Abstract

Introduction: To study possible role of precorneal tear film derived nucleic acids as biomarker in Parkinson’s disease. Material and method: Precorneal tear fluid aspirated with the help of Whatman’s no 41 filter paper from 20 clinically diagnosed cases of Parkinson’s disease and 12 age and gender matched control patients were subjected to chemical extraction, purification, precipitation and finally quantitation by spectrophotometry. Results: The mean DNA Concentration of the patients without Parkinson’s disease was significantly higher than that of the patients with Parkinson’s disease ($t_{28} = 12.67; p < 0.0001$). The mean Yield/µg of tissue sample of the patients without Parkinson’s disease was significantly higher than that of the patients with Parkinson’s disease ($t_{28} = 12.42; p < 0.0001$). Conclusion: Precorneal tear fluid nucleic acid estimation may be used as a biomarker of Parkinson’s disease.

Keywords: Biomarker, Precorneal tear film, Nucleic acid, Parkinsonism.
demonstrated distinct relationship between level of extracellular DNA and human pathology like dry eye diseases\textsuperscript{10}. Protein misfolding or chaperon based Parkinson disease diagnosis are also possible\textsuperscript{11,12}. However this biophysical aspects of Parkinson’s disease has hardly penetrated the clinical niche. There are some other groups who suggested ophthalmological marker for Parkinson’s disease\textsuperscript{13}. We investigated tear fluid as a possible biomarker for Parkinson’s disease as there are some indications of co-occurrence of dry eye disease and Parkinson’s disease\textsuperscript{14}.

**Material and Method:**

To explore whether tear fluid nucleic acid can be utilized as a possible biomarker for the diagnosis of Parkinson’s disease; we did the study involving 20 diagnosed patients of Parkinson’s Disease between January 2016 to February 2017 and 12 age and sex matched normal control subjects. Patients were selected from the Neurology clinic, Medical College, Kolkata by simple random sampling method. Controls were selected from OPD, Regional Institute of Ophthalmology, Kolkata. Patients having atypical parkinsonism, Case with history of surgical intervention, chemical injury, complaints of ocular pain and discomfort, Those on contact lens wear, diabetes mellitus, Persistent epithelial defect were excluded from the study.

**Reagents:**

i. 1X phosphate buffered saline (PBS): 137 mMNaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4.
ii. Liquid nitrogen.
iii. DNA extraction buffer: 10 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA.
iv. 10% sodium dodecyl sulfate (SDS).
v. Proteinase K solution: 10 mg/mL of proteinase K in 50 mM Tris-HCl, pH 7.5; store at 4°C.
vii. Phenol equilibrated with 0.1 M Tris-HCl, pH 8.0.
viii. 3 M Sodium acetate, pH 5.2.
ix. 100% Ethanol.
x. 70% Ethanol.
xi. TE buffer: 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA.
xii. Agarose.
xiii. 1X TAE buffer: 40 mM Tris-acetate, 1 mM EDTA.
xiv. Ethidium bromide (10 mg/mL).
xxv. 10X Gel loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficoll (type 400) in 10X TAE buffer.
xxvi. DNA molecular weight marker.

**Equipments:**

i. Mortar and pestle.
ii. Water baths at 37, 50, and 55°C.
iii. Centrifuge.
iv. Spectrophotometer.
v. Horizontal gel electrophoresis apparatus.
vi. DC power supply.

**Methods:**

**Collection of Tear Specimen:**

Tears was collected using sterile Schirmer strips by making the person seated in a temperate room in comfortable posture with raised head, against any direct source of light or flow of air. The Schirmer strip i.e. No 41 Whatman filter paper 35mm×5mm was folded from one end and was then placed in the lower cul-de-sac region at the junction of medial two-third and lateral one third and was allowed to absorb the tear for 5 min in open eye condition. The length of the paper wetted over a set time of 5 minutes is an indication of tear volume. The strip was then placed in sterile vial at 270uC until processing. While using Schirmer’s the tear collected is considered as reflex tear as it will be collected with no local anaesthesia.

Since adequate amount of DNA was not available from tear fluid samples we had to go for conjunctival impression cytology and biopsy. Conjunctival impression cytology was taken under local anaesthesia with sterile cellulose acetate paper impregnated over bulbar and tarsal conjunctiva under aseptic conditions, in the OPD with the patient sitting comfortably in a chair with electric fans switched off. Conjunctival biopsies were taken in RIO OT under aseptic condition with conjunctival scissors under topical anaesthesia. Topical antibiotics applied after taking specimen.

**Sample Preparation:**

i. Solid tissue samples minced into small pieces (~1-2 mm) with a sterile scalpel blade.
ii. The small pieces of tissue were placed in a clean mortar filled with liquid nitrogen.
iii. Using a clean pestle, the frozen tissue pieces were
grinded to a powder while it is submerged in liquid nitrogen.

iv. The liquid nitrogen was allowed to evaporate, leaving a dry frozen tissue powder in the mortar.

**DNA Extraction:**

**Organic Extraction:**

1. The tear fluid will be subjected to centrifugation at 4000 rpm at 4°C for 15 minutes.
2. Add Proteinase K. to the supernatant from the previous step to digest proteins.
3. DNA that will be released into solution will get extracted with phenol-chloroform to remove proteinaceous material.  
4. DNA is precipitated from the aqueous layer by the additional of ice cold 95% ethanol and salt
5. Precipitated DNA will be washed with 70% ethanol, dried under vacuum and re-suspended in TE buffer.
6. The DNA so obtained will be run in agarose gel for the identification of abnormalities in terms of number of bands and band intensity

**Cell Lysis and Digestion:**

i. 920 µL of DNA extraction buffer was added to the tissue powder in the mortar, and gently mixed with the pestle. Then transferred to a 15-mL falcon tube by gentle pipetting
ii. 50 µL of 10% SDS was added to the mixture and mixed well; the solution became viscous.
iii. 30 µL of proteinase K solution was added to the viscous mixture. The cap was tightly closed and mixed vigorously by vortexing.
iv. Incubated in a 37°C water bath for at least 6 h; The tubes were gently tapped a few times during incubation.

**Phenol/Chloroform Extraction of DNA:**

i. Equal volume of equilibrated phenol was added, and mixed gently by inversion for 1 min with the cap tightly closed.
ii. Centrifuged at 1500rpm in a swinging bucket rotor at room temperature for 10 min.
iii. The upper clear aqueous layer was aspirated and transferred into another clean labeled tube.
iv. The phenol extraction (steps 1–3) was repeated one more time.
vi. The chloroform extraction step was also repeated one more time; to eliminate all of the phenol from the DNA sample.

**Purification and Precipitation of DNA**

vi. The chloroform extraction step was also repeated one more time; to eliminate all of the phenol from the DNA sample.

**DNA Quantitation by Spectrophotometry**

i. DNA sample was gently mixed by vortexing and inversion.
ii. 5 µL of the DNA sample was added to 495 µL of sterile water and mixed well.
iii. Diluted sample was taken in a quartz cuvet and absorbance at 260 and 280 nm was measured against a water blank.

**Gel Electrophoresis to Analyze DNA Quality:**

i. 0.8% agarose gel was prepared in 1X TAE buffer containing 0.5 µg/mL of ethidium bromide.
ii. aliquots of the extracted DNA samples were mixed with 5 µL of loading buffer, and loaded into a submerged well.

iv. Electrophoresed in 1X TAE buffer with 0.5 µg/mL ethidium bromide at 2 V/cm, until the dye front reaches the end of the gel.

Statistical Analysis

Statistical Analysis was performed with help of Epi Info (TM) 3.5.3. EPI INFO is a trademark of the Centers for Disease Control and Prevention (CDC). Using this software, basic cross-tabulation, inferences and associations were performed.

χ² Test was used to test the association of different study variables with the study groups. Z-test (Standard Normal Deviate) was used to test the significant difference between two proportions. t-test was used to compare the means. p<0.05 was taken to be statistically significant.
Results:

10 patients (33.3%) were without Parkinson’s disease and other 20 patients (66.6%) were with Parkinson’s disease. Thus the ratio of patients of the two groups was 1:2 (Table 1). Chi-square ($\chi^2$) test showed that there was no significant association between age and patients of the two groups ($p=0.14$) (Table 2). t-test showed that there was no significant difference between the mean age of the two groups ($t_{28}=0.55; p=0.58$). Thus the patients of the two groups were age matched. Corrected Chi-square ($\chi^2$) test showed that there was no significant association between gender and patients of the two groups ($p=0.78$) (Table 3).

### Table 1: Distribution of the patients in the two groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without Parkinson’s Disease</td>
<td>10</td>
<td>33.3%</td>
</tr>
<tr>
<td>With Parkinson’s Disease</td>
<td>20</td>
<td>66.7%</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>100.0%</td>
</tr>
</tbody>
</table>

### Table 2: Age distribution of the patients of the two groups

<table>
<thead>
<tr>
<th>Age Group (in years)</th>
<th>Without Parkinson’s Disease (n=10)</th>
<th>With Parkinson’s Disease (n=20)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>55 - 64</td>
<td>9(90.0%)</td>
<td>13(65.0%)</td>
<td>22(73.3%)</td>
</tr>
<tr>
<td>65 - 74</td>
<td>1(10.0%)</td>
<td>7(35.0%)</td>
<td>8(26.7%)</td>
</tr>
<tr>
<td>Total</td>
<td>10(100.0%)</td>
<td>20(100.0%)</td>
<td>30(100.0%)</td>
</tr>
<tr>
<td>Mean±s.d.</td>
<td>62.90±5.61</td>
<td>61.90±4.07</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>61.50</td>
<td>61.50</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>55 - 74</td>
<td>56 - 71</td>
<td></td>
</tr>
</tbody>
</table>

$\chi^2=2.13; p=0.14$ NS-Not Significant

### Table 3: Distribution of gender of the patients of the two groups

<table>
<thead>
<tr>
<th>Gender</th>
<th>Without Parkinson’s Disease (n=10)</th>
<th>With Parkinson’s Disease (n=20)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>7(70.0%)</td>
<td>13(65.0%)</td>
<td>20(66.7%)</td>
</tr>
<tr>
<td>Female</td>
<td>3(30.0%)</td>
<td>7(35.0%)</td>
<td>10(33.3%)</td>
</tr>
<tr>
<td>Total</td>
<td>10(100.0%)</td>
<td>20(100.0%)</td>
<td>30(100.0%)</td>
</tr>
</tbody>
</table>

$\chi^2 =0.07; p=0.78$ NS-Not Significant

### Table 4: Distribution of DNA Concentration1 of the patients of the two groups

<table>
<thead>
<tr>
<th>DNA Concentration1 (µg/µl)</th>
<th>Without Parkinson’s Disease (n=10)</th>
<th>With Parkinson’s Disease (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean±s.d.</td>
<td>0.77±0.09</td>
<td>0.16±0.05</td>
</tr>
<tr>
<td>Median</td>
<td>0.78</td>
<td>0.16</td>
</tr>
<tr>
<td>Range</td>
<td>1 - 1</td>
<td>0 - 0</td>
</tr>
</tbody>
</table>

### Table 5: Distribution of DNA Concentration2 of the patients of the two groups

<table>
<thead>
<tr>
<th>DNA Concentration2 (µg/µl)</th>
<th>Without Parkinson’s Disease (n=10)</th>
<th>With Parkinson’s Disease (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean±s.d.</td>
<td>0.78±0.14</td>
<td>0.17±0.05</td>
</tr>
<tr>
<td>Median</td>
<td>0.77</td>
<td>0.17</td>
</tr>
<tr>
<td>Range</td>
<td>0 - 1</td>
<td>0 - 0</td>
</tr>
</tbody>
</table>

### Table 6: Distribution of DNA Concentration3 of the patients of the two groups

<table>
<thead>
<tr>
<th>DNA Concentration3 (µg/µl)</th>
<th>Without Parkinson’s Disease (n=10)</th>
<th>With Parkinson’s Disease (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean±s.d.</td>
<td>0.78±0.10</td>
<td>0.16±0.06</td>
</tr>
<tr>
<td>Median</td>
<td>0.79</td>
<td>0.15</td>
</tr>
<tr>
<td>Range</td>
<td>1 - 1</td>
<td>0 - 0</td>
</tr>
</tbody>
</table>
Thus the patients of the two groups were matched for gender. t-test showed that the mean DNA concentration of the patients without Parkinson’s disease was significantly higher than that of the patients with Parkinson’s disease ($t_{28}=12.67; p<0.0001$) (Table 4). The mean DNA concentration of the patients without Parkinson’s disease was significantly higher than that of the patients with Parkinson’s disease ($t_{28}=10.89; p<0.0001$) (Table 5). t-test showed that the mean DNA concentration of the patients without Parkinson’s disease was significantly higher than that of the patients with Parkinson’s disease ($t_{28}=12.16; p<0.0001$) (Table 6). The mean total DNA yield of the patients without Parkinson’s disease was significantly higher than that of the patients with Parkinson’s disease ($t_{28}=12.40; p<0.0001$) (Table 8, Figure 1). The mean Yield/µg of tissue sample of the patients without Parkinson’s disease was significantly higher than that of the patients with Parkinson’s disease ($t_{28}=12.42; p<0.0001$) (Table 9, Figure 2).
Table 10: Distribution of Schirmer’s test 1 value of the patients of the two groups

<table>
<thead>
<tr>
<th>Schirmer’s test 1 value</th>
<th>Without Parkinson’s Disease (n=10)</th>
<th>With Parkinson’s Disease (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean±s.d.</td>
<td>22.50±6.35</td>
<td>14.80±5.90</td>
</tr>
<tr>
<td>Median</td>
<td>23.00</td>
<td>13.00</td>
</tr>
<tr>
<td>Range</td>
<td>12 - 30</td>
<td>8 - 27</td>
</tr>
</tbody>
</table>

Figure 2). The mean Schirmer’s test1 value of the patients without Parkinson’s disease was significantly higher than that of the patients with Parkinson’s disease (t_{28}=2.45; p=0.025) (Table 10).

Discussion:

Parkinson’s disease (PD) is a neurological disease specifically seen in elderly patients and is characterized by motor symptoms like bradykinesia, cog-wheel rigidity and resting tremor. The disease is also associated with non-motor abnormalities like autonomic and cognitive dysfunction, sleep and mood disorders, all seriously affecting the life quality of patients. Dry eye is a frequently encountered entity in PD due to reduced rate of blinking, which is an important and necessary process for the proper distribution of tears on the ocular surface and the prevention of tear evaporation. Seborrhea is also a common entity in PD which causes meibomian gland dysfunction and subsequently lipid layer abnormality in the tear film. The abnormal lipid layer disturbs the hydrophobic surface characteristics of the tear film, thereby increasing evaporation. Studies have also shown that meibomian gland dysfunction and evaporative dry eye are more prevalent in the elderly population. Therefore, age, reduced blinking rates, seborrhea and meibomian gland dysfunction all contribute to the increased frequency of evaporative dry eye in PD. Apart from evaporative problems of the tear film, studies have also demonstrated reduced tear secretions, probably due to autonomic dysregulation of the lacrimal gland.

According to the International Dry Eye Workshop Study, dry eye is now considered to be inflammation of the ocular surface characterized by tear hyperosmolarity, which is now accepted as the gold standard of objective dry eye diagnosis and the single best marker of disease severity. Blink Rate and Schirmer scores decreased significantly in Parkinson’s disease patients. Although not significant, the demonstrated tear osmolarity increment might be a good indicator to document the dry eye and inflammatory process of the ocular surface in Parkinson’s Disease.

The agarose gel profiles of the isolated genomic DNA from cells obtained by impression cytology and tear fluid using Schirmer strips suggested that the migration of the EtBr tagged DNA bands through the agarose gel are similar in both patients and control cases (Figure 3, 4). Which actually denotes that the size of the DNA obtained from patients as well as control tissue samples is similar. While there was a significant difference in the band widths of test and control which is due to lesser yield of DNA from patient samples than that of the control ones, which was further established by quantifying those samples with respect to their OD on incidence of 260 nm wavelength light on the samples.

Starting with 10 mg of tissue sample for both patients and controls we obtained significantly lesser yield in case of individuals having Parkinson’s disease which may be due to some physical damages occurred under such physiological conditions upon genomic DNA resulting in extrudation of extracellular DNA fragments (Figure 5, 6). Although they are not reported in case of Parkinson’s disease so far but well documented reports are available which have reported the abundance and changes of extracellular DNA in Dry eye patients. Since,
Parkinson’s and dry eye are very closely associated it can well be a concrete explanation with some further studies for the decreased yield of genomic DNA for patients having Parkinson’s disease.

There can also be another alternative explanation for the reduced DNA yield, which can be due to some biochemical changes which are subject to further studies. These biochemical changes which are yet to be identified might bring in some loss of function or loss of integrity of the DNA structure which can actually hinder the isolation procedure (Phenol/chloroform extraction) thus culminating into lower DNA yield starting with patient tissue samples than that from the healthy ones.

References:

Sanyal SK et al: Tear nucleic acid in Parkinson’s disease


Cite this article as:


