

Full Length Research Paper

Expression of MUC2 and MUC5B in ethmoid sinus mucosa of patients with chronic rhinosinusitis

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This study investigated the expression of MUC2 and MUC5B in the ethmoid sinus mucosa of patients with chronic rhinosinusitis (CRS). Immunohistochemistry and fluorescence quantitative reverse transcription polymerase chain reaction (RT-PCR) were performed to detect the expression of MUC2 and MUC5B in the ethmoid sinus mucosa of 32 patients with CRS and 7 control individuals. Immunohistochemistry confirmed the expression of MUC2 and MUC5B in the ethmoid sinus mucosa of both patients and control subjects, and the expression of both proteins in CRS patients was significantly higher than that in control individuals ($p < 0.01$). The mRNA expression of MUC2 and MUC5B was $10.55 \pm 3.91 \times 10^4$ and $18.80 \pm 15.68 \times 10^5$ copies/ μg in CRS patients, and $1.36 \pm 0.46 \times 10^4$ copies/ μg and $1.49 \pm 0.46 \times 10^5$ copies/ μg in control individuals, respectively, with significant differences between the two groups ($p < 0.01$). Statistical analysis demonstrated the markedly increased expression of MUC2 and MUC5B in the ethmoid sinus mucosa of CRS patients, when compared with control individuals. The expression MUC2 and MUC5B in the ethmoid sinus mucosa of CRS patients was up-regulated, and the up-regulated expression may result in mucus hypersecretion and compromised mucociliary clearance in CRS patients.

Key words: chronic rhinosinusitis, mucin, immunohistochemistry, fluorescence quantitative polymerase chain reaction.

INTRODUCTION

Hypersecretion of mucus in the nasal sinuses may result in physiological changes of mucus and is one of the main features of mucosal inflammation in patients with chronic rhinosinusitis (CRS). In normal nasal sinuses, the secreted mucus can be identified into two layers: The inner layer, also called sol layer, is composed of serious fluid and the sinus cilia can swing freely; the outer layer, also called gel layer, consists of mucus and acts as a carrier for mucociliary swing. Recently, mucin plays an important role in maintaining the gel-like characteristics of mucus (Majima et al., 1999), of which the alteration may affect mucociliary clearance, thus resulting in hypersecretion of mucus. MUC2 and MUC5B are secretory proteins and they play an important role in the gel-like

characteristics of mucus (Williams et al., 2001; Pallesen et al., 2002; Chen et al., 2004). In the present study, immuno-histochemistry and fluorescence quantitative reverse transcription polymerase chain reaction (RT-PCR) were performed to determine the expression of MUC2 and MUC5B in the ethmoid sinus mucosa and to explore the potential mechanism involved in the hypersecretion of mucus in CRS patients.

MATERIALS AND METHODS

Clinical data

The ethmoid sinus mucosa samples from 32 CRS inpatients were obtained from January to June 2009 at the authors' department and did not have a history of sinus surgery. The diagnosis of CRS was made according to the symptoms, signs and findings from nasal endoscopy and computed tomography of nasal sinuses. Clinical

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manifestations included nasal obstruction and purulent discharge for more than 12 weeks, compromised by a sense of smell and headache. Nasal endoscopy revealed purulent discharge in middle meatus or olfactory cleft with or without obstruction caused by nasal polyp. CT of paranasal sinus showed lesions in the mucosa of maxillary sinus and ethmoid sinus, or accompanied by lesions in the mucosa of frontal sinus and sphenoid sinus. Patients with allergic rhinitis or asthma were excluded from the present study. There were 20 males and 12 females with a mean age of 33.62 ± 6.17 years. In addition, normal ethmoid sinus mucosa samples from 7 patients who underwent optic canal decompression ($n = 4$) or were with cerebrospinal fluid rhinorrhea ($n=3$) were recruited as controls. There were 5 males and 2 females with a mean age of 31.00 ± 5.51 years. CRS, allergic rhinitis or asthma control subjects were not observed in these control subjects. Informed consent was obtained before the study. The samples were collected under a nasal endoscope and divided into two parts: One part was fixed in 10% paraformaldehyde, paraffin-embedded and cut into 4 μm consecutive sections; another part was stored at -80°C for use. The study was approved by the ethics committee of the Third Affiliated Hospital of Sun Yat-Sun University.

Immunohistochemistry

Two-step immunohistochemical procedures

The tissues were paraffin-embedded and then cut into sections. These sections were de-paraffined and re-hydrated. After antigen retrieval, the endogenous peroxidase was in-activated by 3% hydrogen peroxide in methanol. Then, these sections were incubated with the primary antibody (MUC2: 1:200; MUC5B: 1:100) at room temperature for 1 h followed by incubation with other reagents (ElivisionTM) for 30°C at room temperature. Coloring was performed with DAB and counterstaining was performed with hematoxylin. Sections were dehydrated in absolute ethanol and transparentized in xylene. After mounting, the sections were observed under a light microscope. The primary antibody was replaced with PBS as negative controls.

Light microscopy and assessment

Brown granules were observed in the cytoplasm and on the cell membranes suggesting positive staining for MUC2 and MUC5B. In each section, 5 fields were randomly selected at a magnification of 400, and images were obtained for analysis by NIS-Element Br software (Nikon, Japan). The total area of positive cells, mean absorbance and mean optical density were detected and the values of mean optical density were averaged followed by the statistical analysis. The value of optical density was negatively related with the protein expression.

Fluorescence quantitative RT-PCR

Rationale of fluorescence quantitative RT-PCR

A fluorophore was added to the PCR mixture and the cumulative fluorescence was measured to monitor the PCR progress. Then, the standard curve was delineated, based on which the mRNA expression of target proteins was calculated.

Primers and fluorescent probes

The primers for MUC2: Forward (5'-GGTGGAGACACAGAATTGATTGG-3'), reverse (5'-

TGGCTCTGCAAGAGA TGTTAGC-3'); fluorescent probe: 5'-FAM-ACG TCT GTG GAC CAG GCT GGG C-TAMRA-3'; the anticipated size of amplified products was 70 bp. The primers for MUC5B: forward (5'-GAGGGCCTGATCCTGTTTGA-3'), reverse (5'-AAGCAGCCGTTCTTGCT-3'); fluorescent probe: 5'-FAM-CAAATTCGG GTGAGCAGCGGT-TAMRA-3'; the anticipated size of amplified products was 63 bp. The primers and fluorescent probes were synthesized in Da An Gene Co., Ltd.

RNA extraction and identification

Total RNA was extracted with TRIzol (1 ml) from all samples. The absorbance was determined with a UV spectrophotometer at 260 nm. The amount of mRNA was calculated as follows: mRNA (copies) = $A_{260} \times \text{dilution-fold} (120) \times 40/1000$.

Reverse transcription

Reverse transcription was performed with 4 μl of RNA in a PCR instrument (Perkin Elmer, USA) at 37°C for 1 h and then at 95°C for 3 min.

Fluorescence quantitative PCR

Five-fold dilution of PCR buffer (ABI, USA) was conducted and 10 μl of diluted solution was mixed with forward primers (1 μl , 10 pM/ μl), reverse primers (1 μl , 10 pM/ μl), dNTPs (0.5 μl , 25 mM) (Sigma, USA), fluorescent probes (1 μl , 10 pM/ μl), taq polymerase (1.5 μl ; ABI, USA), cDNA (5 μl) and ddH₂O (30 μl). The final volume of mixture was 50 μl . The PCR buffer contains 10 mM Tris-HCl (pH 8.0), 50 mM KCl and 2 mM MgCl₂. The cycling conditions for PCR were 30 cycles of 93°C for 3 min, 93°C for 45 s and 55°C for 45 s. Fluorescence quantitative PCR instrument was purchased from Perkin Elmer. After PCR, the fluorescence signal was transformed into initial copies and Ct value of MUC2 and MUC5B (The Ct value is defined as the number of cycles required for the fluorescent signal to cross the threshold). Based on the concentration of RNA and results (copies/ μL cDNA) from fluorescence quantitative PCR, the amount of MUC2 and MUC5B in samples was calculated (copies/ μg cDNA).

Statistical analysis

Data were presented as $\bar{x} \pm s$, and statistical analysis was performed with SPSS 9.0 software. Student *t* test was used to compare the difference between the two groups. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Immunohistochemistry

In the ethmoid sinus mucosa of CRS patients, strongly positive staining (brown) for MUC2 was mainly found in the cytoplasm and on the membrane of epithelial cells, especially goblet cells (Figure 1a). No expression of MUC2 was found in submucosal glandular cells (Figure 1b). In the controls, only weakly positive staining (light brown) was noted in the cytoplasm and on the membrane of epithelial cells (Figure 1c), and submucosal glandular cells did not express MUC2 (Figure 1d). The mean optical

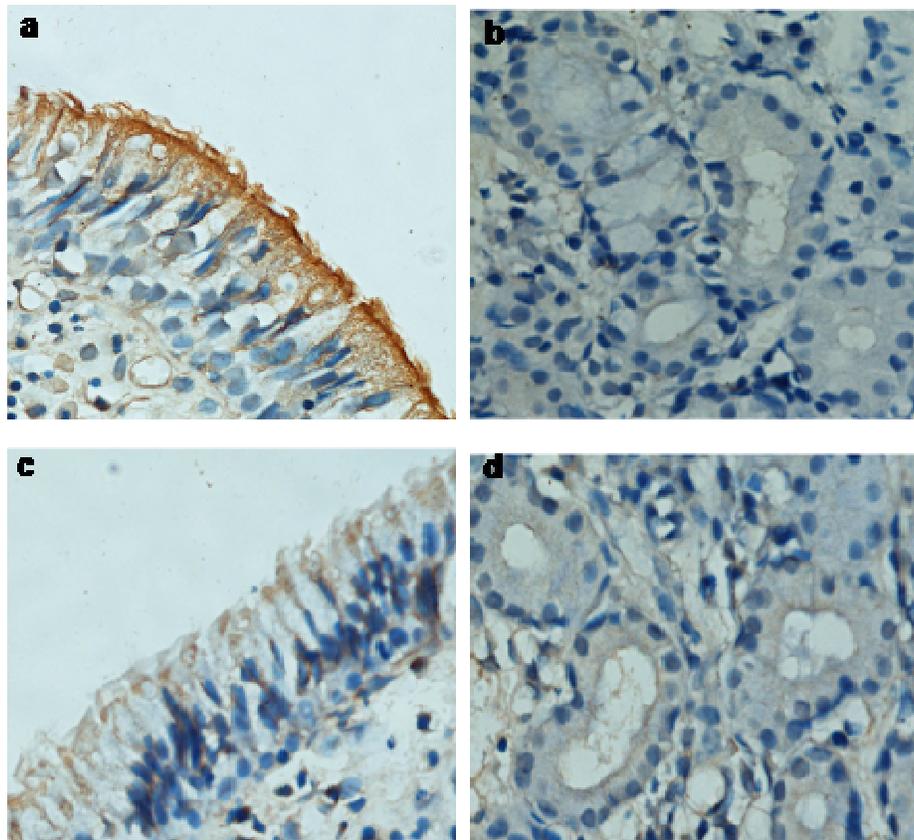


Figure 1. (a) Strongly positive staining (brown) for MUC2 was found in the cytoplasm and on the membrane of epithelial cells of CRS patients ($\times 400$), (b) No expression of MUC2 was found in submucosal glandular cells of CRS patients ($\times 400$), (c) Only weakly positive staining (light brown) for MUC2 was noted in the cytoplasm and on the membrane of epithelial cells of controls ($\times 400$), (d) Submucosal glandular cells did not express MUC2 in control subjects ($\times 400$).

density of MUC2 was 174.52 ± 5.71 in controls and 135.86 ± 20.11 in CRS patients. The statistical analysis that demonstrated the MUC2 expression in the CRS patients was markedly higher than in the controls ($t = 4.99$, $p < 0.01$).

In the ethmoid sinus mucosa of CRS patients, strongly positive staining (brown) for MUC5B was mainly found in the cytoplasm and on the membrane of epithelial cells, especially goblet cells (Figure 2a). The expression of MUC5B was found in the cytoplasm and on the membrane of a fraction of submucosal glandular cells (Figure 2b). In the control subjects, only weakly positive staining for MUC5B (light brown) was noted in the cytoplasm and on the membrane of epithelial cells (Figure 2c) and submucosal glandular cells did not express MUC5B (Figure 2d). The mean optical density of MUC5B was $172.67 + 12.73$ in controls and $143.01 + 22.50$ in CRS patients. The statistical analysis that demonstrated the MUC5B expression in the CRS patients was significantly higher than that in the control subjects ($t = 3.35$, $p < 0.01$).

Fluorescence quantitative PCR

Five microliters of standard templates of different concentrations (4 - 5 concentration) were independently added to reaction tubes and amplification was performed in a PE7000 fluorescence quantitative PCR instrument. Four to five S-shaped kinetic curves were obtained. In the plateau phase of amplifications with templates of different copies, the difference in the copies of products were not proportional. If end point method was used for quantification, the results may be inaccurate. When the critical point was set at the Ct value, the logarithm of initial copies was linearly related to Ct value. The more the copies, the smaller the Ct value. The standard curve was delineated according to standards with known initial copies, in which the x-coordinate represented logarithm of initial copies and y-coordinate represented Ct value. The standard curve was linear and displayed the linear relationship between initial copies and Ct value. The linear correlation coefficient was greater than 0.99. This curve was used as a standard curve. The standard

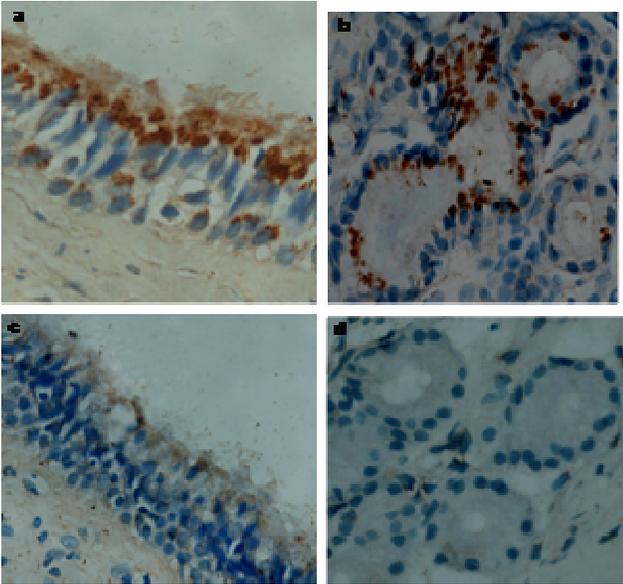


Figure 2. (a) Strongly positive staining (brown) for MUC5B was mainly found in the cytoplasm and on the membrane of epithelial cells of CRS patients ($\times 400$), (b) Expression of MUC5B in submucosal glandular cells of CRS patients ($\times 400$). (c) Only weakly positive staining for MUC5B (light brown) was noted in the epithelial cells of controls ($\times 400$) (d) Submucosal glandular cells did not express MUC2 in control subjects ($\times 400$).

amplification, standard and amplification curves of MUC2 and MUC5B positive samples are displayed in Figures 3 (a, b and c) and 4 (a, b and c) respectively.

Quantitative expressions of MUC2 and MUC5B mRNA

The mRNA expression of MUC2 and MUC5B was found in both CRS patients and control subjects. The mRNA expression of MUC2 was $10.55 \pm 3.91 \times 10^4$ copies/ μg in CRS patients and $1.36 \pm 0.46 \times 10^4$ copies/ μg in controls suggesting significant difference ($t=6.15$, $P<0.01$). The mRNA expression of MUC5B was $18.80 \pm 15.68 \times 10^5$ copies/ μg in CRS patients and $1.49 \pm 0.46 \times 10^5$ copies/ μg in controls, suggesting a significant difference ($t = 2.89$, $p < 0.01$). These results demonstrated markedly mRNA expression of MUC2 and MUC5B in CRS patients.

DISCUSSION

In CRS patients, the hypersecretion of mucus may cause changes in viscoelasticity of the mucus blanket and affect ciliary movement, thus leading to compromised defense function of mucosa. Mucins are a kind of high molecular weight, heavily glycosylated proteins produced by epithelial tissues. They can be divided into membrane-bound mucins and secretory mucins. The secretory

mucins determine the viscosity and elasticity of mucus. Expression of secretory mucins has been found in airway epithelial cells, and MUC2 and MUC5B are the main secretory mucins in the airway mucus (Williams et al., 2001; Pallesen et al., 2002; Chen et al., 2004).

Groneberg et al. (2003) demonstrated MUC5B expression in goblet cells and submucosal glandular cells of normal epithelium. Aust et al. (1997) applied *in situ* hybridization to demonstrate the MUC2 expression in epithelial cells of the inferior turbinate mucosa. Recently, Ali et al. (2005) detected the protein expression of MUC5B and MUC2 in normal nasal mucus. These findings suggest MUC2 and MUC5B are indispensable for physiological viscoelasticity of the nasal mucus blanket. In the present study, the mRNA and protein expressions of MUC2 and MUC5B were also detected in normal epithelial cells of the ethmoid sinus mucosa, which implied MUC2 and MUC5B were also necessary for maintaining physiological viscoelasticity of the nasal mucus blanket.

Our study also showed the expression of MUC2 and MUC5B in the ethmoid sinus mucosa of 32 patients with CRS. In addition, the mRNA and protein expression of MUC2 and MUC5B was markedly higher than in controls ($p < 0.01$). In the study of Jung et al. (2000), the expression of mucins was detected in 8 patients with CRS. Their results showed the increased MUC5B expression in 5 patients but the mRNA expression of MUC2 was not detected. This difference between our study and that of Jung et al may be caused by different experimental methods. Jung et al. used RT-PCR, and qualitative analysis was performed based on the final amplified products. In the present study, fluorescence qualitative PCR was applied and real time fluorescence was monitored at each cycle followed by quantitative analysis. In our procedures, the fluorescence was detected at each cycle and real time amplification curve was delineated. Therefore, the Ct value could be accurately determined, based on which, the initial DNA copies could be calculated.

ELISA was performed by Ali et al. (2005) and results showed significantly increased protein expression of MUC2 and MUC5B in nasal excretions of CRS patients. In our study, the mRNA and protein expressions of MUC2 and MUC5B in CRS patients were dramatically elevated when compared with those in controls ($p < 0.01$). These findings suggested the up-regulation of MUC2 and MUC5B might be related with the hypersecretion of mucus in CRS patients.

Ye et al. (2006) found the proliferation of goblet cells and hyperplasia of mucous glands during CRS, which lasted until post-operative mucosal epithelization. In another study, the post-operative mucosal pathological changes and mucociliary clearance ability were detected at a follow up period of 6 months, and their results showed that the number of goblet cells and mucous glands was not reduced even after complete epithelization.

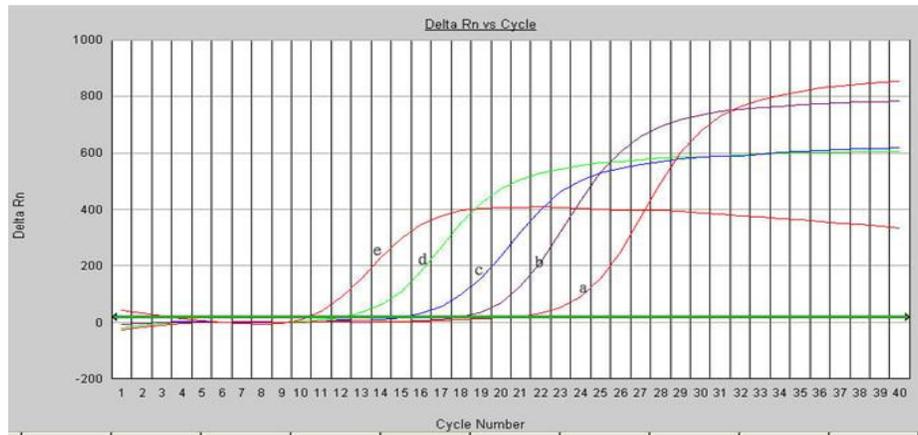


Figure 3a. Standard amplification curve of MUC2 positive samples (a: 1/2 dilution; b: 1/10 dilution; c: 1/100 dilution; d: 1/1000 dilution and e: 1/10000 dilution).

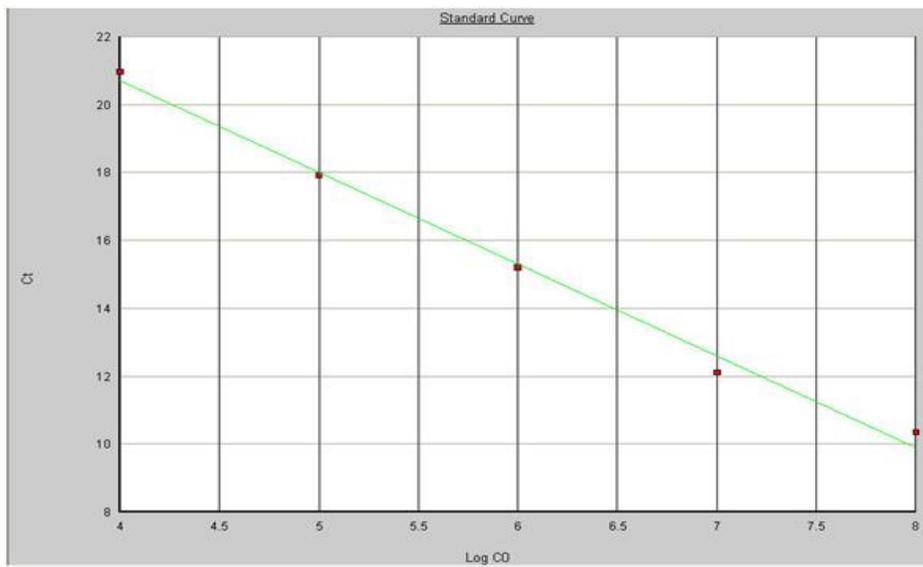


Figure 3b. Standard curve of MUC2.

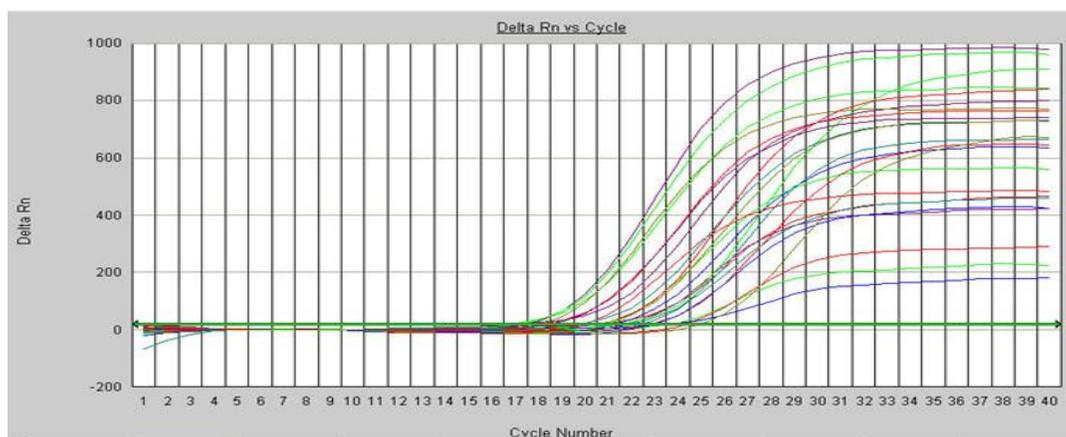


Figure 3c. Amplification curve of MUC2 in ethmoid sinus mucosa.

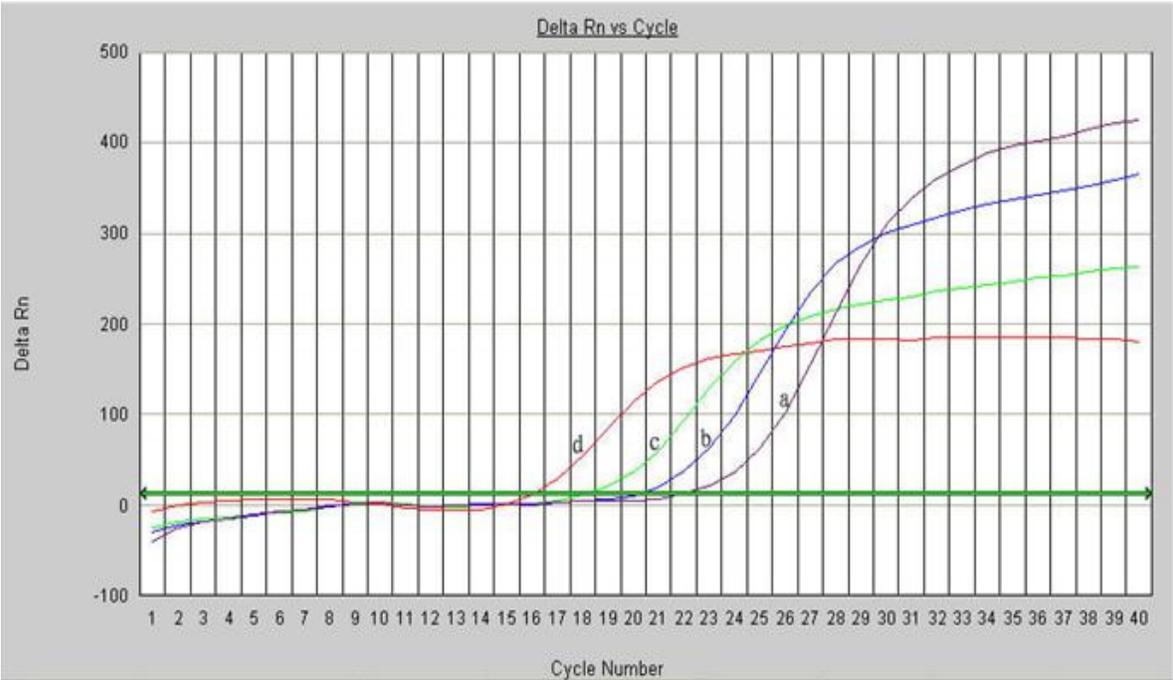


Figure 4a Standard amplification curve of MUC5B positive samples (a: 1/2 dilution; b: 1/10 dilution; c: 1/100 dilution; and d: 1/1000 dilution).

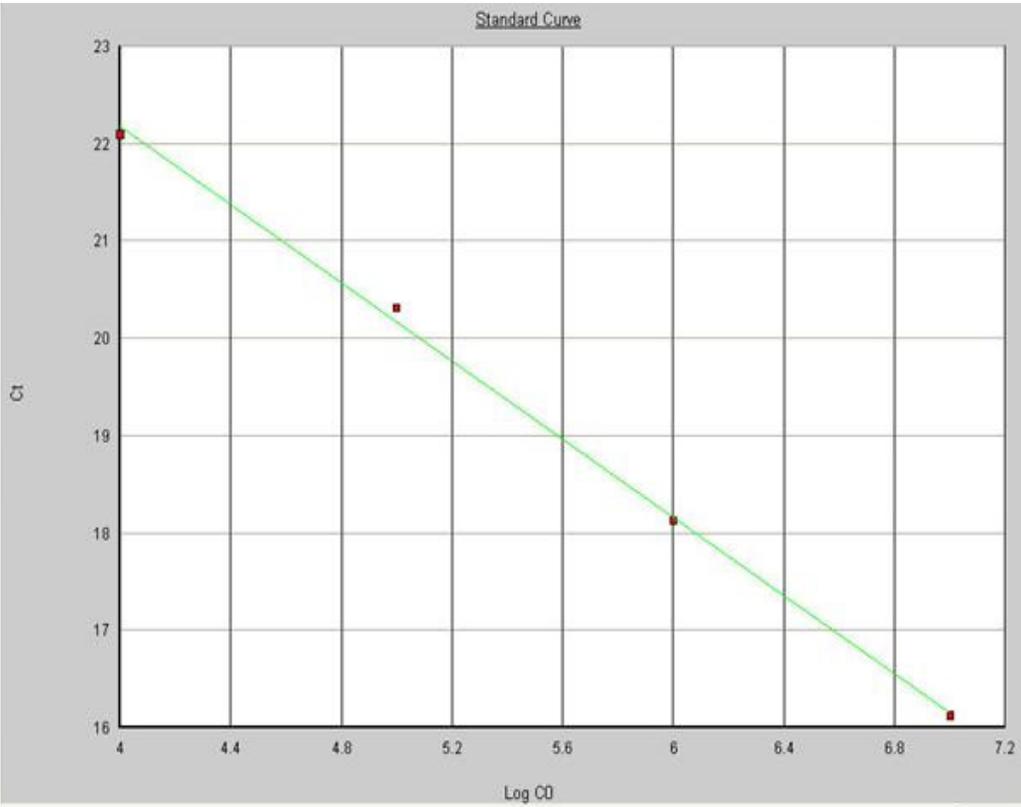


Figure 4b. Standard curve of MUC5B.

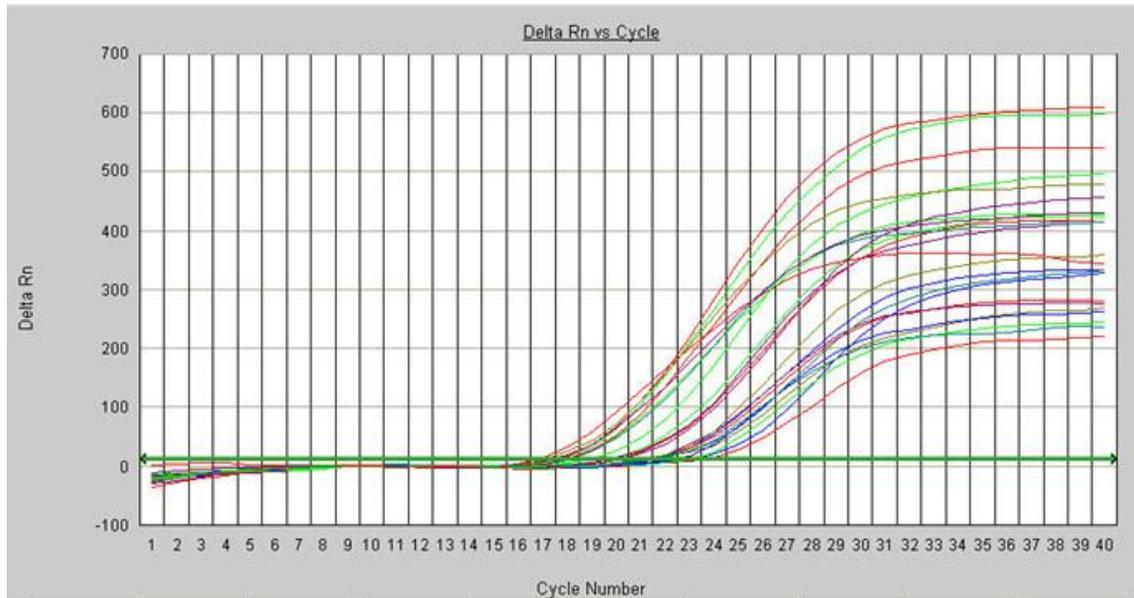


Figure 4c. Amplification curve of MUC5B in ethmoid sinus mucosa.

The hypersecretion of mucus and dysfunction of mucociliary clearance were observed. According to the findings above, we postulate that MUC2 and MUC5B are produced by goblet cells of nasal and sinus mucosa and play an important role in maintaining normal viscoelasticity of the mucus blanket which is necessary for the normal mucociliary clearance. However, in mucosal inflammation, proliferation of goblet cells and hyperplasia of mucous glands occur and cause hypersecretion of MUC2 and MUC5B resulting in dysfunction of the mucociliary clearance. Therefore, the nasal excretion accumulates and finally results in deteriorated inflammation.

Our results demonstrated that the expression of MUC2 and MUC5B in the ethmoid sinus mucosa of CRS patients was markedly elevated. Therefore, it is necessary to further investigate the characteristics of mucin expression and regulation in CRS patients, which may be beneficial for elucidating the mechanism involved in hypersecretion of mucus and improving the therapeutic efficacy.

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