ABSTRACT

Background: Eleven years ago we had described three patients with missing nexin links as a possible cause of primary ciliary dyskinesia (PCD). The assumption was substantiated last year by finding a mutation in these patients.

Materials and Methods: We counted the nexin links, inner (IDA) and outer (ODA) dynein arms and microtubuli in each of, if possible, 50 cilia in 41 patients with normal cilia, 4 patients with deficiency of nexin links only and 4 with deficiency of nexin links and IDA.

Results: In the control group the median number of nexin links was 4.5 per cilium, range 3.4–5.3. In the second group the mean numbers of nexin links per cilium were 1.1–1.4, in the third group 0.8–1.2, per patient. The median number of IDA was in the control group 4.2, range 3.3–5.2. In groups 2 and 3 the numbers were 3.0–3.5 and 0.2–1.0, respectively. Numbers of ODA were normal in all groups.

Conclusions: It is possible to reliably count the number of nexin links in nasal human cilia and to distinguish cases with missing nexin links from normal controls.

Keywords: Electron microscopy, inner dynein arms, nasal cilium, nexin links, primary ciliary dyskinesia

At standard electron microscopy of human nasal cilia, it is not possible to see all details in every cilium. As a rule we find 4–5 IDA in normal cilia. We therefore count the dynein arms in 50 cilia to get a reliable mean value. At IDA deficiency we find <0.6 IDA per cilium [1]. Other authors have previously and later had the same experience [2–5]. More IDA can be found with computer-assisted analysis of the cilia [6]. It is still more difficult to see all nexin links in a cilium. As described earlier, three patients had missing nexin links which is a possible cause of PCD [7]. The suspicion was substantiated by the finding last year of a mutation in these patients, giving a defective assembly of the nexin-dynein regulatory complex [8]. We did not count the number of nexin links neither in the cilia of these three patients, nor in normal cilia in our previous paper [7]. It is therefore the aim of the present paper to count in the transmission electron microscope the number of nexin links, IDA and ODA per cilium in a control group of patients with normal cilia, and in two groups of patients with missing nexin links, namely at deficiency of nexin links only and at combined deficiency of nexin links and IDA.

MATERIALS AND METHODS

Biopsies

Nasal brush biopsies were taken by the clinician. They were fixed in a fresh solution of 2%
glutaraldehyde containing 2 mM MgSO₄ in 0.1 M sodium cacodylate buffer with 0.1 M sucrose at pH 7.2. The biopsies were post-fixed in 2% OsO₄ (dissolved in distilled water) and 0.1 M sodium cacodylate buffer pH 7.2 (1:1). After dehydration in graded ethanol they were embedded in agar resin 100. In semi-thin sections, areas were chosen at light microscopy so the cilia could be cut both parallel and at right angle to the surface. Ultrathin sections of 60 nm were contrasted with 4% uranyl acetate for 25 min at 40°C followed by Reynolds lead citrate for 2 min. They were examined in a Tecnai Spirit BioTWIN (FEI Company, Hillsboro, OR) 120 kV transmission electron microscope at a magnification of 60,000–90,000×. Numbers of nexin links, IDA, ODA and microtubuli were counted in, if possible, each of 50 cilia per biopsy. Down to 30 cilia were accepted. The orientation of the cilia was set down. Spokes and sheaths were also scrutinized. At least two biopsies, taken with an interval of more than six months, were examined in cases with PCD.

Statistics

A 2-tailed paired-samples t-test was conducted at comparison of numbers of nexin links and IDA in group 1. Comparison of number of nexin links and IDA between groups was evaluated with Satterthwaite’s t-test. The calculations were performed using Stata v13.1 (Stata Statistical Software: Release 13. (College Station, TX). All p values are two-sided.

Patients

There were three groups of patients. The first group consisted of 41 patients from 2013 with normal cilia, 23 males and 18 females. Mean age was 12 years, range 2 months – 70 years. The second group comprised totally four patients with deficiency of nexin links, three of them previously described with clinical data in agree with PCD [7] and gene analysis [8]. Two of them were male siblings, age 3 and 5 years at biopsy, the first two in group 2, Table 1. The third patient was a girl aged 14 and the fourth a girl 11-year-old. Four patients with combined deficiency of nexin links and IDA made up the third group. Two of them were siblings, a boy aged 9 and a girl aged 10, the first two in group 3, Table 1. The boy has situs inversus. The two others were a boy aged 8 and a girl aged 10. According to the brief remittances all patients had trouble with repeated respiratory tract infections, also the controls. We had no access to the case histories of the patients except for three patients in group 1 [7,8].

RESULTS

The results are summarized in Tables 1 and 2 and illustrated by Figures 1–3. The nexin links may be difficult to see. Nevertheless we found 4–5 in most cilia considered to be normal (Table 1 group 1, Figure 1). In those cilia deficient in nexin links, one structure judged to be a nexin link was observed in most cilia (Table 1, groups 2 and 3, Figure 2). It was often difficult to be quite sure about their possible presence. There was a statistical difference in number of nexin links in group 1 compared to each of groups 2 and 3 (p < 0.001; Table 2). The number of IDA in group 2 with deficiency of nexin links, mean 3.32 (Table 2), was in the lower range of what may be considered normal (range 3.3–5.2; Table 1), however the difference was statistically significant (p < 0.001; Table 2). The numbers of ODA were normal in all groups, as were the numbers of microtubuli. Spokes were difficult to see in group 2. It was not the aim of the present paper to perform a careful analysis of the spokes. There was an axonemal

| Group and structure | Mean | Standard deviation |
|---------------------|------|--------------------|
| Group 1, nexin links | 4.47 | 0.48               |
| Group 2, nexin links | 4.25 | 0.13               |
| Group 3, nexin links | 1.05 | 0.17               |
| Group 1 versus 2, p < 0.001 | 0.22 |                   |
| Group 1 versus 3, p < 0.001 | 0.36 |                   |
| Group 2 versus 3, p = 0.12 | 0.14 |                   |
| Group 1, IDA | 4.24 | 0.46               |
| Group 2, IDA | 3.32 | 0.24               |
| Group 3, IDA | 0.70 | 0.36               |
| Group 1 versus 2, p < 0.001 | 0.54 |                   |
| Group 1 versus 3, p < 0.001 | 0.66 |                   |
| Group 2 versus 3, p < 0.001 | 0.42 |                   |
| Group 1 nexin links versus group 1 IDA, p = 0.02 | 0.02 |                   |

| Group | Number of |
|-------|-----------|
|        | Nexin links | IDA | ODA | Patients |
| Normal cilia, median and range. | 4.5 | 4.3 | 8.4 | 41 |
| Nexin link deficiency. | 3.4–5.3 | 3.3–5.2 | 7.6–8.7 | 4 |
| Mean of each patient. | 1.2 | 3.0 | 8.7 | |
| 1.1 | 3.5 | 7.9 | |
| 1.3 | 3.5 | 8.6 | |
| Nexin link and IDA deficiency. | 1.2 | 0.2 | 8.4 | 4 |
| Mean of each patient. | 0.8 | 0.9 | 8.1 | |
| 1.1 | 0.7 | 8.3 | |

TABLE 1. Summary of results.

TABLE 2. Statistical data and analysis.
disorganization of the microtubules in most but not all of the cilia in groups 2 and 3, especially in group 3 (Figure 3). Likewise the orientation of the cilia was mostly random in these two groups, especially in group 3.

**DISCUSSION**

Afzelius [9] once listed 13 ultrastructural variants of PCD, one of them: All 9 + 2 microtubules in disarray; maybe the nexin links are defective. He wrote (personal communication in 2001) that it is difficult to see the nexin links in nasal cilia. We have encountered four patients with symptoms of PCD and with lack of nexin links (Table 1 group 2; Figure 2). Three of them, the first 3 in Table 1, were described 11 years ago [7]. With a superior electron microscope and ten more years of experience we have been able to count the nexin links per cilium. There was a large difference in number of nexin links in the deficiency groups (2 and 3) compared to the controls (Tables 1 and 2).

The microtubules were in disarray only in some areas in the patients with deficiency of nexin links, thus contrary to the claim of Afzelius [9] that all axonemes should be disorganized. The spokes were well preserved in the group with deficiency of nexin links only (Figure 2), as is also obvious from our previous figures [7].

Genetic analysis has been performed on the first three cases in group 2. A mutation was found in the gene *CCDC164*, encoding the nexin-dynein regulatory complex 1 [8], giving severe defects in its assembly and an abnormal ciliary movement. No other case has been submitted to gene analysis.

One type of PCD is characterized by combined deficiency of IDA and nexin links, and in some sections microtubular disarray (our group 3; Table 1; Figure 3). The spokes may also be absent [2,10–13]. In one of these papers the nexin links and IDA were counted in three patients. The mean numbers of nexin links per cilium were 0.6, 0.8 and 0.9. The numbers of IDA were 0.1–0.2 per cilium. Not all cilia displayed axonemal disorganization. Patients with other types of PCD and such without PCD had a median number of 2.4 and 2.2 nexin links per cilium, respectively.
The number of IDA in healthy persons was 2.2–4.6, median 3.2 [2].

It is interesting that in 1987 those authors [2] also found, as we did, some nexin links in a group with nexin link and IDA deficiency and fewer IDA than nexin links. We found slightly higher numbers (Table 1) that may be due to a more advanced electron microscope.

A large difference in number of nexin links is thus seen between cases with deficiency of the nexin links and normal cilia. It is generally advised to scrutinize 50 [5,14,15] or even 60 [4] transverse sections of cilia at diagnosis of PCD. In an early study 20 cilia were given as a minimum [2]. That was also sufficient in the present study to get a stable mean value of nexin links. At suspicion of transposition we examine 200 cilia.

Each laboratory has to find its own mean for number of nexin links in normal cilia and cut-off figure at deficiency of nexin links. Therefore median and range figures are important in the diagnostic work with cilia (Table 1). We use 2.0 as maximum figure for nexin link deficiency. More nexin links might be found with computer analysis and tomography. The importance of scrutinizing for nexin links was emphasized in a recent paper, reporting ultrastructurally normal cilia in PCD with mutations affecting the nexin-dynein regulatory complex [16].

The counting of nexin links is influenced by the lengthways distance between them of 96 nm [17], as our ultrathin sections are estimated to have a thickness of 60 nm. The location of the subunits of IDA within the 96 nm axoneme repeat is complex [18,19]. Interestingly in our control material the numbers of nexin links and IDA per cilium were similar (Table 1), perhaps a sign of their close relationship [8,13,20].

**SUMMARY**

It is possible to reliable count the number of nexin links in human nasal cilia. In normal cilia the number was 4.5 per cilium, in two different groups of patients with deficiency of nexin links 0.8–1.4. The difference between deficiency and normal cilia was substantial and statistically significant.

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**DECLARATION OF INTEREST**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper. The study was supported by a grant from the Royal Physiographic Society in Lund.

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