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Comparison of ciliary wave disorders measured by image analysis and electron microscopy

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Abstract

Conclusion. We have developed a simple, reliable method for the simultaneous determination of the ciliary wave disorder (CWD) and ciliary beat frequency (CBF) of actively beating cilia.

Objective. The CBF and the directions of beating cilia are two important components of mucociliary transport. Although lots of studies have been performed on the measurement of the CBF, there have been few studies on the direction of cilia, with the exception of those using electron microscopy (EM). EM takes too long to determine the directions of cilia, and it cannot determine the direction of actively beating cilia. The aim of this study was to develop an image analysis (IA) system to conveniently determine the wave directions of multiple actively beating cilia as well as the CBF.

Material and methods. Sphenoid sinus mucosae obtained from 10 patients undergoing pituitary tumor removal via a trans-septal trans-sphenoidal approach were divided into two 4 x 4 mm²-sized pieces. One piece was studied using IA, the other with EM. Using IA, ciliary wave directions were determined from 5 x 5 mm² regions of interest and the mean of 5 consecutive values was regarded as the CWD of each sample. The CBF was also measured. CWD was also measured using EM. Results. The average number of cilia analyzed by EM was 102.50 (range 48–136). The mean CWDs determined using IA and EM were 28.25 ± 4.84° and 23.59 ± 8.16°, respectively. There was a significant correlation between the CWDs determined using these two methods (Spearman’s correlation coefficient = 0.648; p = 0.043). The mean CBF of sphenoid mucosa was 10.50 ± 2.20 Hz.

Keywords: Cilia, ciliary beat frequency, ciliary wave disorder, electron microscopy, image analysis system

Introduction

The mucociliary clearance system, which is composed of cilia, mucus and periciliary fluid, is an integral part of the airway defense for eliminating inhaled particles. The overall propulsive effect of ciliary beating depends upon the arrangement and length of the cilia, their metachronal relationships and the ciliary beat frequency (CBF) [1].

Lots of methods have been developed for measuring the CBF, including the cinematographic method, the photoelectric method and the laser light-scattering method. Among these, the photoelectric method, which comprises a laser light and a fiberoptic probe, is most widely used [2–4]. However, in contrast to the efforts expended to measure the CBF, relatively little attention has been paid to ciliary wave disorder (CWD), another important prerequisite for effective ciliary movement. There have only been a few studies of CWD [5–7], all of them using electron microscopy (EM).

CWD can be defined as the degree of variation in the direction of beating of individual cilia. A greater than normal value of CWD, i.e. ciliary disorientation, can also lead to impairment of mucociliary transport, as does a decrease in the CBF. It has been reported that ciliary disorientation may occur in patients with various diseases, such as primary ciliary dyskinesia [5,7], asthma [7] and bronchiectasis [7]. The photoelectric method, despite having the advantage of being able to measure ciliary motility in vivo, has difficulty in measuring CWD due to sensor...
limitations [8]. CWD has previously been measured [5] by examining cross-sections of cilia using a transmission electron microscope. However, the EM method has some disadvantages: it is relatively expensive and the procedures involved are complicated. Furthermore, it cannot measure the CWD of actively beating cilia.

We [9] have recently developed a method—which we have called the image analysis (IA) system—that can determine the wave directions of multiple cilia beating in culture media. With IA, the principal axis of inertia is applied to the 2D correlation map calculated from sequential ciliary images to determine the uniphase lines of beating cilia. Based on this, IA can determine the wave direction of individual cilia and the variation in wave directions of multiple cilia, i.e. the CWD. Compared with the EM method, IA is simple, less time-consuming and above all can simultaneously measure the CBF and CWD of actively beating cilia.

The aim of this study was to measure the CBF and CWD in normal human mucosa using IA and to validate the new method by comparing the measured CWDs with those measured by EM.

Material and methods

Subjects

Sphenoid sinus mucosa was harvested during operation from 10 patients (6 males, 4 females; mean age 42.1 years; range 10–78 years) who underwent pituitary tumor removal via a trans-septal trans-sphenoidal approach and immediately immersed in warm saline. None of the patients had any evidence of upper respiratory tract infection. Harvested sinus mucosa was cut into two 4 × 4 mm² pieces.

Preparation of sinus mucosa

One piece of harvested mucosa was stabilized by incubation in Dulbecco’s modified Eagle’s medium (Gibco BRL, Grand Island, NY) and placed in a CO₂ incubator at 37°C for 1 h before recording. The mucosa was then placed on a culture dish. The temperature in the culture dish was maintained at 37°C throughout the experiment using a specially designed heating chamber incorporated into a temperature regulator.

The other piece of mucosa was fixed in 2.5% glutaraldehyde, post-fixed in osmium tetroxide, dehydrated in a graded alcohol series and embedded in Epon. Thin sections (75 nm) were cut using an ultra-microtome and stained with uranyl acetate and lead citrate.

Measurement of CWD by EM

The ciliary beat observed using an immersion microscope at a magnification of ×400 was recorded using a charge-coupled device camera (Digistar; Xomed, Jacksonville, FL). The analog signal recorded on a video monitor was sent to a PC, which digitized and analyzed the signal using a program developed in-house based on the fast Fourier transform [9]. At this magnification, the length of one pixel was equal to 0.025 μm. To extract the gray-intensity variation correlated with the ciliary beat, the whole field was divided into smaller rectangular blocks of equal size (spots). A spot size of 2 × 2 pixels was used. The digitized images for each specimen were acquired at 30 Hz for 4.27 s (128 frames), which is sufficient to extract brightness intensity variations. This change in brightness intensity from the selected region of ciliated mucosa yielded a dominant peak in the power spectrum estimated using fast Fourier transform. The frequency of this dominant peak of intensity variations is the CBF of the corresponding point of the ciliated mucosa (Figure 1).

From this variation of intensities, the phase difference of a spot relative to the reference spot was calculated and represented as a correlation value ranging from 1 [phase difference is 0 (0°)] to −1 [phase difference is π (180°)]. The correlation values of all the spots relative to the reference spot yield a correlation map, which represents the directions of uniphase lines at each spot. According to the principal axes of inertia, the direction of ciliary beating is perpendicular to the uniphase line. The SD of these directions in a region of interest (ROI) was defined as the CWD for the corresponding area (Figure 2).

To determine an appropriate size for the ROI, we measured the CBF and CWD for ROIs of various size in three subjects. The mean values of the CBFs and CWDs for 10 different fields of each specimen were calculated for ROIs ranging from 20 × 20 to 200 × 200 pixels. As the size of the ROI increased, the CWDs increased gradually and converged on the subject-dependent upper limit. The CBFs did not show any significant changes as the size of the ROI increased. CWDs exceeded 90% of their upper limit at a ROI of 80 × 80 pixels (20 × 20 μm²) in all subjects (Figure 3). On the basis of these preliminary data, we measured the CBFs and CWDs of 5 different fields for a ROI of 80 × 80 pixels (20 × 20 μm²) in this study.

Measurement of CWF by IA

Photomicrographs of cilia sectioned near the root were taken at a magnification of ×10 000. The
sectioned level was confirmed by the presence of microvilli. The ciliary orientation was determined for all cilia in which two central microtubules could be seen. The ciliary axis was determined by drawing lines through the centers of two central microtubules of each cilium (Figure 4). Ciliary angles were measured automatically with the aid of computer software (Matlab version 6.5; The MathWorks Inc., Natick, MA). The software was programmed to calculate the angle \( \theta \), defined as the ciliary angle, between the ciliary axis and an arbitrary reference line. The direction of ciliary beating is known to be perpendicular to the ciliary axis. The SD of ciliary angles can be defined as the CWD [6]. A mean of 102.5 (range 48–136) cilia were analyzed in each specimen.

**Statistical analysis**

Differences between CBFs and CWDs according to age or sex were analyzed using the Kruskall–Wallis test. Correlations between the CBF and CWD measured using IA and between the CWDs measured by IA and EM were analyzed using

Figure 1. Schematic illustration of the IA system. Ciliary motility is observed and recorded using a microscope and VCR. A PC equipped with an image-grabbing board converts the VCR signals into digital images and the CBF and CWD are measured using analysis software.

Figure 2. (A) A color-scaled map of the CBF distribution, a specific color representing a specific beat frequency. The histogram shows the CBF distribution in this field. (B) The correlation map shows the color-coded phase differences relative to the reference spot. Spots of the same color have the same phase difference (uniphase line). **Upper panel:** the white line perpendicular to the uniphase line is the direction of wave propagation at the red spot.
Spearman’s rank correlation. For all statistical procedures, SPSS for Windows version 10.0 (SPSS Inc., Chicago, IL) was used. \( p < 0.05 \) was considered being statistically significant.

Results

The mean value of the CBF measured using IA was \( 10.50 \pm 2.20 \) Hz (Table I), and there was a large variation in the CBFs measured (4.93–13.14 Hz). There was no significant difference in CBF according to the age or sex of the subjects.

The mean values of CWD measured by IA and EM were 28.25 \( \pm 4.84 \) and 23.59 \( \pm 8.16 \), respectively (Table I). CWD measured by EM showed a relatively high inter-subject variation ranging from 16.82 to 40.45°. CWD ranged from 22.42 to 35.92° when measured by IA.

CWDs measured by IA and EM were almost the same in most cases. There was a significant correlation between the CWDs measured by these two methods: Spearman’s correlation coefficient = 0.648; \( p = 0.043 \) (Figure 5).

There was no significant difference in CWD in relation to the age or sex of the subjects and there was no significant correlation between the CBF and CWD determined for each subject.

Discussion

In this study, the mean CWD measured by IA was greater than that measured by EM. This difference might be due to the difference in the size of the ROIs used in these two methods. The average number of cilia in each \( 8 \times 8 \) \( \mu \text{m}^2 \) area of ciliated epithelium is known to be \( \approx 300 \). The mean number of cilia analyzed by EM in this study was 102.5, equivalent to an ROI of \( <4 \times 8 \) \( \mu \text{m}^2 \), which is less than one-tenth the size (20 \( \times \) 20 \( \mu \text{m}^2 \)) of that analyzed by IA. This result suggests that a sufficiently large ciliary population is important to obtain a meaningful value of CWD, as proposed previously [10]. EM, which analyzes CWD using photomicrographic images of cilia, determines structural aspects of ciliary orientation, compared to IA, which measures the CWD of actively beating cilia. Another possible explanation for the discrepancy between CWDs measured by the two methods is the presence of interference between the actively beating cilia in vivo when measured by IA. Such interference may change the direction of beating cilia. It may be suggested that CWD measured by IA is a better representation of the real state of actively beating cilia than that measured by EM. IA is superior to EM in that it can also measure the CBF, another important factor in ciliary motility, of actively beating cilia in an easier and simpler manner.

The mucociliary clearance system can be evaluated functionally or structurally. The CBF, CWD
and mucociliary clearance time are functional parameters of the mucociliary system. Ciliary ultrastructural defects, including compound cilia, absence of a dynein arm and central and peripheral microtubule defects, are structural abnormalities which can cause a decrease in ciliary beating, ciliary disorientation and prolongation of the mucociliary clearance time. Following the first report [11] of defective ciliary function and structure as an etiologic factor in chronic airway infection and male sterility, various ciliary ultrastructural defects in patients with primary ciliary dyskinesia or Kartagener’s syndrome have been identified [12–14]. Although lots of studies have focused on structural abnormalities, it was revealed that abnormal ciliary function does not always seem to accompany abnormal ciliary structure [15–17]. As suggested by Rutland and de Iongh [17], ciliary disorientation can also be a factor in ciliary dyskinesia, independent of ultrastructural defects. Hence, in addition to a structural study, analysis of ciliary function, including the CBF and CWD, is needed for an exact evaluation of respiratory tract diseases resulting from abnormal mucociliary clearance.

It was reported [18] that there is little variation in CWD as measured by EM according to the level of the ciliary shaft. Theoretically, however, when the cilia are sectioned far from the surface of the epithelia, the cut surface can be oblique due to deflection of the cilia and there can be some bias in the CWD determined. To reduce this possible error, we tried to measure CWD from photomicrographs taken as near to the base of the ciliary shaft as possible.

The CBF and CWD for each subject did not show any significant correlation. However, Joki et al. [19] showed a correlation between ciliary orientation and CBF in a semi-quantitative manner using scanning electron microscopic images. This discrepancy can be attributed to the different methods used to measure ciliary disorientation.

**Conclusions**

We measured the CBF and CWD using the newly developed IA system. We demonstrated a strong positive correlation between CWDs measured by IA and conventional EM. IA, a simpler and easier method than EM and one that can measure both the CBF and CWD of actively beating cilia, would seem to be a powerful tool for evaluating the mucociliary clearance system of the respiratory mucosa.

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