Ocular Mucin Gene Expression Levels as Biomarkers for the Diagnosis of Dry Eye Syndrome

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Purpose. To evaluate mRNA levels of the ocular mucins *MUC1*, *MUC2*, *MUC4*, *MUC5AC*, and *MUC7* in conjunctival impression cytology samples from patients with moderate to severe dry eye syndrome (DES) compared with a population of healthy subjects; and to investigate the use of the levels of these mucin genes as biomarkers of DES and subsequently as a potential diagnostic test for DES.

Methods. This prospective study commenced in the year 2000 and ended in the year 2009. Thirty-eight patients with DES and 43 age- and sex-matched healthy subjects completed the initial part of the study. Investigations were repeated at a later stage in 16 healthy subjects and 30 patients with DES, which were used as external validation data. Conjunctival impression cytology was performed in all subjects to test gene expression of ocular mucin genes *MUC1*, *MUC2*, *MUC4*, *MUC5AC*, and *MUC7*. Statistical analysis was performed to determine whether there was a difference in the levels of mucin gene expression between the two groups of subjects. Sensitivity and specificity of mucin gene expression for the diagnosis of DES was calculated.

Results. Expressions of *MUC1*, *MUC2*, *MUC4*, and *MUC5AC* ($P < 0.0001$) were significantly lower in conjunctival epithelium of patients with DES compared with that in normal subjects. These results were replicated in the external control subject and patient groups. *MUC1* expression levels demonstrated the greatest sensitivity (83.3%) and specificity (87.5%) among all genes tested.

Conclusions. The data strongly suggest that the expression levels of *MUC1* may be used as a diagnostic test in DES for investigational and selective clinical trials. (*Invest Ophthalmol Vis Sci.* 2011;52:8363–8369) DOI:10.1167/iovs.11-7655

Dry eye syndrome (DES) has recently been redefined as "a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface. Dry eye is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface."[1] It is a disease frequently observed among ophthalmic patients, prevailing in approximately 7–25% of the population.[2–5] Based on severity, DES can cause symptoms from simple ocular irritation to blindness.[6] Although there are several clinical tests available for the diagnosis of DES, no single test can accurately differentiate a patient with DES from a healthy subject, and thus a battery of tests is routinely used.[6–8] One recent study in the Spanish population (similar to the participants of this present study) also demonstrated no correlation between subjective dry eye questionnaires and objective clinical testing.[9] For this reason, investigative studies or clinical trials performed on DES often resort to using multiple tests for the diagnosis of the DES study group, which increase the time course of patient visits during the study period, preventing subjects from having a uniform evaluation endpoint.[6,10,11]

Mucins are high molecular weight proteins, with tandem repetitions in the central position of the protein.[12] The ocular surface expresses at least 9 of the 18 human mucin genes: *MUC1*, *MUC2*, *MUC4*, *MUC5AC*, *MUC7*, *MUC13*, *MUC15*, *MUC16*, and *MUC17*.[13–24] Mucins keep the ocular surface wet and protected from adverse environmental conditions. Based on their amino acid sequences, mucins are categorized in three distinct families: gel forming (MUC2, MUC5AC, MUC5B, MUC6, and MUC19), soluble (MUC7 and MUC9), and transmembrane (MUC1, MUC3A, MUC3B, MUC4, MUC12, MUC13, MUC15, MUC16, MUC17, MUC20, and MUC21); other mucins remain unclassified (MUC8 and MUC11).

There is some evidence that DES leads to an alteration in ocular mucins.[23,25–33] A previous study showed that the mucin *MUC5AC* transcripts in the conjunctival epithelium of patients with DES associated with Sjögren syndrome were significantly lower than those in normal individuals, and no significant changes in the expression of *MUC1* and *MUC4*.[23] One recent study showed that patients with Sjögren's syndrome dry eye expressed greater amounts of soluble MUC-1 protein and mRNA in the tear fluid and conjunctiva compared with patients with non-Sjögren's dry eye and healthy controls.[29] They also demonstrated that MUC4 correlated well with clinical findings such as lid-parallel conjunctival folds and lid-wiper epitheliopathy.[29] In a separate study, Caffery et al.[30] also noted increased mRNA and protein levels of MUC16 in conjunctival and tear fluid samples. Berry et al.[31] demonstrated that MUC5AC levels were significantly decreased in patients with DES. One recent

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study reported that the frequency of patients with non-Sjögren's aqueous-deficient dry eye, expressing only the *MUC1/A* splice variant of the mucin *MUC1* in conjunctival samples, was lower than that of a normal control group.[32] Furthermore, another study from the same group[33] showed genotypic differences in *MUC-1* expression between DES and healthy patients that could promote the ocular surface damage observed in patients. *MUC-1* expression has also shown to be variable in diseased human corneas compared with that of healthy corneas.[34] MUC16 and MUC5AC were correlated with corneal staining levels.[31] The above-mentioned previous studies suggest that mucin expression levels not only correlate well with DES clinical findings but can also be an important distinguishing feature between patients with DES and healthy subjects.

Conjunctival impression cytology (CIC) is a well-established, noninvasive method to obtain epithelial cells from the ocular surface in normal and pathologic conditions.[13,17,21–24,35–46] The collected cells may be used in many different ways, including the detection of mRNA levels of ocular mucin genes in healthy individuals.[13,17,21–24,35]

This study was designed to evaluate mRNA levels of the ocular mucins *MUC1*, *MUC2*, *MUC4*, *MUC5AC*, and *MUC7* in CIC samples from patients with moderate to severe tear-deficient DES compared with those from a population of healthy subjects. The data from this study will identify the potential use of mucin gene levels as important biomarkers for the diagnosis of DES.

Methods

Selection of Patients and Normal Donors

Informed consent, based on the tenets of the Declaration of Helsinki and Ethics Committee approval, was obtained from all participants before commencement of the investigations. Adult patients were eligible for participation if they presented with a diagnosis of moderate to severe DES nonassociated with Sjögren's syndrome, with documented signs and symptoms in the worse eye (see the following text) despite conventional management, which may include artificial tears, gels, and ointments, defined by the following criteria:

1. Sum of corneal and nasal and temporal rose bengal conjunctival staining, using the Oxford Scheme[47] of $\geq +5$ in the same eye where fluorescein corneal staining was $\geq +2$ in the worse eye.
2. Schirmer's tear tests with topical anesthesia >0 mm but <5 mm/5 minutes in the worse eye.
3. The presence of at least one symptom of discomfort: sensitivity to light, dryness, sandy or gritty feeling, burning/stinging, pain, itching, and blurred vision.

Patients were excluded in they had a previous diagnosis of Sjögren's syndrome. In the absence of this diagnosis, patients with symptoms of dry mouth were also excluded. Because blood alterations typical of Sjögren's syndrome are not ordered unless a patient with dry eye complains of concomitant dry mouth, we did not order blood work, preferring to exclude that potential candidate. Patients were excluded who, in the previous 6 months, had systemic diseases (other than hypertension or hypercholesterolemia) and were using systemic medications (other than those to treat the two mentioned conditions). Patients who had any ocular surgery or any recurrent or chronic ocular disease (other than dry eye) or patients who had ever worn contact lenses were also excluded. Allergies of any kind constituted other exclusion criteria; artificial tears and lubricants were the only ocular medications allowed.

Specimens were obtained from only one eye and always from the worse eye of patients who met the entry criteria and qualified for randomization. The worse eye was defined as the one showing the highest degree of corneal staining, or the lowest Schirmer's test result when both eyes had the same corneal scores. If the two criteria were equal in both eyes, the right eye was chosen for CIC.

Healthy donors were excluded from the study if they had ever used or were presently using contact lenses, had any ocular or general disease, or used any ocular or systemic medication for the last 6 months that could affect mucin gene expression, including contraceptive agents.

Three groups of patients were studied:

Group 1 (G1): 38 DES (range: 28 to 82 years) patients and 43 (range: 20 to 84 years) age- and sex-matched healthy donors. These samples were obtained and analyzed in the year 2000.

Group 2 (G2): 16 healthy myopic subjects (range: 18 to 35 years), as previously defined. These samples were obtained and analyzed in the year 2002.

Group 3 (G3): 30 patients with DES (range: 38 to 78 years). These samples were obtained and analyzed in the year 2006.

Conjunctival Epithelial Cell Collection by CIC

CIC specimens were collected as previously described, between 1 to 2 hours after diagnostic tests had been performed, including instillation of diagnostic vital dyes.[21,22,35] After the instillation of topical anesthetic drops, two halves of one polyethersulfone filter (Supor 200, pore size 0.20 μm, 13 mm in diameter; Gelman Laboratory, Ann Arbor, MI) were applied to the superior and superotemporal bulbar conjunctiva of one eye (the above defined as "worse eye") of each individual. This area is where CIC samples are routinely taken in our clinic as an aid to diagnosis (i.e., degree of squamous metaplasia and goblet cell density), offering enough space for the two needed samples and avoiding the superior tarsal conjunctiva, where more intraepithelial lymphocytes reside in normal conditions, which could mask results. Routinely, this area never stained positive for either fluorescein or rose bengal stainings before sampling, which is typically the case in patients with DES. The filters were applied with the subject looking far down and as close as possible to the superior fornix and, therefore, approximately 4 to 5 mm form the limbal area. Filters were kept in place for 10 seconds before careful removal. The filters were then suspended in 1 mL lysis buffer (Buffer RLT; Qiagen, Hilden, Germany) containing 1% 2-mercaptoethanol (Merck KGaA, Darmstadt, Germany) and kept at -80°C for total RNA isolation.

RNA Isolation and Reverse Transcription

Total RNA was extracted using a commercial kit (RNeasy Mini Kit; Qiagen) under standard conditions and treated with RNase-free DNase following the manufacturer's instructions.[19,21,35] Agarose gel electrophoresis and ethidium bromide staining checked the integrity and size distribution of the purified RNA. The first strand of cDNA was synthesized from the total RNA with random hexamer (M-MuLV Reverse Transcriptase Ready-To-Go You-Prime First-Strand Beads; GE Healthcare Europe GmbH [formerly Amersham Biosciences], Munich, Germany).

Real-Time Polymerase Chain Reaction

Real-time PCR was performed using a dye detection kit (SYBR Green PCR Master Mix, Applied Biosystems, Foster, CA) and a commercial system (GeneAmp 5700 Sequence Detection System; Applied Biosystems) according to the manufacturer's recommendations.[21,22,35] Assays were performed in duplicate. A nontemplate control and total RNA without retrotranscription were included in all the experiments to evaluate PCR and DNA contamination of the reagent used. We designed primers for amplification of the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene and the nontandem repeat region MUC genes used in this study (Table 1) from GenBank sequences (GenBank is provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD, and can be found at http://www.ncbi.nlm.nih.gov/GenBank), using software available on the World Wide Web.[48]

TABLE 1. Relative Expression Levels of Mucin *(MUC)* mRNAs in Healthy Donors and Dry Eye Syndrome (DES) Patients

	Mean ± SEM		
Mucin Level	**Healthy Donors**	**DES Patients**	***P*** **Value**
MUC1	0.650 ± 0.036	3.864 ± 0.077	<0.0001
MUC2	2.561 ± 0.200	4.943 ± 0.260	<0.0001
MUC4	0.654 ± 0.067	2.318 ± 0.144	<0.0001
MUC5AC	1.142 ± 0.090	1.964 ± 0.144	<0.0001
MUC7	1.269 ± 0.104	1.534 ± 0.206	0.2393

Mucin levels are shown using a negative logarithmic transformation [−log *MUC/GAPDH*)]. Thus, there is an inverse relationship between the expression of each *MUC* gene and the value that represents it; e.g., low levels of *MUC* gene expression have relatively high values, and high levels of *MUC* gene expression have relatively low values. Consequently, as the ratio increased, the −log (*MUC/GAPDH*) value decreased. The *P* values were calculated using the Student's *t*-test for independent samples.

The *GAPDH* gene was used as an endogenous reference for each reaction to correct for differences in the amount of total RNA added. To verify the validity of using the *GAPDH* gene as an internal standard control, the efficiencies of the *MUC* genes and *GAPDH* amplifications were compared. The PCR products were quantified by comparing the test samples with standard curves made with samples of known quantities. To verify the identity of the mucin gene amplification product, all samples were tested for correct amplification by dissociation curves that were specific for each fragment analyzed by their melting temperatures and they were sequenced and compared against nucleotide databases by a publicly available program (BLASTn is provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD, and is available at http://www.ncbi.nlm.nih.gov/blast).

Mucin Level Measurements

The final amounts of PCR products were expressed as the ratio of amplification for each mucin gene to that of the housekeeping gene GAPDH (MUC/GAPDH). This accounted for any differences in the initial amounts of RNA. The data were analyzed using a negative logarithmic transformation [−log (*MUC/GAPDH*)] to obtain a distribution closer to the normal. The mucin gene and *GAPDH* gene transcript levels were expressed as the mean ± SEM of the transformed values. Thus, there is an inverse relationship between the expression of each *MUC* gene and the value that represents it (e.g., low levels of *MUC* gene expression have relatively high values; high levels of *MUC* gene expression have relatively low values). Consequently, as the ratio increased, the −log (*MUC/GAPDH*) value decreased. The *P* values between healthy donors and DES samples were calculated using the Student's *t*-test for independent samples. For clarification purposes, these data are presented in Figure 1 as (*MUC1/GAPDH*) values on the *y*-axis, which means low levels of MUC gene expression will have low values and vice versa.

Reference Intervals

Reference intervals were determined by fractiles from the 43 healthy donor samples of G1, following the recommendations of the International Federation of Clinical Chemistry.[49] The central 0.95 interfractile interval was estimated by parametric statistical methods as the 0.025 and 0.975 fractiles. Parametric estimations are theoretically more precise with smaller samples sizes, provided the assumption about distribution type is valid. Our data were normally distributed after the negative logarithmic transformation. Parametric 90% confidence intervals (CIs) for the estimated fractiles were calculated.

To validate the reference intervals we have used two methods. First, we classified each observed value as "usual" if between or equal to either of the reference limits, or "unusual" if otherwise. Then, we evaluated the relationship between the classification class and the observed group, healthy subjects or patients with DES, by bivariate cross-tabulation. Statistical significance was estimated by the χ^2 test or Fisher's exact test (if the χ^2 test could not be applied). Associations were quantified as the odds ratio (OR) and 95% CI. The second tool used was the atypicality index,[50] a measurement that determines the probability that an observed value does not belong to the reference population.

Classification Models

A stepwise logistic regression procedure was used to identify mucin levels significantly associated with diagnosis of DES. Three classification models (M) were constructed using the mucin levels in the G1 data set as explanatory variables: (M1) *MUC1*, (M2) *MUC4*, and (M3) *MUC2* + *MUC5AC*. *MUC7* was not selected by any of the models and thus *MUC7* results have not been shown. The three models were internally validated by a cross-validation procedure. Sensitivity (SE) and specificity (SP) were calculated to evaluate their predictive capability. The models were also externally validated using two new data sets (G2 and G3), which were different from those used to fit the classification models. Receiver operating characteristic (ROC) curves were constructed as a measure of assessing discrimination.[51] The curves were compared by computing the areas under the ROC curves (AUC).[52] The partial AUC (pAUC)[53] was also calculated to compare the performance of the three models, ensuring a minimum acceptable specificity. We fitted 80%, which means that at least 80% of healthy subjects will be correctly classified.

RESULTS

Reference Intervals

All conjunctival samples offered adequate material to proceed with the planned assays. *MUC1*, *MUC2*, *MUC4*, *MUC5AC*, and *MUC7* mucin transcripts were detected in all healthy donor samples of G1 (n = 43) and in the most of the patients with DES (n = 38). The relative quantitation of mucin gene expression could be performed because the slope of the standard curve for each gene showed similar efficiencies of amplification. The data had a normal distribution after the logarithmic transformation. The negative logarithmic values for normal

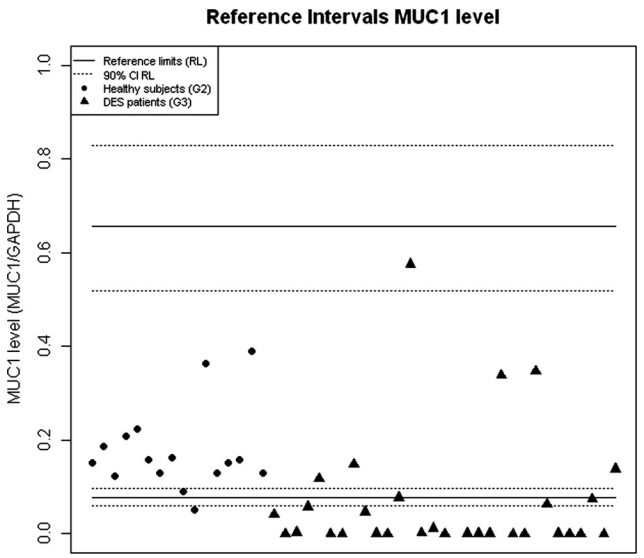

FIGURE 1. Reference intervals for *MUC1* in DES patients of group 3 (G3) and healthy subjects of group 2 (G2). *MUC1* levels demonstrated significant direct association with unusual values occurring more frequently in patients with DES.

Table 2. 95% Reference Intervals for Each Ocular *MUC* Level Accompanied and 90% Confidence Intervals (CI) around Each Reference Limit

Mucin Level	Inferior Reference Limit		Superior Reference Limit	
	Value	90% CI	Value	90% CI
MUC1	0.1832	0.0811 to 0.2853	1.1174	1.0153 to 1.2195
MUC2	−0.0046	−0.5654 to 0.5563	5.1256	4.5647 to 5.6864
MUC4	−0.2016	−0.3887 to −0.0145	1.5098	1.3227 to 1.6969
MUC5AC	−0.0143	−0.2670 to 0.2384	2.2977	2.0450 to 2.5504
MUC7	−0.0698	−0.3625 to 0.2229	2.6076	2.314 to 2.9003

mucin gene expression ranged from 0.65 (*MUC1* and *MUC4*) to 2.56 (*MUC2*) relative to *GAPDH* expression. The negative logarithmic values for patient samples ranged from 1.5 (*MUC7*) to 4.9 (*MUC2*). Expressions of *MUC1*, *MUC2*, *MUC4*, and *MUC5AC* were significantly lower in the DES samples (Table 1) than those in healthy donor samples. We used this data set as a reference sample to determine reference intervals. The average age in this group of 43 subjects was 61.14 ± 2.31 years (range: 20–84 years). In this group, 44.3% (19/43) were females with a mean age of 60.63 ± 3.80 years (range: 26–84 years) years. Although the mean age of the males was slightly higher (61.54 ± 2.91; range: 20 to 79 years), it was not statistically significant (Student's *t*-test; *P* value = 0.8475).

The 95% reference interval for each mucin level and the 90% CIs around each reference limit are given in Table 2. To validate the reference intervals we considered two data sets of observed values, G2 and G3. These data were collected following the same procedures for the reference sample. Table 3 shows the classification of observed values into one of two reference interval classes. There was a statistically significant inverse relationship for *MUC2* (OR 0.0805; 95% CI 0.0072, 0.8949) but not for *MUC5AC* levels (χ^2 *P* value = 0.2699). *MUC2*, *MUC5AC*, and *MUC7* levels demonstrated an inverse relationship, with more frequent unusual values occurring in the healthy subjects (OR < 1). *MUC1* levels had the most significant direct association (OR 49.2857; 95% CI 5.4942, 442.1152), with more frequent unusual values occurring in the patients with DES (Fig. 1). The summary of the atypicality index for each mucin level is shown in Table 4. In general, the index values were larger and higher for mucin levels in patients with DES. The lower value was the *MUC1* level in the healthy subject sample: an approximately 60% chance that the observed profile came from the reference population. The higher value was *MUC1* in the DES subject sample: an approximately 93% chance that the observer profile did not come from the reference population.

Table 4. Atypicality Index for Each Mucin Level

Mucin Level	*n*	Mean	SEM
G1, healthy subjects			
MUC1	16	0.4402	0.0652
MUC2	10	0.7787	0.0578
MUC4	16	0.7655	0.0696
MUC5A	16	0.8852	0.0383
MUC7	16	0.8052	0.0514
G3, DES patients			
MUC1	30	0.9245	0.0278
MUC2	30	0.6289	0.0484
MUC4	30	0.9188	0.0440
MUC5AC	30	0.9023	0.0406
MUC7	30	0.8756	0.0426

Atypicality index values were generally larger and higher for mucin levels in patients with DES. G1 consisted of CIC samples from 43 healthy patients (20–84 years old). G3 consisted of CIC samples from 30 patients with DES (38–78 years old).

Classification Models

The three classification models constructed using the mucin levels in the G1 data set as explanatory variables had SE and SP values > 80%: (M1: *MUC1*) SE = SP = 100%; (M2: *MUC4*) SE = 95.10%, SP = 95.30; and (M3: *MUC2* + *MUC5AC*) SE = 83.70%, SP = 91.10%.

Additional model validation was performed with the G2 and G3 data sets. The results of this validation are given in Table 5. The AUC was not statistically different from 0.5 in M3 (AUC = 0.640; *P* value = 0.190). In addition, pAUC with an 80% to SP was zero. M1 was the best classificatory model (Fig. 2). Al-

Table 3. Classification of Observed Values into One of Two Reference Interval Classes

Mucin Level	Observed Values			Odds Ratio	
	G2, Healthy Subjects	G3, DES Patients	*P* Value*	Value	95% CI
MUC1					
Unusual	1	23	<0.0001	49.2857	5.4942 to 442.1152
Usual	15	7		1	-
MUC2					
Unusual	3	1	0.0417†	0.0805	0.0072 to 0.8949
Usual	7	29		1	-
MUC4					
Unusual	7	25	0.0084†	6.4286	1.6213 to 25.4896
Usual	9	5		1	-
MUC5AC					
Unusual	8	10	0.2699	0.5000	0.1448 to 1.7271
Usual	8	20		1	-
MUC7					
Unusual	12	9	0.0035	0.1429	0.0361 to 0.5649
Usual	4	21		1	-

* Bivariate cross-tabulation analysis. Chi-squared *P* value or Fisher's exact *P* value (†) G2 consisted of conjunctival impression cytology (CIC) samples from 16 healthy subjects (18–35 years old); G3 consisted of CIC samples from 30 DES patients (38–78 years old).

TABLE 5. External Validation of the Data Performed with Two Different Patient Data Sets

Model	AUC	pAUC (80%)	*P* Value H_0: AUC = 0.5	SE (95% CI) (%)	SP (95% CI) (%)
M1: *MUC1*	0.871	0.1542	<0.001	83.3 (66.4 to 92.7)	87.5 (64.0 to 96.5)
M2: *MUC4*	0.698	0.1225	0.028	63.3 (45.5 to 78.1)	93.8 (71.7 to 98.9)
M3: *MUC2* + *MUC5AC*	0.640	0.0000	0.190	76.7 (59.1 to 88.2)	70.0 (39.7 to 89.2)

Additional model validation was performed with G2 (CIC samples from 16 healthy subjects [18–35 years old]) and G3 (CIC samples from 30 DES patients [38–78 years old]) data sets. *MUC1* data constituted the best model to classify DES versus healthy patients. AUC, area under the curve; pAUC, partial area under the curve; SE, sensitivity; SP, specificity; M1, M2, M3, models 1, 2, and 3.

though it seemed to be slightly worse than M2 to classify healthy subjects (M1 SP = 87.5% vs. M2 SP = 93.8%), this difference was not statistically significant (M1 95% CI SP = 64%, 96.5% vs. M2 95% CI SP = 71.7%, 98.9%).

DISCUSSION

Our study has clearly demonstrated that levels of *MUC1* expression in superior bulbar conjunctival cells are significantly lower in patients with aqueous-deficient DES compared with that in healthy patients. We have also shown that expressions of *MUC2*, *MUC4*, and *MUC5AC* are lower in these patients with DES. The implications of these results are discussed in the following text.

As mentioned earlier, DES is a common clinical condition causing ocular irritation that is diagnosed and treated by eye-care practitioners around the world. The common nature of prevalence of DES in the population, lack of objective and subjective correlation, and the lack of a "cure" has prompted several basic-science and clinical investigations into the nature of this disease. Clinical dry eye tests face problems such as varying sensitivities and specificities, lack of consensus on cutoff values, poor reproducibility, and poor correlation with subjective signs.[6,8,54] This is similar to other diseases with tests of self-perception such as cataract[55] and benign prostate hypertrophy,[56] where the symptoms experienced by the patient demonstrated a low correlation to the objective findings. The goal of the present study was to investigate if studying gene expression levels of certain ocular surface mucins will help diagnose patients with dry eye more accurately. Mucins play a major role in maintaining ocular surface health.[57] The clinical relevance of mucins is evident from the facts that the expressions of mucins *MUC1, MUC5A, MUC16*, and *MUC19* are altered in evaporative DES, contact lens wearers and ocular allergy, and non-Sjögren's DES and autoimmune DES, respectively.[23,28–31,58]

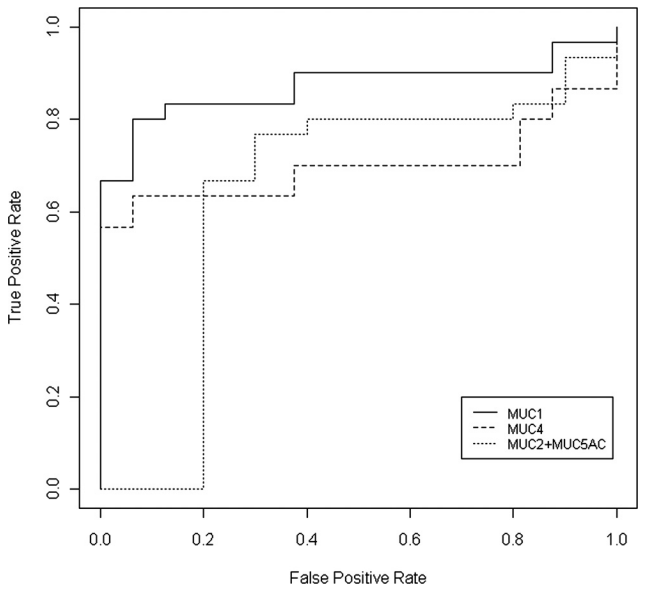

FIGURE 2. Receiver operating curve analysis. The MUC1 model (M1) was the best to distinguish patients with DES from healthy subjects. The MUC4 model (M2) was better at classifying healthy subjects alone.

Interestingly, a recent study found an increase of soluble MUC1 in Sjögren's DES and non-Sjögren's DES compared with that in healthy patients.[29] They showed *MUC1* mRNA expression to be similar in non-Sjögren's DES and healthy patients, whereas membrane-bound *MUC1* expression was different only between the Sjögren's DES and healthy patients.[29] Our study found decreased conjunctival *MUC1* mRNA expression in non-Sjögren's DES compared with that in healthy subjects. The participants with DES in our study are comparable (with respect to the disease) to the patients with non-Sjögren's DES in the earlier study.[29] The difference in results for *MUC1* expression between our study and the previous study[29] could be attributed to several factors. The previous study[29] pooled the conjunctival impression cytology samples from different patients, whereas we analyzed individual samples on each patient. Pooling impression cytology samples may mask the variability between individual patients. Some recent studies[32,33] suggest conjunctival *MUC1* polymorphism, in the variable number of tandem repeats (VNTR) region, which is the region that elaborates the soluble form of MUC1. Patients with dry eye syndrome demonstrate different genotypes in this region compared with that in healthy patients. In our present study, we designed our probes to be far away from the VNTR region to amplify the transmembrane part of conjunctival *MUC1*. A different primer between our present study and that in the previous study[29] can be one factor in the difference in results. In our present study, experiments were performed at two different periods of time (years 2000 and 2009), demonstrating that our results are consistent and repeatable over time. One previous study also demonstrated results similar to ours.[32] The level of expression of mucins (whether decreased or increased) as a clinical marker for DES has been studied, thus appearing to be the next logical step in the quest to identify a gold standard for the diagnosis of this multifactorial disease.

The membrane-associated mucins such as MUC1 play a major role in lubrication, apical surface barrier, and osmosensing.[29,57–59] Previously it has been shown that the frequency of patients with non-Sjögren's aqueous-deficient DES, expressing only the *MUC1/A* splice variant of the mucin *MUC1*, may be lower than that of a normal control group.[32] Thus, a longer repeat sequence of amino acids on *MUC1* variants may play a role in susceptibility to DES.[32] Differences in *MUC1* genotype can explain loss of ocular surface integrity that is often observed in patients with DES.[60] A recent study[61] demonstrated that molecules such as neutrophil elastase and tumor necrosis

factor-alpha (TNF-α) induce the release of membrane-associated mucins MUC1, MUC4, and MUC16 in human corneal–limbal epithelial cells. Similar release of membrane-associated mucins can be induced in patients with DES who often demonstrate increased levels of inflammatory cytokines such as TNF-α and other inflammatory cytokines.[62] The release of these mucins from the corneal epithelial surface can lead to loss of integrity of the ocular surface in DES (evidenced in clinical practice as vital dye positive staining). Therefore, a decrease in *MUC1* expression not only indicates increased risk for DES but also appears to be diagnostic for the disease.

The present study seems to be the first one to propose using *MUC1* expression levels as a diagnostic marker for diagnosis of dry eye syndrome. The validity of our study is greatly increased by using external data sets. Internal validation of prediction models may not be sufficient and indicative for the model's performance in future patients because the classifiers tend to perform better on data on which the model was constructed than on new data. Accordingly, external validation is essential before implementing prediction models in clinical practice. The *MUC1* expression level has been shown to be the best predictor with our validation sets.

Therefore, from the data proposed in this study, it appears that expression levels of the *MUC1* gene can form a major diagnostic aid for DES. Although measuring *MUC1* expression is a laboratory procedure, it can still be widely used in investigational studies where patients are monitored while on therapy for DES over a period. It is well known that having a good test to clinically diagnose DES is essential for ophthalmic physicians as well as scientists conducting drug trials or clinical trials in DES. Based on the data presented in this present study we foresee that *MUC1* gene expression levels will be used as a successful diagnostic biomarker to accurately distinguish patients with DES from normal, healthy subjects in the near future.

This study did not investigate ocular mucin expression in other ocular surface diseases and thus we cannot state at this time whether these findings would be widespread and seen in other ocular surface conditions. These studies are therefore warranted.

In the present study, although the level of expression of *MUC4* was second best (compared with *MUC1*) in identifying patients with DES, the *MUC4* model was the best to classify healthy patients. A recent study demonstrated that levels of *MUC4* correlated well with temporal lid-parallel conjunctival folds and lid-wiper epitheliopathy, which are clinical signs observed in patients with DES.[31] It has been suggested that *MUC4* levels are increased in ocular allergies as an ocular surface defense mechanism to compensate the loss of other mucins at the ocular surface.[63] Thus it can be hypothesized that a decrease in *MUC4* levels can challenge the integrity of the ocular surface.

The present study is novel in nature due to the fact that we have compared the expression of several different conjunctival mucins in DES and healthy patients and repeated the experiments at two different periods in time, separated years apart. The data appear consistent over time and can be used for external validation. In summary, our data have demonstrated that *MUC1* levels can be used to detect patients with DES with very high accuracy. Thus, we propose the use of *MUC1* levels as a diagnostic marker for DES since this will not only increase the accuracy of classifying patients correctly, but also significantly aid in clinical trials for the development of therapeutic agents for this prevalent ocular disease.

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