. Proteomic analysis

#### 2.4.1. 2-D electrophoresis (2-DE)

Immobilized pH gradient (IPG) strips (17 cm, pH 3–10 nonlinear; Bio-Rad) were passively rehydrated for 1 h with 300 μL protein solution (150 μg for analytical gels and 1500 μg for preparative gels, respectively), covered with mineral oil and transferred to an isoelectric focusing (IEF) cell (Bio-Rad). IEF was performed with constant power (50 μA/IPG strip) at 50 V for 12 h of rehydration; 250 V for 30 min, linear; 1000 V for 1 h, rapid; linear ramping to 10,000 V for 5 h; and finally 10,000 V for 6 h.

The IPG strips were equilibrated with a buffer containing 50 mM Tris/HCl (pH 8.8), 6 M urea, 2% SDS, 20% glycerol, and 10 mM DTT for 15 min, washed with wash buffer (50 mM Tris/HCl, pH 8.8, 6 M urea, 2% SDS, 20% glycerol, and 200 mM iodoacetamide) for 15 min, transferred to 12% SDS-PAGE gels ($18.5\times20$ cm, manually poured) and separated at constant current (10 mA for the initial 40 min and then 30 mA to the end). 2-DE was performed at least twice for each sample.

#### 2.4.2. ESI-Q-TOF MS/MS and protein identification

The peptides from the tryptic digestion were analyzed with use of fused silica tubing (75 μm × 100 mm) packed with symmetry 300 $C_{18}$, 3.5-μm spherical particles with pore diameter 100 Å (Waters) on a Waters Capillary liquid chromatography system including 3 pumps A, B and C (Waters). Samples were injected at a flow rate of 20 μL/min with pump C and salts were removed on the precolumn ($0.35\times5$ mm) packed with symmetry 300 $C_{18}$, 3.5-μm spherical particles with pore diameter 100 Å (Waters). The precolumn was connected in the 10-port switching valve and switched to the analytical column after the sample was desalted. Mobile phase A consisted of water/ACN (95/5, v/v) with 0.1% aqueous formic acid (FA). Mobile phase B consisted of water/ACN (5/95, v/v) with 0.1% FA. The separation was

performed by running on gradient: 3% B, for 0.1–3.5 min for injection; 5–40% B, for 3.5–40 min; 40–60% B, for 40–60 min; 60–90% B, for 60–65 min; 90–5% B, for 65–70 min; 5–5% B, for 70–90 min. The Cap LC was coupled online with a Q-TOF Ultima Global mass spectrometer (Waters) for protein detection and identification. Peptide mixtures were dissolved with 2 μL of 0.1% FA and injected by atmosphere on the precolumn with use of a Cap LC system. Peptides were directly eluted into a Q-TOF mass spectrometer (Q-TOF Ultima Global mass spectrometer; Waters) at 250 nL/min on the analytical column. After being analyzed by Mass Lynx 4.0, data from MS/MS analysis underwent identification with use of the MASCOT search engine (http://www.matrixscience.com/) with database Swiss-Port, taxonomy Homo sapiens (human), trypsin for the digestion enzyme, one missed cleavage site, fixed modifications of carbamidomethyl (C), variable modifications of oxidation (M), peptide tolerance of 0.2 Da, MS/MS tolerance of 0.1 Da.

### 2.5. Western blot analysis

In total, 5 μg secretion protein or 10 μg tissue protein was mixed with 1× sample buffer (50 mM Tris pH 6.8, 2% SDS, 10% glycerol, 5% β-mecaptoethanol and 0.002% bromophenol blue), heated at 100 °C for 2 min and cooled on ice for 2 min, separated by 12% SDS-PAGE, transferred to a polyvinylidene fluoride membrane (Millipore), and probed with polyclonal rabbit anti-SPLUNC2 antibody (1:2000) or monoclonal goat anti-CA VI antibody (1:2000). The blots were incubated with horseradish peroxidase-conjugated secondary antibodies (1:10,000) and visualized by use of an ECL detection system (Pierce). The membrane was stripped for anti-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal reference for tissue samples only. Because of no internal reference for saliva, we used an SDS gel of secretion samples with Coomassie blue staining to show equal sample volumes.

### 2.6. Statistical analysis

Statistical analysis involved use of SPSS v12.0 for Windows (SPSS Inc., Chicago, IL). Analysis of 2 groups involved unpaired Student's *t* test and more than 2 groups one-way ANOVA followed by Bonferroni's post hoc test. Data are presented as mean ± SD. A $P<0.05$ was considered statistically significant.

## 3. Results

### 3.1. Protein concentrations of saliva from non-transplanted or transplanted submandibular glands and tear fluid

The concentration of total protein in saliva from non-transplanted and transplanted submandibular glands did not differ (0.77 ± 0.13 *vs* 0.86 ± 0.09 mg/mL, $P>0.05$). However, the protein concentration was higher for tear fluid than saliva from non-transplanted or transplanted submandibular glands (2.78 ± 0.27 mg/mL, $P<0.05$).

### 3.2. Proteome of saliva from transplanted submandibular glands

The representative 2-DE gel map for saliva from transplanted submandibular glands is in Fig. 1. We detected more than 150 protein spots in the 2-DE gel. We cut 80 protein spots that appeared repeatedly in all 5 patient samples for tryptic digestion and ESI-Q-TOF-MS/MS analysis.

We identified 34 distinct proteins from the 80 protein spots (Table 1). Among them, α-amylase was the most abundant, at about 58 kDa (spots 20–31), which differs from its predicted molecular weight of 45 kDa (pI 5.0). We found similar phenomena for CA VI (spots 36–41) and SPLUNC 2 (spots 43–47), and the others. Among the others, mucin-7 (spot No. 64) was especially in a location where the molecular weight was much lower than what was predicted for mucin-7 (MW 39 kDa and pI 8.9). This type of distribution is similar to what was observed in 2-DE maps for other body fluids [14]. Post-translational modifications (PTM) such as glycosylation and phosphorylation may influence the molecular weight and pI of proteins [15]. The observed molecular weights higher than those predicted for α-amylase, CA VI and SPLUNC 2 spots could be due to glycosylation, because saliva proteins are usually heavily glycosylated [16]. As well, degradation or cleavage may contribute to change in molecular weight and pI of the proteins. Glycosylation and cleavage may be the main reasons for the inconsistency in observed and predicted molecular weight for Mucin-7. Mucins are susceptible to cleavage by saliva protease to a smaller form [17]. Moreover, peptides derived from mucin-7 show non-specific tryptic cleavage sites after saliva is digested with trypsin, which suggests that some proteases also cleaved mucin-7 [18]. Mucin-7 may be cleaved by endogenous proteases in saliva before and after saliva collection. Therefore, spot 64 was likely the cleaved mucin-7.

### 3.3. Proteome of tear fluid

The representative 2-DE gel map for tear fluid is in Fig. 2. We detected more than 100 protein spots in the 2-DE gel of the tear fluid and excised the 32 distinguishable protein spots with intense staining, which underwent identification by ESI-Q-TOF MS/MS. We identified 11 distinct proteins (Table 2). The most abundant protein was lactotransferrin (spots 7–11), with the largest protein spot about 80 kDa and pI 8.5. Immunoglobulins, albumin, zinc-alpha-2-glycoprotein, cystatins, lipocalin, and lysozyme were also abundant.

### 3.4. Functional categories of proteins in saliva from transplanted submandibular glands

We classified the identified proteins in saliva from transplanted submandibular glands into functional categories by their annotations in the NCBI database. The largest group (23%) was immune response-related proteins, including immunoglobulins and polymeric immunoglobulin receptor. The second group (17%) was anti-bacterial related proteins, including SPLUNC 2, lactotransferrin, lysozyme, and histatin-1. We found some of the cystatin family members (cystatin C, S and SN), which possess a cysteine protease inhibitory property with multi-functions. In addition, some proteins were involved in metabolism, such as α-amylase, and reversible hydration of $CO_2$, such as CA VI.

### 3.5. Identification of differentially expressed proteins in saliva of non-transplanted and transplanted submandibular glands

For each patient, two secretion samples, one collected from the non-transplanted submandibular gland and the other from the transplanted submandibular gland, were paired for 2-DE gel analysis. A total of 5 pairs of secretion samples were subjected to 2-DE gel analysis. We compared the protein spot patterns for secretion from the paired non-transplanted and transplanted glands to rule out inter-individual difference. The protein spot patterns for the paired samples were in general similar (Fig. 3).

However, we found 19 spots on the 2-DE gel that were differentially stained, with at least 2-fold change in staining intensity, in secretions from transplanted and non-transplanted submandibular glands (10 spots upregulated and 9 downregulated). In total, 17 spots underwent MS, with the exclusion of spots 68 and 69 because they seemed to consist of more than one protein. We identified 7 proteins by MS (Table 3). The expression of SPLUNC 2 and CA VI appeared to be higher in saliva from transplanted than the paired non-transplanted submandibular gland (Fig. 4). The staining of SPLUNC2 and CA VI also showed large inter-individual variation


**Fig. 1.** Representative 2-D electrophoresis (2-DE) gel of saliva from transplanted submandibular gland to replace lacrimal gland in patients with severe keratoconjunctivitis sicca. 1500 μg protein was subjected to 2-D electrophoresis, then Coomassie brilliant blue staining. Numbered arrows indicate the distinguishable protein spots.

between the 5 patient samples of transplanted submandibular glands. This variation could be due to the content of SPLUNC2 and CA VI showing differences among individuals and to the procedure of silver staining, with its several steps, which could influence the staining intensity. The difference in staining intensity was confirmed by Western blot analysis to validate the expression changes of SPLUNC2 and CA VI.

### *3.6. Confirmation of upregulation of SPLUNC2 and CA VI in secretion from transplanted submandibular glands*

Consistent with changes seen on silver staining of 2-DE gels, Western blot analysis revealed the expression of SPLUNC 2 and CA VI markedly higher in secretions from transplanted than non-transplanted submandibular glands (density $12469.25 \pm 442.06$ *vs.* $7147.25 \pm 1560.86$, $P<0.01$; $5234.25 \pm 497.76$ *vs.* $1695.50 \pm 619.67$, $P<0.01$, respectively) (Fig. 5). In addition, the expression of SPLUNC 2 was significantly higher in tissue of transplanted than non-transplanted submandibular gland (relative intensity $2.90 \pm 0.43$ *vs* $1.93 \pm 0.44$, $P<0.01$), but the expression of CA VI did not differ between the 2 tissue types (relative intensity $0.90 \pm 0.19$ *vs* $0.92 \pm 0.37$, $P>0.05$) (Fig. 6).

## 4. Discussion

In this study, we proteomically identified 34 distinct proteins from the saliva of transplanted submandibular glands in patients with severe KCS and 11 distinct proteins from tear fluid of normal persons. The saliva from transplanted submandibular glands contained almost all the abundant proteins of the tear fluid, which supports that the

**Table 1**
Identification of proteins in saliva from transplanted submandibular gland.

| Spot no. | Protein name | Gene name | Swiss-prot accession no. | Mr | *pI* | Molecular function |
|---|---|---|---|---|---|---|
| 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 | Polymeric immunoglobulin receptor | PIGR | P01833 | 84429 | 5.58 | Immune response and defense |
| 9, 12 | Ig mu chain C region | IGHM | P01871 | 49960 | 6.35 | Immune response and defense |
| 11, 12 | Lactotransferrin | LTF | P02788 | 80014 | 8.50 | Anti-bacterial |
| 13, 14, 15, 16, 17, 18, 19, 26 | Serum albumin | ALB | P02768 | 71317 | 5.92 | Regulate the colloidal osmoti pressure |
| 20, 21, 23, 24, 25, 27, 28, 29, 30, 31 | α-amylase 1 | AMY1A | P04745 | 58415 | 6.47 | Metabolism |
| 22 | Keratin, type I cytoskeletal 13 | KRT13 | P13646 | 49898 | 4.91 | Regulate the activity of kinases |
| 25, 26, 27 | Ig alpha-2 chain C region | IGHA2 | P01877 | 37301 | 5.71 | Immune response and defense |
| 26, 27 | Ig alpha-1 chain C region | IGHA1 | P01876 | 38468 | 6.08 | Immune response and defense |
| 32, 33, 34, 35 | Zinc-alpha-2-glycoprotein | AZGP1 | P25311 | 34079 | 5.57 | Stimulates lipid degradation |
| 36, 37, 38, 39, 40, 41 | Carbonic anhydrase 6 | CA6 | P23280 | 35459 | 6.51 | Reversible hydration of carbon-dioxide |
| 42 | Chitinase 3-like 2 variant | CHI3L2 | Q15782 | 42321 | 7.27 | Extracellular matrix protein |
| 43, 44, 45, 46, 47 | Short palate, lung and nasal epithelium carcinoma-associated protein 2 | SPLUNC2 | Q96DR5 | 27166 | 5.35 | Anti-bacterial |
| 48, 49, 50 | Cysteine-rich secretory protein 3 | CRISP3 | P54108 | 28524 | 8.09 | Function unkonwn |
| 49 | Annexin A1 | ANXA1 | P04083 | 38918 | 6.57 | Phospholipase A2 inhibitory activity |
| 51 | Zymogen granule protein 16 homolog B | ZG16B | Q96DA0 | 22725 | 6.74 | Tansport |
| 52 | Immunoglobulin J chain | IGJ | P01591 | 18543 | 5.12 | Immune response and defense |
| 52 | Salivary acidic proline-rich phosphoprotein 1/2 | PRPC | P02810 | 17006 | 4.63 | Function unkonwn |
| 53, 54, 55, 56, 57, 58, 59, 60, 61, 62 | Ig kappa chain C region | IGKC | P01834 | 11773 | 5.58 | Immune response and defense |
| 58, 59, 60, 61, 62 | Ig lambda chain C regions | LAC | P01842 | 11401 | 6.92 | Immune response and defense |
| 59, 61 | Ig kappa chain V-III region SIE | KV302 | P01620 | 11882 | 8.70 | Immune response and defense |
| 63, 64, 65, 66, 67, 68 | Prolactin-inducible protein | PIP | P12273 | 16847 | 8.26 | Regulation of water transport |
| 64 | Mucin-7 | MUC7 | Q8TAX7 | 39432 | 8.99 | Anti-bacterial |
| 68 | Lipocalin-1 | LCN1 | P31025 | 19409 | 5.39 | Bind lipophiles to minimize solvent contact |
| 69, 70 | Cystatin-S | CST4 | P01036 | 16489 | 4.95 | Cysteine protease inhibitory |
| 69 | Cystatin-SA | CYTT | P09228 | 16719 | 4.85 | Cysteine protease inhibitory |
| 71 | Histone deacetylase 10 | HDAC10 | Q969S8 | 11449 | 5.14 | Transcriptiont and cell cycle |
| 72 | Protein S100-A9 | S100A9 | P06702 | 13291 | 5.71 | Anti-bacterial |
| 73, 74 | Cystatin-SN | CST1 | P01037 | 16579 | 6.82 | Cysteine protease inhibitory |
| 75 | Cystatin-C | CST3 | P01034 | 16017 | 9.00 | Cysteine protease inhibitory |
| 76 | Lysozyme C | LYZ | P61626 | 16982 | 9.38 | Anti-bacterial |
| 77 | Histatin 1 | HTN1 | P15515 | 6958 | 9.11 | Anti-bacterial |
| 78 | Keratin, type II cytoskeletal 1 | KRT1 | P04264 | 66170 | 8.15 | Regulate the activity of kinases |
| 79 | Hemoglobin subunit alpha | HBA | P69905 | 15305 | 8.72 | Transport |
| 80 | Cystatin-D | CST5 | P28325 | 16355 | 6.70 | Cysteine protease inhibitory |

Mr: relative molecular weight; *pI*: isoelectric point.

saliva of transplanted submandibular gland could have a somewhat similar function as tear fluid for the eyes. Most of the identified proteins in saliva from transplanted submandibular glands had immune-response-related and anti-bacterial-related functions, which suggests that the saliva from transplanted glands may protect ocular structures. These results may help clinicians understand the possible effects of the secretion from transplanted submandibular glands on ocular structures. To the best of our knowledge, this is the first report on the proteomic study of the secretion from transplanted submandibular glands from patients with severe KCS.

Given that the saliva from transplanted submandibular glands contained all the abundant proteins of tear fluid, such as lactotransferrin, albumins, immunoglobulins, zinc-alpha-2-glycoprotein, cystatins, lipocalin, lysozyme and mucins [19], with comparable abundance for some, the saliva from transplanted glands may have some functions similar to that of tear fluid in ocular structures. Indeed, most patients with severe KCS show significantly improved clinical symptoms and signs after transplantation [3–5,8]. However, we found that the saliva from transplanted submandibular glands contained many specific saliva proteins, such as α-amylase, SPLUNC2, and CA VI. Among these proteins, α-amylase, which is mainly responsible for breaking down complex carbohydrates into maltose, was the most abundant protein in saliva from transplanted submandibular glands. Whether excessive salivary amylase in the eyes would have any non-desired effects on ocular structures is of interest. Although the effect of salivary amylase on ocular structures remains to be evaluated, salivary amylase may not harm the eyes. Amylase was also detected in normal tears in previous studies [20–22], but we could not detect it by 2-D electrophoresis in this study due to its low content. An *in vitro* study showed no histopathological change to the cornea after incubation with parotid secretions which contain high concentration of amylase [23]. And the long-term clinical follow-up of KCS patients after transplantation of submandibular gland showed improvement or at least stabilization of ophthalmologic structures [8].

Analysis of the functional categories of the identified proteins in saliva from transplanted submandibular glands revealed that they may be favorable to the eyes: most were immune-response-related or anti-bacterial-related proteins. The immunoglobulins, including α-chain and J-chain, for forming sIgA, can provide acquired immune protection to the mucosa [24] and help lysozyme lysis bacteria [25], and so should have similar effects on ocular structures when secreted into the eyes. Mucin-7 is a monomeric mucin glycoprotein mainly coded by the Mucin-7 gene localized on chromosome 4q13-21 [26], which has been detected in saliva from minor salivary glands, as well as submandibular and sublingual glands [27]. It can limit the adhesion of bacteria and represent part of an innate host defense system that controls infection by inhibiting the growth of a number of pathogenic microbes [28]. Clinical observations support that the saliva of transplanted submandibular gland is in general favorable to the eyes, without non-desired side effects on the ocular structures [3,5,8].

The efficiency of our identification of proteins from the 2-DE gel for saliva from transplanted submandibular gland was 34 proteins from 80 spots, which was similar to results of a previous study, in which 64 proteins were identified from 105 spots in whole saliva [10]. About half of the spots on the 2-DE gel of the saliva of transplanted gland did not show distinct proteins, which reflects that post-modification such as glycosylation or cleavage of saliva proteins may be frequent events, so some saliva proteins presented as more than one spot and even up to 10 spots, like α-amylase. The efficiency of identification of proteins from the 2-DE gel for tear fluid was higher

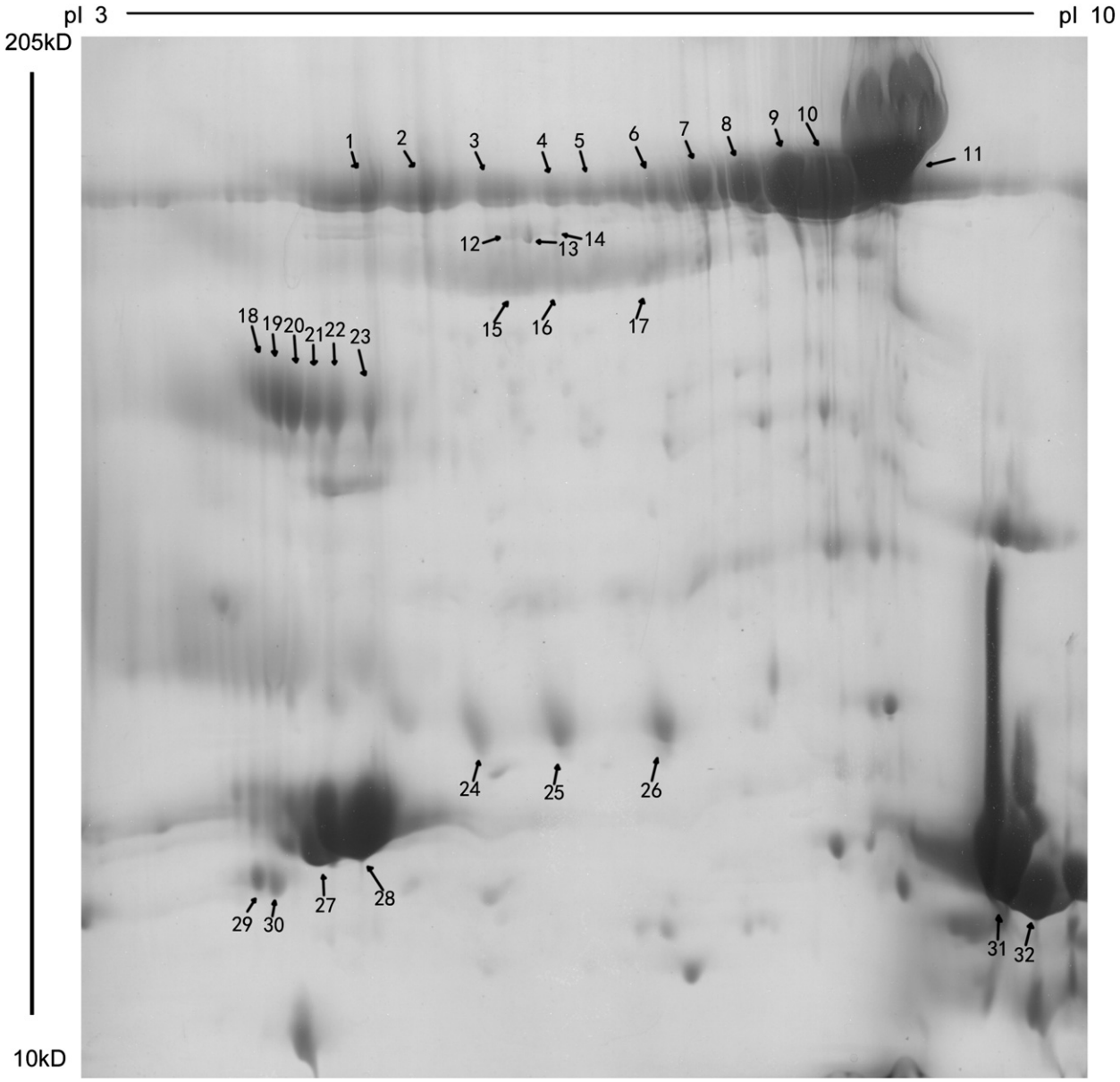


**Fig. 2.** Representative 2-DE gel of human tears from normal subjects. 1500 μg protein was subjected to 2-D electrophoresis, then Coomassie brilliant blue staining. Numbered arrows indicate the high abundance protein spots.

than that from a previous study, in which 6 proteins were identified from 30 spots [29], and the 6 proteins were all identified in our research. However, use of high-resolution MS identified 60 proteins from human tear fluid [19]. The discrepancies could be due to different methods used for preparing the samples. We cut spots on 2-DE gels from tear samples from 3 subjects for MS, whereas the previous study pooled samples from 6 patients and fractioned the pooled sample by high-performance liquid chromatography, then performed

**Table 2**
Identification of proteins in tear fluid from normal subjects.

| Spot no. | Protein name | Gene name | Swiss-prot accession no. | Molecular function |
|---|---|---|---|---|
| 1, 2, 3, 4, 5, 6 | Polymeric immunoglobulin receptor | PIGR | P01833 | Immune response and defense |
| 7, 8, 9, 10, 11 | Lactotransferrin | LTF | P02788 | Anti-bacterial |
| 12, 13, 14 | Serum albumin | ALB | P02768 | Regulate the colloidal osmoti pressure |
| 15, 16, 17 | Ig alpha-2 chain C region | IGHA2 | P01877 | Immune response and defense |
| 16, 17 | Ig alpha-1 chain C region | IGHA1 | P01876 | Immune response and defense |
| 18, 19, 20, 21, 22, 23 | Zinc-alpha-2-glycoprotein | AZGP1 | P25311 | Stimulates lipid degradation |
| 24, 25, 26 | Ig kappa chain C region | IGKC | P01834 | Immune response and defense |
| 25, 26 | Ig lambda chain C regions | LAC | P01842 | Immune response and defense |
| 27, 28 | Lipocalin-1 | LCN1 | P31025 | Bind lipophiles |
| 29, 30 | Cystatin-S | CST4 | P01036 | Cysteine protease inhibitory |
| 29 | Cystatin-SA | CYTT | P09228 | Cysteine protease inhibitory |
| 31, 32 | Lysozyme C | LYZ | P61626 | Anti-bacterial |

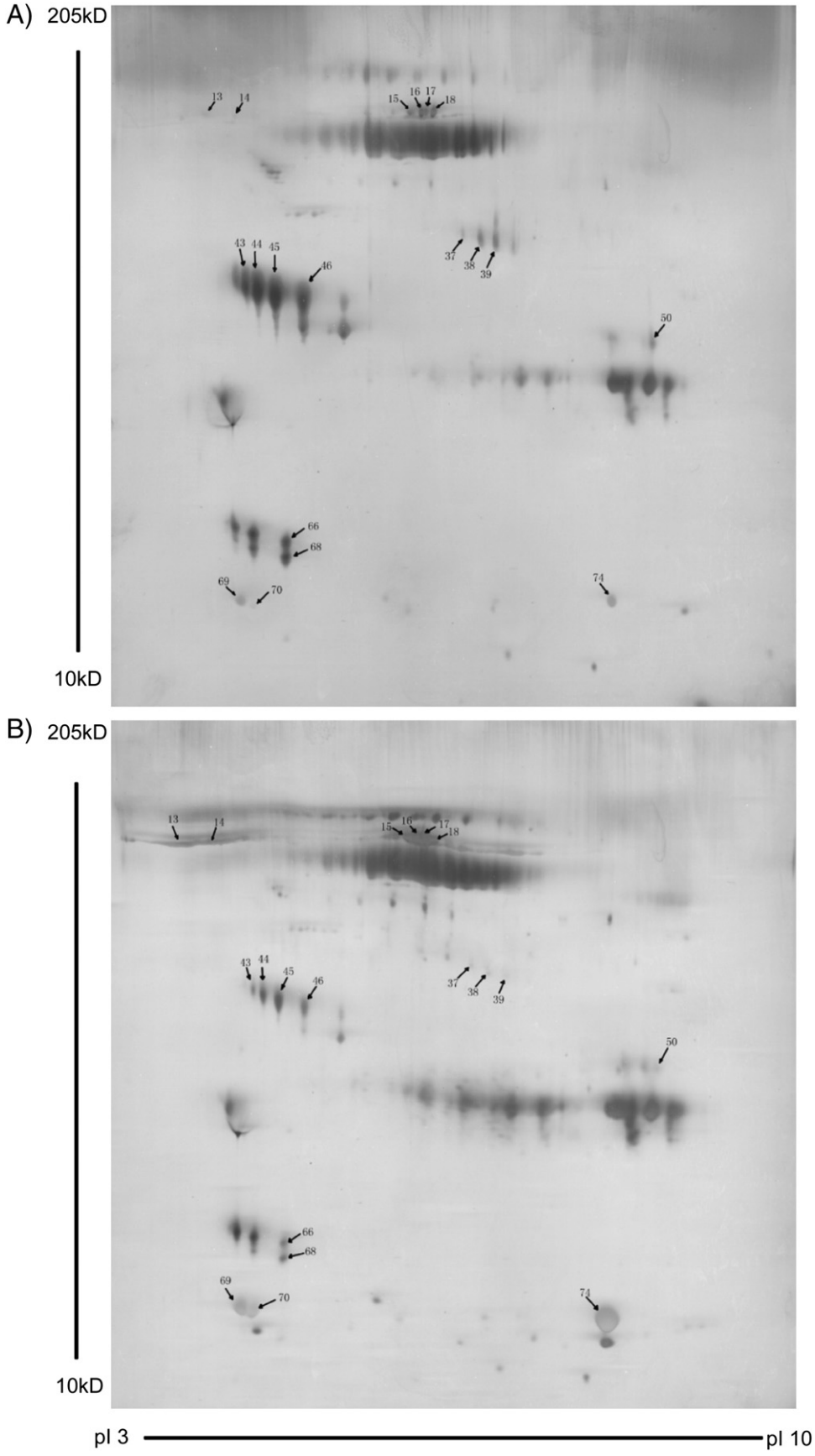


**Fig. 3.** 2-DE profiling (silver staining) of saliva from transplanted (A) and non-transplanted submandibular glands (B) from one patient. Numbered arrows indicate identified protein spots significantly and consistently altered between saliva from transplanted and non-transplanted submandibular glands.

**Table 3**
Identification of proteins differentially expressed in saliva from transplanted submandibular glands to replace lacrimal gland in patients with severe keratoconjunctivitis sicca.

| Spot no. | Protein name | Gene name | Swiss-prot accession no. | Mr | *pI* | Fold variation[a] | Sequence coverage (%) | Matched peptides | Score |
|---|---|---|---|---|---|---|---|---|---|
| 13, 14, 15, 16, 17, 18 | Serum albumin | ALB | P02768 | 71317 | 5.92 | Down 2.1 | 72, 70, 57, 49, 61, 67 | 99, 92, 67, 55, 111, 142 | 3405, 3247, 3180, 2516, 5241, 6239 |
| 37, 38, 39 | Carbonic anhydrase 6 | CA6 | P23280 | 35459 | 6.51 | Up 3.0 | 24, 28, 30 | 22, 24, 27 | 552, 680, 1419 |
| 43, 44, 45, 46 | Short palate, lung and nasal epithelium carcinoma-associated protein 2 | SPLUNC2 | Q96DR5 | 27166 | 5.35 | Up 3.8 | 6, 30, 51, 45 | 9, 37, 68, 52, | 112, 772, 1755, 1137 |
| 50 | Cysteine-rich secretory protein 3 | CRISP3 | P54108 | 28524 | 8.09 | Up 2.3 | 12 | 17 | 292 |
| 66 | Prolactin-inducible protein | PIP | P12273 | 16847 | 8.26 | Up 2.2 | 67 | 69 | 3013 |
| 70 | Cystatin-S | CST4 | P01036 | 16489 | 4.95 | Dowm 2.0 | 72 | 59 | 2013 |
| 74 | Cystatin-SN | CST1 | P01037 | 16579 | 6.82 | Down 2.7 | 34 | 20 | 715 |

[a] The fold variation was measured from the summed intensity of the corresponding spots. Mr: relative molecular weight; *pI*: isoelectric point.

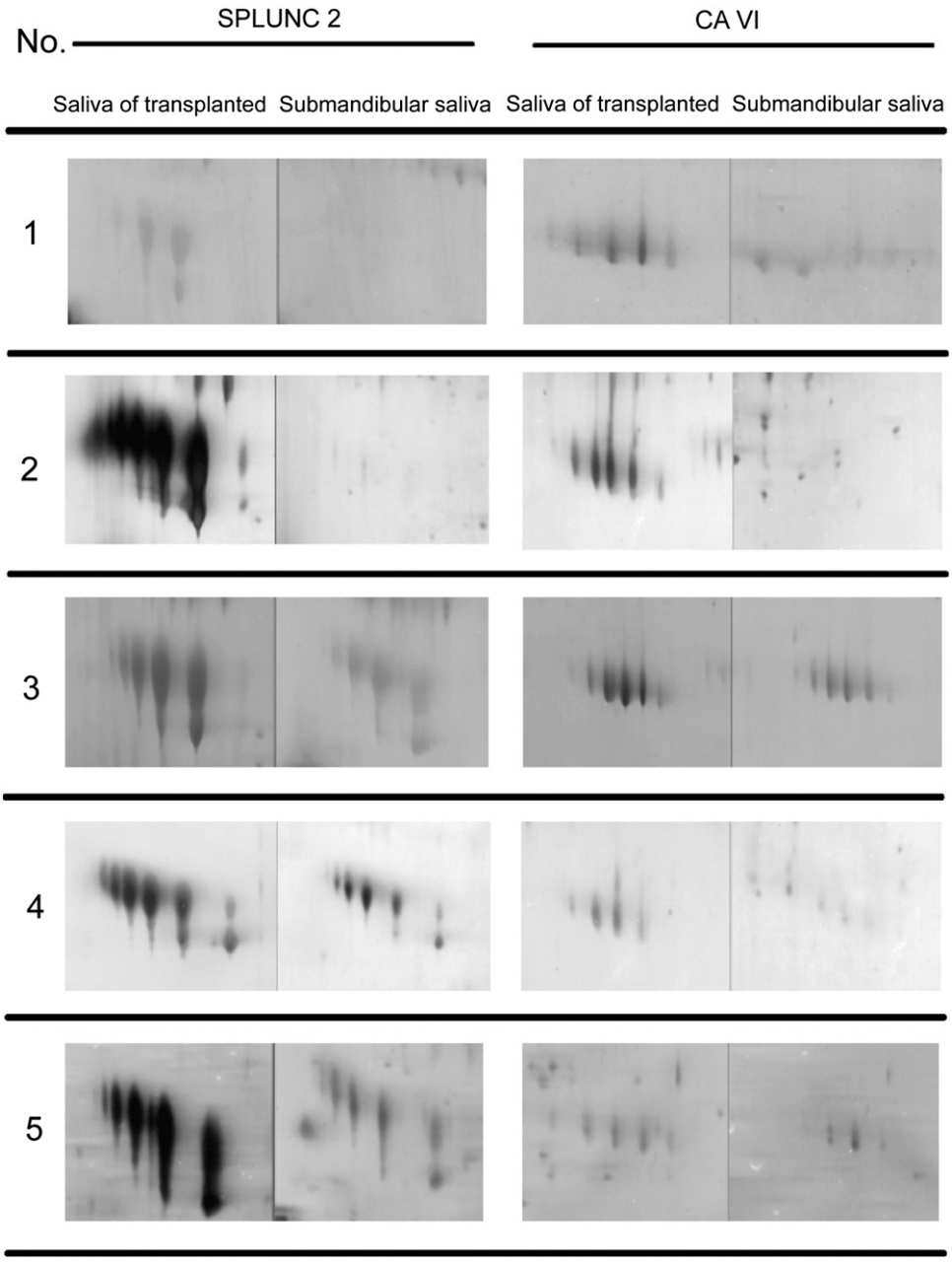


**Fig. 4.** Intensity of spots for SPLUNC 2 and CA VI on 2-DE gels for saliva from transplanted submandibular glands from 5 patients. Enlarged images of silver stained 2-D gels of 5 paired samples of transplanted and non-transplanted submandibular glands from 5 patients show higher intensity of spots for SPLUNC2 and CA VI in saliva from transplanted than non-transplanted submandibular glands.

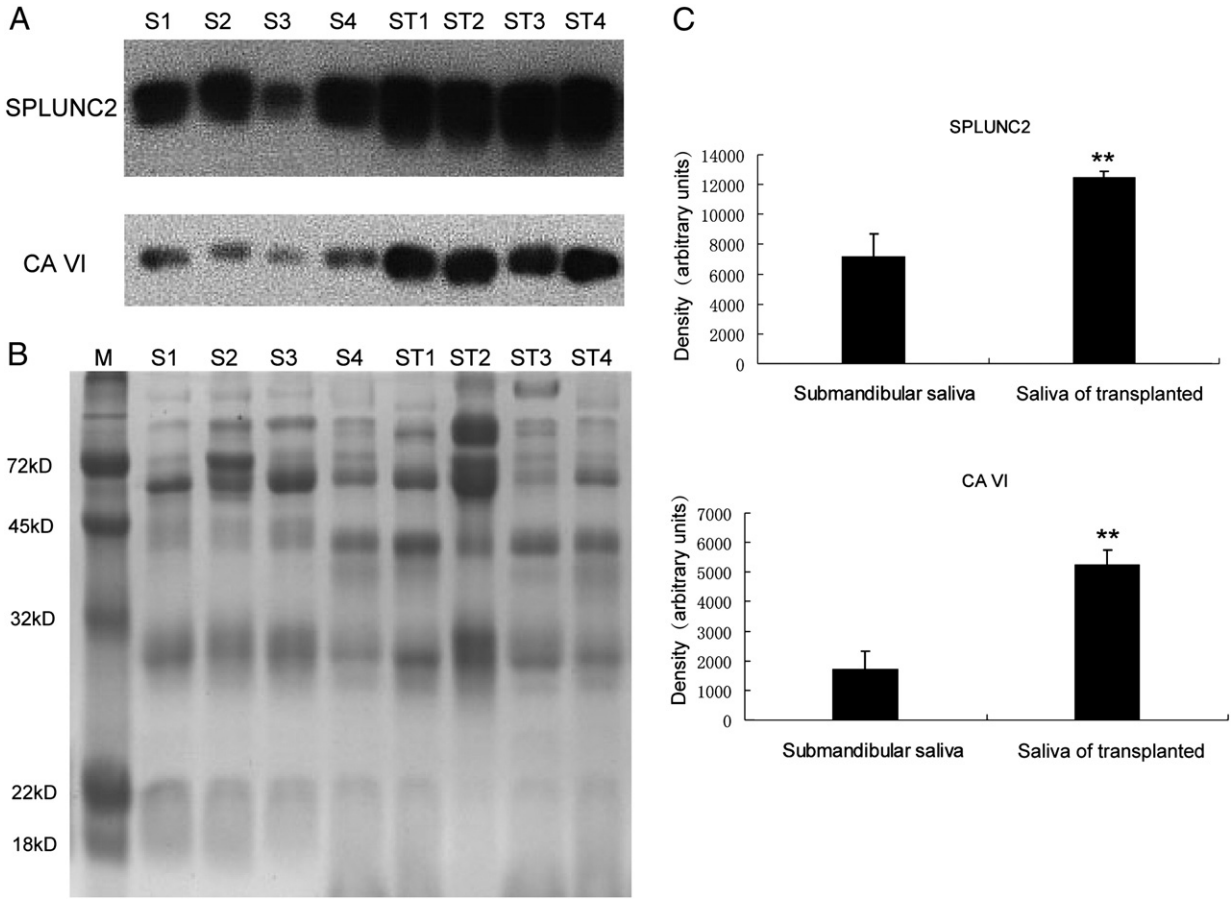


**Fig. 5.** Confirmation of upregulation of CA VI and SPLUNC2 in saliva from transplanted submandibular gland by Western blot analysis. (A) Upregulation of CA VI and SPLUNC2 in saliva from transplanted submandibular glands. We examined 4 paired samples from transplanted and non-transplanted submandibular glands. (B) SDS-PAGE gel stained with Coomassie brilliant blue as an internal control. (C) Quantification of protein expression of SPLUNC 2 and CA VI. Data are mean ± SD. $^{**}P<0.01$. S1–S4: saliva from non-transplanted submandibular glands from patients 1–4. ST1–ST4: saliva from transplanted submandibular glands from patients 1–4. M: marker.

MS for the concentrated fractions. Therefore, the proteins we identified in tear fluid should be abundant enough for sensitivity of Coomassie blue staining, which could identify abundant proteins, including the 11 proteins we identified and also proteins with much less abundance in the eye fluid. However, our results of tear fluid also help in understanding the abundant proteins in the eye fluid and for comparing with that of saliva from transplanted submandibular glands with the same 2-DE-MS method.

Although the protein spot pattern of the saliva of transplanted submandibular gland on 2-D gel was in general similar to that of non-transplanted submandibular glands, 7 proteins showed differential expression in saliva from transplanted glands. SPLUNC2 and CA VI were upregulated in saliva from transplanted submandibular glands, and the upregulation of SPLUNC2 in the transplanted gland tissue was further confirmed by Western blot analysis. Previous studies also showed concentrations of ions changed in saliva from transplanted submandibular glands [8,9]. These differences between the saliva from transplanted and non-transplanted glands reflect that some function of the gland changed after transplantation. The differential expression of proteins in saliva from transplanted glands may be mainly due to the denervation [9] and the changes in the micro-environment of the transplanted gland, we previously found cytoskeletal proteins all upregulated in transplanted glands and the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) increased [30]. The differential expression may be also due to the transplanted gland receiving different signals from surrounding tissue. Reinnervation of the transplanted gland has been reported [31], and later reinnervation may have different control over the transplanted gland as compared with the original innervation. However, the differential expression may not have been due to the mixture of residual tears from the eyes because most of the upregulated proteins (SPLUNC2, CA VI, cysteine-rich secretory protein 3; Tables 2 and 3) were not identified in tear fluid. Further study is needed to answer when the differential expression occurs in saliva from transplanted gland.

Although the mechanism underlying the upregulation of SPLUNC2 and CA VI in saliva from transplanted submandibular gland is not clear, the upregulation of SPLUNC2 and CA VI may be favorable to eyes. SPLUNC2, also known as parotid secretory protein, is a major saliva protein [32,33]. A number of studies suggest that SPLUNC2 may play a role in antimicrobial infection [32,34,35]. SPLUNC2 has a structure similar to bactericidal/permeability-increasing protein (BPI) [32,34,35], an important member of the innate immune system. SPLUNC2 can also inhibit the growth of *Pseudomonas aeruginosa in vitro*, serving as a BPI-like antibacterial protein [36]. Moreover, peptides derived from SPLUNC2 can directly inhibit the binding of lipopolysaccharide (LPS) to LPS-binding protein and decrease the release of inflammatory factors induced by LPS [37]. Therefore, upregulation of SPLUNC2 in saliva from transplanted submandibular gland could provide anti-bacterial protection for the ocular structures of the eyes. CA VI is one of the carbonic anhydrases that can catalyze the hydration of $CO_2$. Although no study shows CA VI in the human lacrimal gland, it exists in the lacrimal gland of goats [38]. CA VI could inhibit the decrease in pH in dental plaque caused by decomposition of sugar [39]. Therefore, CA VI may be related to maintaining pH of secretions [40]. Interestingly, levels of salivary CA VI were found lower in women than in men [41] and were decreased in patients with Sjögren's syndrome but increased in type-2 diabetes patients [40]. The expression of SPLUNC2 in human gingival keratinocytes can be

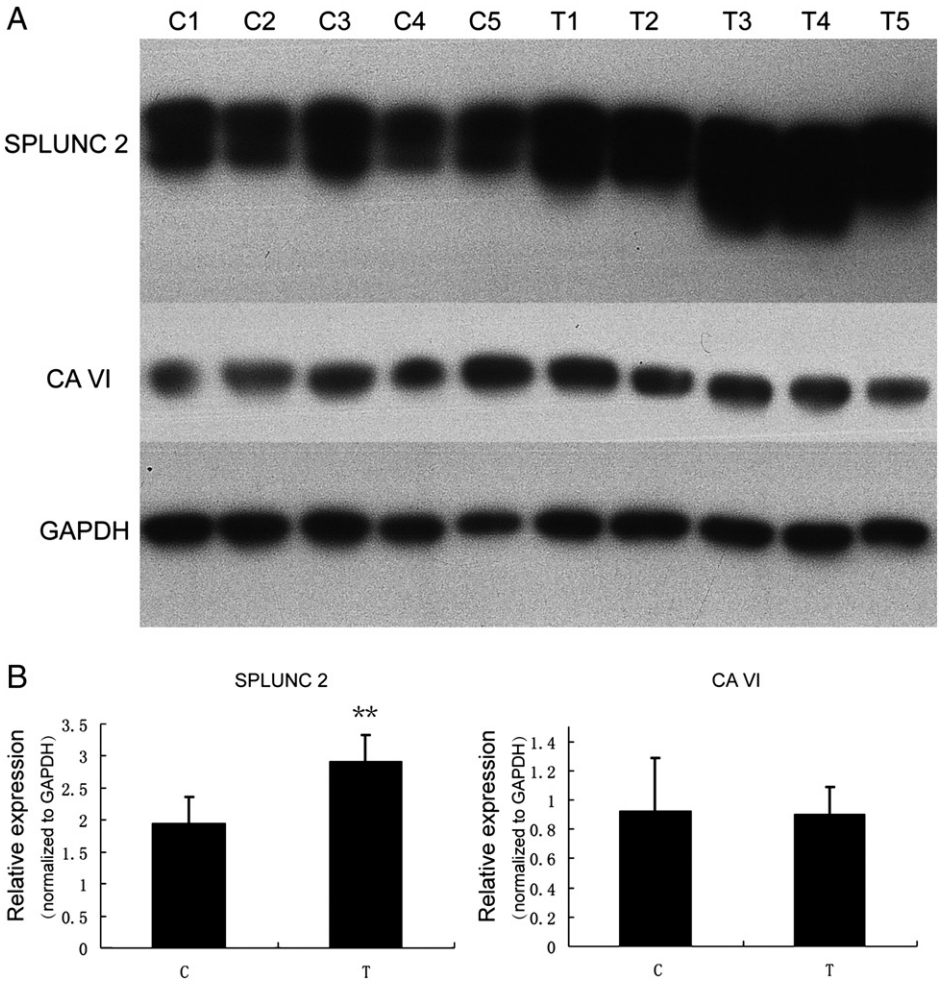


**Fig. 6.** Upregulation of SPLUNC2 but not CA VI in tissue from transplanted submandibular glands. (A) Western blot analysis of protein expression of SPLUNC 2 and CA VI in tissue from transplanted and normal submandibular glands. GAPDH was used as the internal reference. C1–C5: tissue from normal submandibular glands from the 5 patients collected during surgery for partial glandular transplantation of submandibular gland served as a control. T1–T5: tissue from transplanted submandibular glands from the 5 patients collected during surgery for reducing the size of transplanted submandibular glands 1 year after the transplantation. (B) Quantification of protein expression of SPLUNC 2 and CA VI. The levels of SPLUNC 2 or CA VI were normalized to that of the GAPDH. Data are mean ± SD. $^{**}P<0.01$.

upregulated by bacteria and humoral factors [42]. It indicated that SPLUNC2 and CA VI may be potential biomarkers for many diseases. Because the upregulation of SPLUNC2 and CA VI in saliva from transplanted submandibular glands was significant and constant, they could be used as biomarkers to monitor the activity of transplanted submandibular glands.

Because Schirmer test values for all our patients were <1 mm, the lacrimal glands and accessory lacrimal glands of the eyes would hardly secrete tear fluid, although we could not rule out that the residual lacrimal glands and the accessory lacrimal glands would not secrete some eye fluid. Therefore, the samples collected from the eyes of patients after transplantation should be mainly from the transplanted submandibular glands. Even if there was residual eye fluid, it had no influence on the 2-DE gel analysis because the protein spot pattern of saliva from transplanted submandibular gland was similar to that from non-transplanted submandibular gland. For collection of samples from non-transplanted submandibular gland, it was difficult to prevent samples from mixing with saliva of the sublingual glands because the ducts of submandibular and sublingual glands open into the oral cavity through the sublingual caruncle. However, most of the unstimulated saliva collected from the submandibular gland should still be mainly from the submandibular gland: previous study showed that 90% of the submandibular/sublingual saliva collected in the resting state is secreted from the submandibular gland [43]. However, to improve the comparability of samples and to ensure that secretions were newly secreted, all samples were collected with the first several drops of the secretion discarded. Results for our samples showed good repeatability and comparability. In addition, due to the limitation that the number of our samples was low, our proteomic results of the saliva of transplanted submandibular gland were still preliminary.

In summary, our results of proteomic analysis showed that saliva from transplanted submandibular gland contained almost all the abundant proteins of tear fluid and that most of the identified proteins in the saliva from transplanted submandibular gland could be favorable to the eyes. The saliva from transplanted submandibular gland was in general similar to that of normal submandibular gland, with some proteins differentially expressed. These findings can help in understanding the functional status of transplanted submandibular glands replacing the lacrimal gland in patients with severe KCS.

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