Myrtol standardized affects mucociliary clearance
Ying Ying Li, BM, PhD1, Jing Liu, BM, MS1, Chun Wei Li, BM, PhD1, Somasundaram Subramaniam, MD2, Siew Shuen Chao, MD1, Feng Gang Yu, PhD1, Noam A. Cohen, MD, PhD3, Shi Li, MD, PhD4 and De Yun Wang, MD, PhD1

Background: Myrtol standardized (Gelomyrtol forte) has been shown to be effective in controlling nasal symptoms of rhinosinusitis by promoting mucociliary clearance. Our aim was to evaluate the short- and long-term effects of myrtol on ciliated columnar cells and goblet cells in an in-vitro setting.

Methods: Nasal epithelial cells were harvested (42 days) from an air-liquid interface (ALI) culture of human nasal epithelial stem/progenitor cells (hNESPCs), which was derived from biopsies of nasal inferior turbinate mucosa. Myrtol 0.1% was applied to the ALI culture system at 2 different time-points (day 0 and day 35) on progenitor and differentiated cells. Ciliary beat frequency (CBF), supernatant fluid, and ciliated and goblet cell markers were evaluated after short- (7 days) and long-term (42 days) treatment.

Results: In the long-term treatment with myrtol, there was an increase in cilia area (type IV β-tubulin+, 1.53-fold, p = 0.031) and ciliogenesis-related markers (Foxj1 and CP110) with no change in CBF, as compared with control. In addition, the short-term myrtol treatment group exhibited greater mucin secretion compared with control.

Conclusion: This study demonstrates, through cellular and molecular mechanisms, that myrtol standardized enhances the mucus production from goblet cells in the short term, and promotes ciliated cell differentiation in the long term.

Key Words: ciliogenesis; differentiation; human nasal epithelial stem/progenitor cells; long-term treatment; mucus secretion; myrtol standardized; short-term treatment

from China). However, the exact mechanism of action of myrtol standardized on nasal epithelium, especially on epithelial cell differentiation and its barrier function, is not well understood. Although studies from clinical trials and primary cell culture models have so far demonstrated that myrtol standardized can reduce symptoms of acute and chronic rhinosinusitis by enhancing airway mucociliary clearance, no studies to date have examined the drug’s impact on the repair of MCC after injury from infection and/or inflammation.

Recently, we have been successful at isolating adult human nasal epithelial stem/progenitor cells (hNESPCs) from nasal biopsy specimens. Single cell–derived colonies stain uniformly for basal cell markers, such as p63 and keratin 5, and about 80% of colonies show long-term self-renewal potential. The lineage potential of these cells has been assessed through multiple differentiation assays. Pedigree lines developed from a single cell can differentiate into mature stratified mucociliary airway epithelium, composed of both ciliated columnar cells and goblet cells. This in-vitro cell model can be used for testing of myrtol standardized and assessing its impact on the biophysiologic process of progenitor cell differentiation into mature ciliated and goblet cells. The aim of this study was to utilize this in vitro model to investigate the effects of myrtol standardized on ciliated and goblet cell differentiation through the analysis of the differential expression of ciliogenesis-associated markers.

**Patients and methods**

**Study population**

Approval for conducting this study was obtained from the National Healthcare Group Domain-Specific Review Board of Singapore (Singapore) and from the institutional review boards of Qilu Hospital, Shandong University (China). Eleven healthy adult subjects (9 males and 2 females) were recruited from the Department of Otolaryngology of the National University Health System (Singapore) and from the Department of Otolaryngology at Qilu Hospital. The clinical characteristics are listed in Table 1. Biopsies were taken from the inferior turbinate of these patients with septal deviation, who were scheduled for septoplasty surgery. The hNESPCs derived from all patients were used for nasal epithelial cell differentiation ability testing. Among these, hNESPCs from 10 of the patients were used for the myrtol study, but not for the remaining 1 patient, due to insufficient cell numbers. The hNESPCs were harvested for baseline analysis of un-differentiated status to compare with differentiated status.

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*The hNESPCs from 10 patients were used for Myrtol study, but not for 1 patient (patient 11), due to insufficient cell numbers. hNESPCs = human nasal epithelial stem/progenitor cells.*

**Myrtol standardized preparation**

Myrtol standardized, obtained from G. Pohl-Boskamp GmbH & Co. (Hohenlockstedt, Germany), was first diluted in 100% ethanol (Fisher Scientific, Singapore) to a concentration of 10%, and then further diluted in B-ALI differentiation medium (Lonza, Walkersville, MD) to the final working concentration of 0.1%. B-ALI differentiation medium without myrtol standardized was used as control.

**Human nasal epithelial stem/progenitor cells culture and differentiation in an air-liquid interface culture system**

Primary epithelial cells isolated from fresh nasal specimens and human nasal epithelial stem/progenitor cells (hNESPCs) were cultured in our progenitor cell and air-liquid interface (ALI) culture system. The progenitor cells consisted of fully differentiated ciliated and goblet cells with 35 days of culture (Figure 1A). Details are presented in the Supplementary Material associated with this article (available online).

To observe the effects of myrtol standardized on progenitor cells, 0.1% myrtol standardized was added at day 0 to the ALI culture and changed every 48 hours (progenitor cell—treated group, PTG). To study the effects of the drug on differentiated epithelial cells, 0.1% myrtol standardized was applied on Day 35 only and changed every 48 hours in a separate group (differentiated cell—treated group, DTG). Before transferring into ALI culture system (hNESPCs) cells were harvested for baseline analysis of undifferentiated status to compare with differentiated status. Both control and cells treated with myrtol were harvested at day 42 for quantitative real-time polymerase chain reaction (PCR) and immunostaining (Figure 1A).

**Immunofluorescence staining**

Epithelial cell markers (MUC5AC, type IV β-tubulin, Foxj1, and CP110) were detected by immunofluorescence
Myrtil standardized in MCC

FIGURE 1. Effect of myrtil standardized on mucus secretion. Flowchart of the study showing the experimental design (A). Mucus secretion of in the control group and the PTG and DTG for ALI after 42 days (B). MUC5AC shown by IF staining (C) (100× amplifications; scale bar =100 μm) and ELISA (D). Median values with 25th and 75th percentiles indicated by scale bar for the 3 groups. The fold change of MUC5AC expression was compared for control, PTG and DTG (E). Median values with 25th and 75th percentiles indicated by scale bar. mRNA level of MUC5AC was evaluated in the cells before and after differentiation (F) (n = 11; Wilcoxon signed rank test), as well as in the 3 groups (G) (median values with 25th and 75th percentiles as indicated by scale bar). ALI = air-liquid interface; hNESPCs = human nasal epithelial stem/progenitor cells.

Evaluation of staining results

To ensure standardization of the staining for histologic evaluation, at least 2 researchers independently assessed all specimens, with each researcher blinded to the other’s findings. MUC5AC, type IV β-tubulin, and 4′,6-diamidino-2-phenylindole (DAPI)–positive area (in arbitrary units) in Transwell inserts (Corning, Corning, NY) from untreated/treated cells (5 measurements per individual) were evaluated at 100× amplification using ImageJ software (National Institutes of Health, Bethesda, MD). Foxj1, located specifically in the ciliated cell nuclei, was evaluated for positive cell numbers at 400× amplification (3 measurements per individual). CP110 cells, observed through staining of the ciliary structure, were evaluated by positive area (in arbitrary units, with 3 measurements...
Effect of myrtol standardized on ciliated cells (n = 10). Type IV β-tubulin was evaluated by IF staining (A) (100× amplifications, scale bar = 100 μm). Median values with 25th and 75th percentiles as indicated by scale bar in the 3 groups (B). CBF was also measured in the 3 groups (C) (median values with 25th and 75th percentiles, indicated by scale bar). MUC5AC (red) and type IV β-tubulin (green) with DAPI (nuclear staining, blue) findings are presented for the 3 groups (D) (200× amplifications, scale bar = 50 μm). Total cell number (DAPI + cells) assessed for the 3 groups (E) (median values with 25th and 75th percentiles, indicated by scale bar). DAPI = 4′,6-diamidino-2-phenylindole; hNESPCs = human nasal epithelial stem/progenitor cells; IF = immunofluorescence.

Measurement of mucus secretion in apical washes

At day 42 after ALI culture, the apical surfaces of the Transwell inserts from control and treated cells were incubated with prewarmed phosphate-buffered saline (PBS; 100 μL) at 37°C for 15 minutes, then collected and stored at −80°C. MUC5AC protein level was measured in diluted supernatant (1:50) using enzyme-linked immunosorbent assay (ELISA). Apical washes were diluted in carbonate bicarbonate coating buffer (Sigma, Ronkonkoma, NY) and incubated at 4°C overnight on MaxiSorp microtiter 96-well plates (Nunc, Rochester, NY). After washing with PBS plus 0.1% Tween-20 (PBS-T; Labconsult, Brussels, Belgium), wells were blocked for 1 hour at 37°C with 1% bovine serum albumin (BSA; Sigma) in PBS-T. After 3 washes with PBS-T, wells were sequentially incubated overnight at 4°C with mouse monoclonal anti-MUC5AC (45M1; Thermo Fisher Scientific, Waltham, MA). After washing with PBS-T, the samples were incubated for 1 hour at 37°C with horseradish peroxidase–conjugated goat antimouse antibody (Abcam, Cambridge, UK). After washing, 3,3′, 5,5′-tetramethylbenzidine substrate (Life Technologies, Carlsbad, CA) was added and incubated for 15 minutes at room temperature, followed by stop solution (Life Technologies) to terminate the reaction. Absorbance was read at 450 nm on a microplate reader (Synergy/HT; Biotek, Winooski, VT).

CBF measurement

CBF was analyzed by using Sisson-Ammons Video Analysis (SAVA, Omaha, NE). The sample IDs were coded confidentially and blinded to the 2 investigators who performed the CBF and data analysis. Whole-field analysis was performed using software that automatically analyzed the entire captured video of all ciliated cells in a given field. For all experiments, the digital sampling rate was set at 100 frames/second (fps). The predominant frequency of a small group of cilia from each sample was viewed and recorded at a minimum of 5 separate fields every minute for up to 4 minutes per individual at 400× amplification by ImageJ. The ratio of positive staining area before and after myrtol standardized treatment was calculated for each individual.
Myrtol standardized in MCC

FIGURE 3. Effect of myrtol standardized on Foxj1 (n = 10). Foxj1 (red) was evaluated by double staining with type IV β-tubulin (green) and total area for hNESPCs in the control group, PTG, and DTG, respectively (A) (400× amplifications). Median values with 25th and 75th percentiles are indicated by the scale bar (B). mRNA level of Foxj1 for cells in the 3 groups (C) (Wilcoxon signed rank test, with median values with 25th and 75th percentiles indicated by scale bar), as well as in the groups before and after differentiation (D) (n = 11; Wilcoxon signed rank test). Correlations are shown between total area of Foxj1 and cilia length (one-to-one) (E). The x-axis scale is logarithmically (base 10) transformed. DTG = differentiated cell–treated group; hNESPCs = human nasal epithelial stem/progenitor cells; PTG = progenitor cell–treated group.

5 minutes while being maintained at a constant temperature (23 ± 0.5°C). All frequencies from each sample were expressed as the mean from each field over 5 minutes.

RNA extraction and quantitative real-time PCR

Details of RNA extraction are provided in the Supplementary Material. Real-time PCR analysis (StepOnePlus™ System; Applied Biosystems) was performed in duplicate to evaluate the expression levels of Foxj1, CP110, and MUC5AC after long- and short-term treatment with myrtol standardized by using the TaqMan assays (Foxj1, Hs00230964_ml; CP110, Hs00206922_m1; MUC5AC, Hs00873651_ml; Applied Biosystems). Phosphoglycerate kinase-1 (PGK1) was used as an endogenous control gene. For comparing the expression level of Foxj1, CP110, and MUC5AC before and after differentiation, the absolute value of $2^{-\Delta\Delta C_t}$ was presented. For comparing the effect of myrtol on progenitor and differentiated cells, after normalizing control as 1, the fold change of $2^{-\Delta\Delta C_t}$ was presented.

Statistics

All data were analyzed using SPSS version 18.0 (SPSS, Inc., Chicago, IL). Wilcoxon signed rank test was used to compare expression of MUC5AC, Foxj1, and CP110 between cells before and after differentiation, and was then used to analyze the effect after myrtol standardized treatment. Correlation analysis between ciliogenesis-associated markers and cilia area (type IV β-tubulin+) was performed using Spearman’s rank correlation coefficient ($r$). $p < 0.05$ was considered significant in all analyses.

Results

Effect of myrtol standardized in mucus secretion

To assess the changes of cells in response to myrtol standardized, 0.1% myrtol standardized was treated with progenitor cells in one group (PTG) and differentiated cells in another group (DTG) (Figure 1A). Consistent with our previous studies, the differentiated cells changed into a compacted pattern and smaller size compared with the progenitors. The DTG showed an increase in mucus
Effect of myrtol standardized on CP110 (n = 10). CP110 (red) was evaluated by double staining with type IV β-tubulin (green) and total area of hNESPCs in the control group, PTG, and DTG, respectively (A) (400× amplifications). (B) Median values with 25th and 75th percentiles are indicated by the scale bar. mRNA level of CP110 assessed in all 3 groups (C) (Wilcoxon signed rank test, with median values with 25th and 75th percentiles are indicated by scale bar), as well as in the groups before and after differentiation (D) (n = 11; Wilcoxon signed rank test). Correlations are shown between total area of CP110 and cilia length (one-to-one) (E). The x-axis scale is logarithmically (base 10) transformed.

secretion (Figure 1B). The ELISA (1.2-fold; p = 0.01) and IF (2.52-fold; p = 0.016) staining results showed a higher expression level of MUC5AC in DTG with myrtol standardized compared with control and PTG (Figure 1C-E). MUC5AC mRNA level showed a dramatic increase after differentiation in ALI culture (133.58-fold; p < 0.0001; Figure 1F), strongly suggesting that the goblet cells in the samples were developed from hNESPCs in this culture system. In contrast, the DTG had only slightly higher expression of MUC5AC mRNA (1.05-fold), whereas the PTG had a lower expression of this marker (Figure 1G). Meanwhile, the ethanol group (without myrtol standardized) did not show a significant change in MUC5AC protein (Figure 1C) and mRNA levels (see Figure S1A in Supplementary Material). These results indicate that short-term treatment with myrtol standardized had affected the secretory property of the mature goblet cells.

Effect of myrtol standardized in ciliated cell differentiation

There was a significant increase in cilia area (type IV β-tubulin+ area; 1.53-fold; p = 0.031) after differentiation in the PTG, as compared with the control and ethanol-treated groups. This effect was not observed in the PTG (Figure 2A and B). Also, there was a minor decrease in CBF in both the PTG (0.84-fold) and DTG (0.92-fold; Figure 2C) the ethanol-treated group (Figure S1B in Supplementary Material). After quantification by DAPI staining (nuclear staining of all cells), we did see an increase in total number of cells in the PTG (Figure 2D and E). The progenitor cells of myrtol standardized may promote hNESPC differentiation into ciliated cells.

Effect of myrtol standardized in ciliogenesis-associated markers

Both protein and mRNA expression levels of the ciliogenesis-associated markers Foxj1 and CP110 were measured in the control group and in the PTG and DTG.

**Foxj1**

Foxj1 was typically expressed (by IF staining and quantitative real-time PCR) in the nucleus of ciliated cells, but not in hNESPCs (Figure 3A and D). The expression level
FIGURE 5. Effects of myrtol standardized on mature and nasal epithelium after injury. The effect of myrtol standardized on mature epithelial cells (short-term treatment) may trend to increase goblet cell secretion while promoting ciliated cell differentiation by regulating ciliogenesis-related genes in a physiologic manner. Thus, it may contribute to restoring efficient mucociliary clearance by balancing the structured interaction between the cilia and the nasal mucus.

Recent reports have shown that myrtol standardized can relieve respiratory symptoms by increasing the secretion of mucin, decreasing the viscosity of mucus, and CBF. Our data demonstrate, for the first time, that myrtol standardized enhances goblet cell secretion and can also promote greater differentiation of progenitor cells to ciliated cells by regulating ciliogenesis-associated markers (Foxj1 and CP110). This is a clinically important process as it assists in restoring MCC after injury from infection and/or inflammation.

CP110

CP110 was located in the nucleus before differentiation and translocated to the root of the cilia after 42 days of ALI culture (Figure 4A), with no changes in mRNA level (Figure 4D). Translocated CP110 showed a higher expression level in the PTG (2.47-fold; \( p = 0.002 \)) and a slight increase in the DTG (Figure 4B), but no change in mRNA level (1.10-fold; Figure 4C). Translocated CP110 also showed a positive correlation with cilia area (\( r = 0.838, p < 0.0001 \); Figure 4E). In addition, there was no change in translocated CP110 protein and CP110 mRNA in the ethanol-treated group (Figure 4A, and Figure S1C in the Supplementary Material). These data suggest that myrtol standardized promotes differentiation of ciliated cells, possibly in association with translocation of CP110.

Discussion

Recent reports have shown that myrtol standardized can relieve respiratory symptoms by increasing the secretion of mucin, decreasing the viscosity of mucus, and CBF. Our data demonstrate, for the first time, that myrtol standardized enhances goblet cell secretion and can also promote greater differentiation of progenitor cells to ciliated cells by regulating ciliogenesis-associated markers (Foxj1 and CP110). This is a clinically important process as it assists in restoring MCC after injury from infection and/or inflammation.

Based on the 2012 European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS 2012) guidelines, myrtol standardized was recommended for patients with acute rhinosinusitis. Another study showed that the main components of myrtol standardized (300 mg) reached almost 100% systemic absorption, with a maximum concentration in plasma reaching 2382 ng/mL. In an experimental study (rat tracheal explants), it was found that only high concentrations of myrtol standardized (0.05% to 0.1%) were effective in stimulating CBF. However, this effect was not found in our study in either short- or long-term in-vitro treatment of human ciliated cells with myrtol. The concentration was much higher than clinical plasma concentrations.

In this study, we attempted to evaluate the effect of myrtol standardized on mucociliary function, including CBF, level of mucus production, and cell growth and differentiation,
using our in-vitro nasal epithelial cell model. In agreement with a previous study, our data show that DTG can significantly elevate the MUC5AC, which is the main element of mucus, but only at the protein level. Therefore, we hypothesize that myrtol may mainly affect the secretion of mucus. In another recent in-vitro study, treatment with a distillate of rectified essential oils (with the main active component being myrtol) suggested an increase in airway surface liquid hydration, thereby accelerating mucociliary transport velocity in human sinonasal epithelial cultures. By increasing the mucus secretion as well as liquid hydration, short-term treatment with myrtol standardized can potentially enhance the secretory function of goblet cells (Figure 5), thereby promoting MCC. However, we did not study the molecular mechanism underlying the effect of myrtol on goblet cells, which could be a limitation of this study.

Interestingly, our study has demonstrated that long-term treatment with myrtol standardized promotes the differentiation of progenitor cells to ciliated cells. This was confirmed by the increased expression levels of 2 important ciliogenesis-related markers (Foxj1 and CP110). Foxj1 is well known as a regulator of cilia differentiation and promotes the formation of motile cilia. CP110 is a centrosomal protein that can participate in ciliogenesis.

Our results show that progenitor cells treated with myrtol standardized appear to promote the growth of ciliated cells by upregulating the expression of Foxj1 (both at protein and mRNA levels), and supporting the translocation of CP110. Unlike the pathologic situation in our previous study, long-term treatment with myrtol standardized shifted the fate of progenitor cells by regulating ciliogenesis-related genes in a physiologic manner, with no effect on cilia function (eg, CBF; Figure 5).

Cells from 4 patients with allergy did not show any difference in mucus secretion and ciliogenesis after myrtol standardized treatment as compared with cells from patients without allergy. A limitation of our study is that the investigation was performed using a healthy hNESPC model, and did not test diseased or inflamed mucosa. Therefore, we were unable to identify whether myrtol standardized has similar effects on diseased nasal epithelium.

In conclusion, our data suggest that myrtol standardized may have a therapeutic effect in promoting MCC by restoring the balance between the cilia (long-term treatment) and nasal mucus production (short-term treatment). We hope that the results of this study will support further investigation into the underlying mechanisms of action of this herbal remedy within the nasal cavity and paranasal sinuses.

References