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Original Article

Evaluation of ciliary functions and ciliary beat frequency via cell culture method in patients with primary ciliary dyskinesia

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ABSTRACT

Background. Cell culture increases both diagnostic specificity and sensitivity of primary ciliary dyskinesia (PCD) and the most important reason to use cell culture for definitive diagnosis in PCD is to exclude secondary ciliary defects. Here we aimed to evaluate the cilia functions and cilia ultrastructural abnormalities after ciliogenesis of cell culture in patients with definitive diagnosis of PCD. We also aimed to compare high speed videomicroscopy (HSVM) results of patients before and after ciliogenesis and to compare them with electron microscopy, genetic and immunofluorescence results in patients with positive diagnosis of PCD.

Methods. This study was conducted as a cross-sectional study in patients with PCD. HSVM, transmission electron microscopy (TEM) and immunofluorescence staining results of the nasal biopsy samples taken from patients with the definitive diagnosis of PCD were evaluated and HSVM findings before and after cell culture were described.

Results. Ciliogenesis and regrowth in the cell culture occurred in the nasal biopsy sample of eight patients with PCD. The mean age of the patients was 15.5±4.2 years (8.5-18 years). Mean beat frequency was found to be 7.54±1.01 hz (6.53-9.45 hz) before cell culture, and 7.36±0.86 hz (6.02-7.99 hz) after cell culture in the nasal biopsy of patients. There was no significant difference in the beat frequency of PCD patients before and after cell culture. Ciliary function analysis showed the similar beating pattern before and after cell culture in patients with PCD.

Conclusions. This study showed us that there was no difference between cilia beat frequency and beat pattern before and after cell culture in patients with definitive diagnosis of PCD and repeated HSVM would be a useful diagnostic approach in patients who have no possibility to reach other diagnostic methods.

Key words: primary ciliary dyskinesia, cell culture, high speed videomicroscopy, cilia function analysis.

Primary ciliary dyskinesia (PCD) is a rare genetic disease caused by congenital abnormalities in both structure and function of the motile cilia characterized with recurrent upper and lower airway infections. Ciliary dysfunction leads to impairment of the mucociliary transport and this is the leading cause of chronic respiratory infections and progressive lung disease since the first years of life. Early diagnosis and treatment of the disease can prevent the development of bronchiectasis.[1-3]

There is no "gold-standard" test for PCD and guidelines recommend to use the combination of different methods for early diagnosis of these

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patients. According to European Respiratory Society (ERS) taskforce, in patients with strong clinical suspicion, it is necessary to demonstrate the hallmark of ciliary ultrastructural defects on transmission electron microscopy (TEM), or pathogenic biallelic mutations in PCD causing genes for positive diagnosis of PCD. Otherwise, low nasal nitric oxide (nNO) results plus abnormal high-speed video microscopy analysis (HSVM) findings on three occasions or following cell culture even with normal TEM make PCD 'highly likely'.[4-6] In a subset of patients, the features of ciliary dysmotility only became apparent after ciliogenesis in cell culture. Thus, cell culture increases both diagnostic specificity and sensitivity of PCD and this method may help to reduce false-positive diagnosis in patients with secondary ciliary dysfunction.[7-9] The use of cell culture for PCD diagnosis has been developed by Jorissen et al. using a submerged cell culture system.[10-12]

The most important reason to use cell culture for definitive diagnosis in PCD is to exclude secondary ciliary defects. Here, we aimed to evaluate the cilia functions and cilia ultrastructural abnormalities after ciliogenesis of cell culture in patients with definitive diagnosis of PCD. We also aimed to compare HSVM results of patients before and after ciliogenesis and to compare them with electron microscopy results in patients with a positive diagnosis of PCD.

Material and Methods

This study was conducted as a cross-sectional study in patients with PCD. In clinical practice, nasal NO measurement, ciliary functional analysis with HSVM and genetic tests are being used in the evaluation of patients with PCD in our Department of Pediatric Chest Diseases. This study was approved by the local institutional review board and supported in part of the University Scientific Research Committee Project with number THD-2016-9044. Informed consents were obtained from the children and parents.

Children between the ages of 6-18 years who had definitive PCD diagnosis based on clinical, radiological findings, nasal NO, HSVM and genetic analysis according to ERS guidelines from September 2016 to December 2017 were included to study. Children with other chronic lung diseases and who were highly likely to be diagnosed with PCD according to ERS guidelines were excluded.

At the same time, two different nasal biopsies and nasal brushing samples were obtained from the inferior nasal concha into the isotonic saline and gluteraldehyde solution by punch biopsy method in the Ear Nose and Throat Department from children who were symptom free for two weeks before the date of nasal biopsy.

In this study, HSVM, TEM and Immunofluorescence staining results of the nasal biopsy samples taken from patients who were followed up with the definitive diagnosis of PCD were evaluated and obtained results were analyzed through Matlab software. Additionally, HSVM findings before and after cell culture were described and these findings were compared with TEM and genetic or Immunofluorescence staining results.

Cell culture method

After the nasal biopsies were obtained, the tissues were washed with saline in a petri dish, to remove debris and blood. Cells were first grown in a monolayer to expand the basal cell population without cilia (dedifferentiation). After three weeks the cells reached confluency and ciliated cells disappeared, cells were then transferred to a suspension medium to induce redifferentiation into ciliated epithelial cells (ciliogenesis). After two weeks of suspension culture functional cilia reappear on the spheroids and these ciliated aggregates can be kept in culture for more than several months. Jorissen et al.[13] developed this method and this process was performed according these rules.

Cilia Function Analysis with High Speed Videomicroscopy

Ciliary functions including cilia beating pattern and beat frequency (CBF) were analyzed by HSVM before the cell culture process started. After six weeks of cell culture, cilia beating pattern was assessed with HSVM and cilia beat frequency was measured. An inverted microscope was used and images were acquired by a high speed camera, connected to the microscope. For every sample at least three or four regions with a colony of ciliated cells were included. The CBF value was computed using Matlab software. The CBF value was expressed as a histogram and the mean CBF value of this histogram was used as the result for one CBF measurement (reference values obtained in our laboratory were CBF 12 Hz, SD 0.8 at 37°C). Cilia beat pattern was categorized as hypokinetic cilia, hyperkinetic cilia, stiff pattern, and abnormal circular movement according to HSVM motion analysis.

Electron Microscopy Analysis

The biological samples from patients (both cultured cells and biopsy samples) were fixed in 2.5% phosphate buffered glutaraldehyde solution for 1 hour at room temperature. Samples were postfixed with 1% osmium tetroxide in the dark for 30 minutes. After washing and centrifuging, pellets of cells were embedded in 37°C warm agar. Eventually, both agar-embedded cells and tissue samples were dehydrated in graded alcohols. The samples were cleared using propylene oxide and embedded into araldite. The samples were polymerized at 60°C for 48 hours. Semi-thin and thin sections were obtained from the plastic blocks. Sections stained with uranyl acetate and lead citrate were analyzed under transmission electron microscopy (TEM) (JEOL, JEM 1400 attached with a Gatan Orius SC 1000 CCD camera). Defects in the outer dynein arms, outer and inner dynein arms, inner dynein arms with microtubule disorganization, radial spokes, or central apparatus provided confirmation of PCD diagnosis according to ERS guidelines.[14]

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 23.0 (SPSS Inc., Chicago, IL, USA). Continuous variables were presented as a mean ± standard deviation (SD) for normal distribution or median (min-max) for non-normal distribution.

Results

A total of 43 patients were included in the study. The cell culture process was terminated in seven patients due to infected cells during the procedure. Cilia regrowth did not occurr in 28 patients during the cell culture procedure at the sixth week.

Ciliogenesis and regrowth in the cell culture occurred in the nasal biopsy sample of eight patients with PCD. Clinical characteristics of these patients are shown in Table I. The mean age of the patients was 15.5 ± 4.2 years (8.5-18 years). Mean beat frequency was found to be 7.54 ± 1.01 hz (6.53-9.45 hz) before cell culture, and 7.36 ± 0.86 hz (6.02-7.99 hz) after cell culture in the nasal biopsy of eight patients with proliferation in cell culture. There was no significant difference in the beat frequency of PCD patients before and after cell culture. Ciliary function analysis showed a similar beating pattern before and after cell culture in patients with PCD. Table II and Figure 1 show the distribution of ciliary beat frequency in these patients.

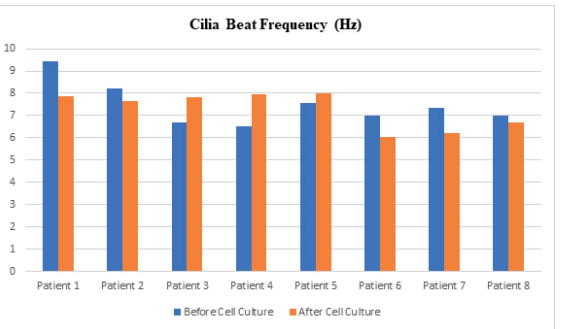

Fig. 1. Distribution of cilia beat frequency in patients with PCD.

Table I. Clinical characteristics of patients with PCD.

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8
Age at diagnosis (years)	12	14	8	12	11	8	13	12
Gender	Female	Male	Female	Female	Female	Female	Female	Female
Neonatal respiratory distress	Positive	Positive	Negative	Positive	Positive	Positive	Positive	Positive
Chronic rhinitis	Positive	Positive	Positive	Negative	Positive	Positive	Positive	Positive
Recurrent sinusitis	Positive	Positive	Positive	Negative	Negative	Negative	Positive	Positive
Recurrent otitis	Positive	Negative	Negative	Positive	Negative	Negative	Negative	Positive
Situs inversus totalis	Positive	Negative	Negative	Positive	Negative	Negative	Positive	Positive
Bronchiectasis	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Nasal NO (ppb)	12 ppb	10 ppb	8 ppb	6 ppb	15 ppb	15 ppb	5 ppb	5 ppb

Table II. HSVM and electron microscopy results of PCD patients grown in cell culture.

	Electron Microscopy				HSVM			Genetic and IF findings
Case	Central complex defect	Microtubular disorganization	Inner dynein arm defect	Outer dynein arm defect	CBF before cell culture (Hz)	CBF after cell culture (Hz)	Cilia beat pattern	
1	Negative	Negative	Negative	Positive	9.45±1.22	7.85±1.86	Hypokinetic	DNAH5
2	Positive	Positive	Positive	Positive	8.2±1.02	7.64±0.79	Hypokinetic, stiff pattern	New mutation
3	Negative	Negative	Positive	Positive	6.69±0.81	7.83±0.73	Hypokinetic	DNAI1
4	Negative	Negative	Negative	Positive	6.53±0.68	7.96±0.29	Hypokinetic	DNAH5
5	Positive	Positive	Negative	Negative	7.58±0.56	7.99±0.21	Hypokinetic, stiff pattern	RSPH4A
6	Positive	Positive	Negative	Negative	7.01±0.51	6.02±0.61	Hypokinetic	RSPH4A
7	Negative	Negative	Negative	Positive	7.33±1.03	6.22±0.91	Hypokinetic	DNAH5
8	Negative	Positive	Positive	Negative	7.01±1.02	6.68±0.84	Hypokinetic	CCDC40

HSVM: high speed videomicroscopy, IF: immunofluorescence

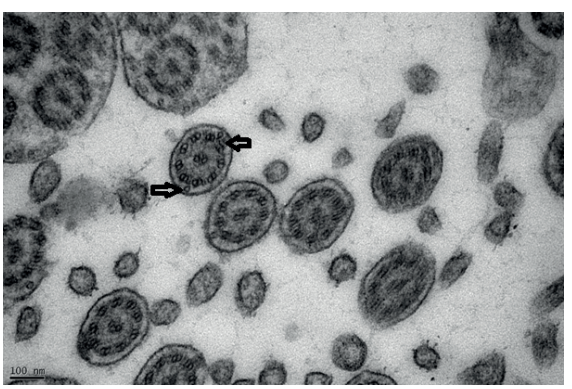

Fig. 2. In the sample of a patient, cilia ultrastructure was observed: The central and peripheral microtubule structures (9 + 2) were not in the normal structure with extratubules (marked by arrow) located in the periphery; electron micrograph (X100000 magnification; Uranyl acetate & Lead citrate).

In TEM, while some of the ciliated cells had no inner or outer dynein protein arms or were deficient, in others there was no microtubule placement (9 + 2) in the periphery and central. It was observed that some of them had an extra tubule structure or the missing tubules (Fig. 2).

TEM findings and HSVM findings in nasal biopsy of cell culture compared with genetic results and Immunofluorescence staining are also indicated in Table II.

Discussion

Cell culture method of nasal biopsy specimens may help to reduce false-positive diagnoses in patients with secondary ciliary dysfunction

(SCD) and confirm the diagnosis of PCD. Recent data demonstrate that cell culture method has almost 100% sensitivity and specificity in differentiating PCD and acquired ciliary dyskinesia.[12,15] This study showed that there was no difference between cilia beat frequency and beat pattern before and after cell culture in patients with definitive diagnosis of PCD. In all cases, dyskinesia associated with PCD was unchanged or became more prominent. This is the first study in our country using a highly specialized and time-consuming method for evaluating the cilia functions and electron microscopy results of PCD patients after the ciliogenesis of cell culture.

Previous reports which used the cell culture method introduced by Jorissen et al.[16] had different success rates.[7,13,16] In this method, monolayer culture and suspension culture procedures were used which takes a longer time of almost six weeks. Abnormalities secondary to respiratory infection and toxic agents disappear and SCD and PCD could be clearly distinguished. Boon et al.[9] found that the success rate of the culture developed by Jorissen et al.[16] was 75%, which was higher compared with other cell culture methods. However, using a monolayer culture technique, Pifferi et al.[17] could not culture sufficient ciliated tissue for a PCD diagnosis. Because of these reasons, alternative methods for growing ciliated cells in culture by exposing cells to an air-liquid interface have been developed and used. The air-liquid interface culture of nasal samples yields more cilia than the suspension culture technique that enables the cilia growth for definitive diagnosis. Hirst et al.[12] found 54% of success rate in their investigation with an air liquid interface culture method. Hirst et al.[12] also reported 43% of successful cilia regeneration in their patients with PCD and 26% success rate in their non-PCD samples on exposure to an air-liquid interface. The success rate of our study was 28% in patients with PCD which may be related with the vast majority of patients referred for diagnostic testing of PCD have chronic nasal symptoms and this increases the chance of losing the culture growth to a secondary infection and ciliary cell shedding.

It was also shown that similar beating patterns were revealed in patients with PCD before and after ciliogenesis. Hirst et al.[12] and Pifferi et al.[18] reported that ciliary function was shown as abnormal before and after cilia cell culture in all subjects with PCD similar to our study. However Boon et al.[9] reported that initial evaluation of the ciliary coordination and ciliary beat frequency in the biopsy was normal in 10.2% of patients with a final diagnosis of PCD. In our study all of the patients with PCD had abnormal beating patterns before the cell culture. Jorissen et al.[16] also found beat frequency results similar to our study before and after ciliogenesis. They found that the mean CBF was 8.4±1.6 Hz in the nasal biopsy materials before cell culture; after the suspension culture the mean CBF was 8.6±0.9 Hz.[16] There was no difference within the cilia beat frequency before and after cell culture. Our results also support these analysis which we found that the mean CBF was 7.54 ± 1.01 Hz before cilia cell culture, and 7.36 ± 0.86 Hz after cilia cell culture. However, Hirst et al.[19] found that CBF decreased in patients with PCD after the cell culture which was different from the previous studies.

TEM of cilia is a time-consuming method and needs experienced people in this area and also cilia ultrastructural analysis requires expensive equipment; but it is not available in all centers. This method is highly contributive to diagnosis of PCD although ultrastructure of cilia is normal in 21% of PCD patients.[20] This study showed that cilia functions were compatible with cilia ultrastructural defects in patients with PCD, here we also confirmed the results with genetic analysis of these patients. Despite the small number of patients in this study, these results showed that repeated HSVM would be a useful diagnostic approach in patients who have no possibility to reach other diagnostic methods. However, when HSVM is performed in specialized centers, in conjunction with

TEM evaluation, it will increase the diagnostic accuracy. Also we compared these results with the genetic and Immunofluorescence staining findings which is one of the strengths of our study.

A combination of functional and ultrastructural evaluation of the cilia before and after ciliogenesis seems to be the best approach for a PCD diagnosis.[8] The most important limitation of this study was the termination of cell culture procedure in some of the nasal biopsy samples due to infection-related problems. Because of recurrent infections in patients and low proliferation of ciliated cells in the cell culture, the success of this procedure was lower compared with previous reports. Furthermore during the ciliary deterioration and ciliogenesis, the most important thing is the regrowth of cilia again. Also, this technique is time consuming, invasive, requires significant expertise in cell culture, and as such is unlikely to be widely available outside of specialist diagnostic centers.[12] During TEM analysis, among the evaluated patients with cytoplasmic cilia like extensions in small microscope magnification, these findings were consistent as metaplasia in the respiratory epithelium and thickening of the stromal layer in these patients, which was another limitation of this study.

In conclusion, this study has provided an important diagnostic method for patients who are positive to be definitive PCD if ciliary function analysis is available and suggestive for the disease, but cannot be diagnosed with the causes of difficulties in diagnostic methods. Also, successful ciliated cell culture from the nasal biopsy samples will reduce the need to perform repeated biopsies in a number of patients. The results obtained from this study will, then, be used for early diagnosis of patients with PCD to prevent progression of disease complications such as bronchiectasis and respiratory failure, increasing the life span and quality of life of the patients, decreasing hospitalization and drug use costs.

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Ethical approval

This study was approved by Hacettepe University local institutional review board with number GO 15/638-14.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: NE, RH, UÖ, ÖG; .data collection: BK, EB, PA, GDT, SEP, MGH; analysis and interpretation of results: NE, UÖ, DD, EY, NK, ÖG; draft manuscript preparation: NE; RH, UÖ, DD, EY, NK, PA. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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