

DNA damage studies in cases of Trisomy 21 using Comet Assay

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Vol. 14, No. 1 (2010-01 - 2010-06)

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Abstract

Objective: To study the DNA damage levels among the cases of Down's syndrome(DS) with that of the age and sex matched controls using single cell gel electrophoresis (SCGE) or Comet assay. Methods: Phytohaemagglutinin stimulated lymphocyte cell culture was carried out using RPMI 1640. G banded metaphase spreads obtained following harvesting was screened using automation software "Ikaros metasystem, Carl Zeiss", Germany. Alkaline comet assay was carried out with the lymphocytes isolated using Histopaque. The lymphocytes were layered on the agarose (NMA,LMA) coated slides. They were then subjected to alkaline medium (PH>13) followed by submarine gel electrophoresis (300mA current), neutralisation (Tris buffer PH-7.5), fixation and staining with silver nitrate. Comet metrics values were obtained using the Comet score software. Results : All 40 cases of DS which was subjected for automation karyotyping showed pure trisomy 21 pattern. The results of Comet assay showed statistically significant elevated levels among cases as compared to controls. Comet length in cases was found to be $45.38 \pm 12.5 \mu\text{m}$ as compared to $24.48 \pm 3.2 \mu\text{m}$ in controls. Similar observations were made for comet tail length ($18.35 \pm 8.9 \mu\text{m}$ for cases and $2.61 \pm 1.1 \mu\text{m}$ for controls) and head diameter ($27.35 \pm 5 \mu\text{m}$ for cases and $23.08 \pm 2.6 \mu\text{m}$ for controls). Conclusion: There is increased DNA damage among the cases of Down's syndrome which can be attributed to oxidative stress. DNA damage may be responsible for the clinical manifestations and long term outcome among the cases of Down's syndrome.

Key words: Down's syndrome, DNA damage, Comet Assay
Accepted October 10 2009

Introduction

Down syndrome (DS) individuals develop multi-systemic involvement of clinical expression due to partial or total extra copy of chromosome 21. Mental retardation, skeletal anomalies, increased incidence of congenital heart disease, leukaemia and infections are a few. About 95% have a complete extra chromosome 21 (47, XY, +21)[1]. Oxidative stress induced DNA damage plays a major role in the clinical manifestations of DS due to over expression of genes which are frequently encountered on the extra copy of 21st chromosome[2]. The level of DNA damage is usually assessed by Single cell gel electrophoresis or "Comet assay". This assay is based on the ability of negatively charged loops/fragments of DNA to be drawn through an agarose gel in response to an electric field. The extent of DNA migration depends directly on the "damaged DNA" present in the cells[3]. The present study was undertaken to compare the extent of DNA damage among the cases of DS with that of age and sex matched controls.

Methods

Forty children suspected to have DS were selected for the study. The chromosomal analysis was carried out by lymphocyte cell culture using the whole blood with RPMI 1640 and Phytohaemagglutinin as a mitogen[4]. The cultures were terminated after colchicine(SIGMA) and hypotonic KCl treatment followed by fixation with methanol and acetic acid. The slides made out of cell suspension were stained with conventional Giemsa and scanned under Olympus BX51 microscope using the software : Ikaros -Metasystem,Carl zeiss,Germany for automation karyotyping.

Comet Assay was carried out in all the Karyotypically confirmed cases of DS and seventeen age and gender matched controls for the assessment of DNA damage. In the present study “alkaline comet assay” technique [3] was used for the evaluation of DNA damage. Lymphocyte cell suspension obtained by using Histo-paque (SIGMA) was sandwiched between normal/low melting point agarose and were immersed in lysis buffer, followed by submarine electrophoresis in alkaline buffer of pH 13 for 30 min with a current strength of 300mA. Neutralisation was done using Tris buffer of pH 7.2. After fixation with Trichloroacetic acid, the slides were stained using silver nitrate [5]. During electrophoresis, the negatively charged DNA fragments migrate from the nucleus of each cell towards the anode producing the images which appear like comets. In other words, a comet thus formed with the tail of the comet being directly proportional to the extent of DNA damage in a single cell. The images were captured using the Olympus BX51 fluores-cent microscope and screened using Comet score™ Software. The values of comet parameters such as mean comet length (CL), comet head –diameter (HD), mean comet tail length (TL) were measured. The results were tabulated and analysed using one way ‘t’ test.

Results

Forty cases of DS children below five years of age formed the cases for the current investigation; which was compared with 17 normal age and sex matched children as controls. Among 40 DS cases investigated 23 were males and the rest female. Out of 17 controls, the number of males and female were 8 and 9 respectively. Out of 40 cases, 26 children were below one year of age which formed the major group, while the rest were between 1 -2 yrs and above 2 yrs with 7 cases in each group – (Table : 01). All 40 cases of DS were confirmed cytogenetically as pure trisomy 21 through automated Karyotyping system.

Table 1: Showing distribution of cases and controls age wise

| | Cases | | | Controls | | |
|-----------|-------|--------|-------|----------|--------|-------|
| | Male | Female | Total | Male | Female | Total |
| < 1 year | 13 | 13 | 26 | 5 | 5 | 10 |
| 1-2 years | 5 | 2 | 7 | 1 | 2 | 3 |
| >2 years | 6 | 1 | 7 | 2 | 2 | 4 |
| Total | 24 | 16 | 40 | 8 | 9 | 17 |

Table 2: Comparison of the comet parameters between cases and controls.

| | Comet Length (µm) * | Tail length (µm)** | Head Diameter(µm)*** |
|---------|---------------------|--------------------|----------------------|
| Case | 45.38999±12.5 | 18.35357±8.9 | 27.35465 ±5 |
| Control | 24.48601±3.2 | 2.614701±1.1 | 23.08357±2.6 |

* P – value <0.0001

** P – value <0.0001

*** P – value <0.0081

The Comet length, Tail length and head diameter showed statistically significant elevated levels among the cases when compared to controls indicating increased DNA damage among cases.

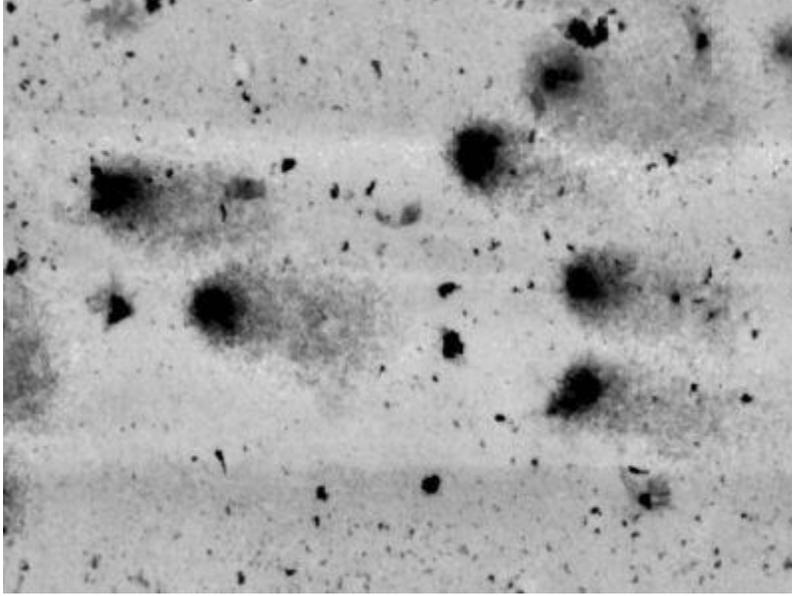


Figure 1: Showing parts of comet – comet length, comet tail length and Head Diameter 20X Silver Nitrate

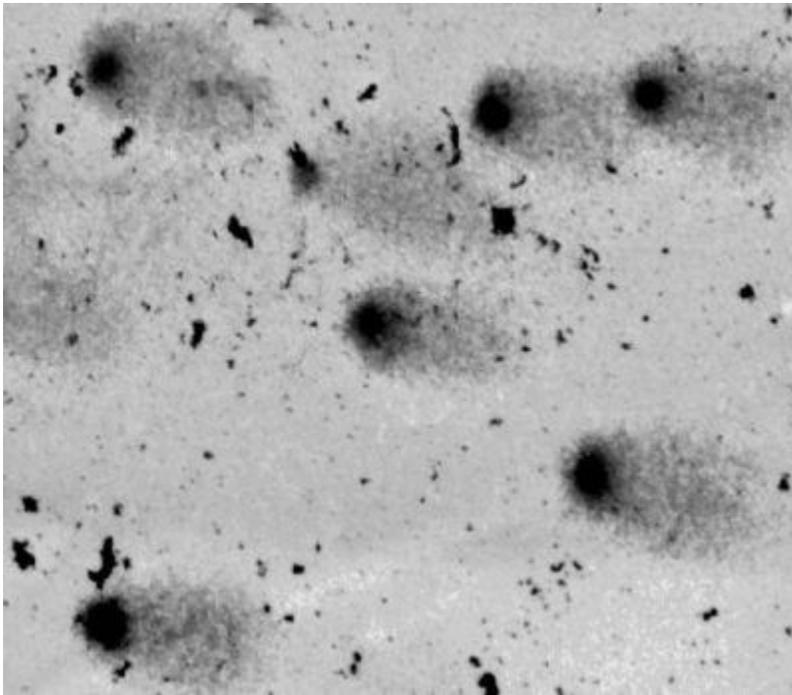


Figure 2A: Comets observed in Cases of Down's syndrome 20X Silver Nitrate

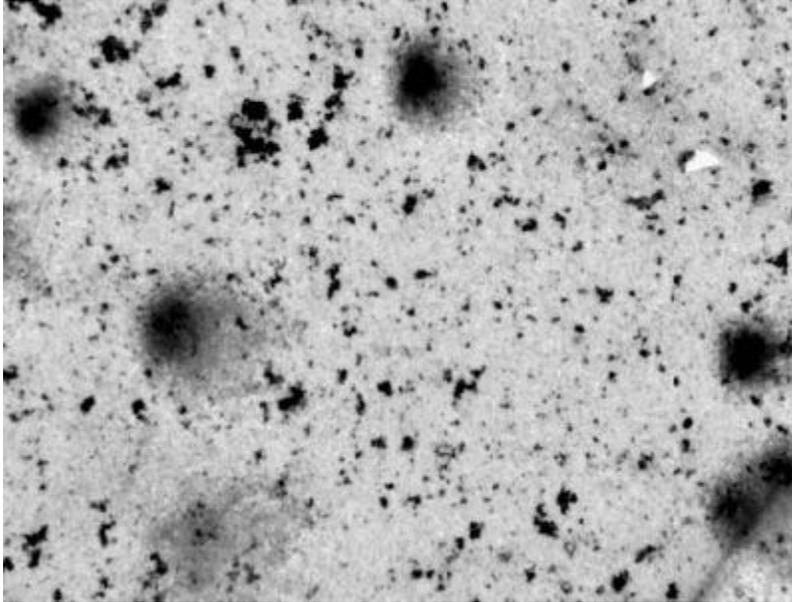


Fig 2B: Comets observed in Controls 20X Silver Nitrate

Discussion

DS is now regarded as a genomic instability condition with over-expression of genes present on chromosome 21. Around 52 genes are identified on chromosome 21. Over-expression of these genes due to extra copy of 21st chromosome leads to increased level of oxidative stress leading to DNA damage which results in various clinical manifestations. Some of these are :Superoxide Dismutase (SOD1)- overexpression may cause premature aging and decreased function of the immune system; its role in Senile Dementia of the Alzheimer's type or decreased cognition is still speculative [6]. COL6A1 overexpression may be the cause of heart defects[7]. ETS2 - overexpression may be the cause of skeletal abnormalities[8]. CAF1A interacts with other Polypeptide units to promote the assembly of histones to the replicating DNA [9]. Cysta-thione Beta Synthase (CBS) – overexpression may disrupt metabolism and DNA repair [10].DYRK1A and RCAN1- overexpression may cause learning and memory deficit [11]. DSCAM- it is expressed in nervous system and a gene for Heart defects [12,13]. GARS-AIRS-GART—is an important candidate gene in studies of DS-related Alzheimer's disease (AD), due to its chromosomal localization (21q22.1) in the Down syndrome critical region, involvement in de novo purine biosynthesis, and over-expression in DS brain [13].

It is claimed that increase in dose of Cu/Zn Superoxide Dismutase due to gene dosage effect in Trisomy 21 leads to disturbance in metabolism of Oxygen radicals which are responsible for the occurrence of comets due to damage of DNA which is shown in SCGE[14]. In the present investigation, all 40 cases of DS were of pure trisomy 21 and one anticipates high levels of extra chromosomal “over dose gene effect” with elevated levels of SOD and Catalase leading to imbalance between antioxidants and free radicals causing cell injury due to oxidative stress phenomenon. Cu/Zn SOD and biochemical profile for oxidative stress were not carried out in the current investigation. However, the increase in the values of comet metrics are indicative of oxidative stress.

Over expression of genes present on the extra copy of chromosome 21 (Gene dosage effect) leading to oxidative stress results in production of various Reactive oxygen species (ROS) [15].The results revealed elevated numbers of Single Strand Breaks and oxidized bases (Purines and pyrimidines) in the cases of DS compared to controls.

Results of oxidative DNA damage in lymphocytes demonstrated elevated DNA damage in DS children in both the stress-induced state and after the repair period [15]. The elevated levels of DNA damage in cases with DS observed in the current study can be attributed to the “Gene dosage effect” due to the presence of the extra copy of chromosome 21.

The interference in the repair capacity of DNA in the cell cycle leading to the accumulation of the damaged DNA substantiates the formation of a comet [14]. In the current study comets with increased tail length

were observed in cases of DS suggestive of increased levels of DNA damage which can be attributed to impaired DNA repair mechanism. The comet tail length values among the DS individuals between 4-13 years of age and the controls were 25 ± 10.6 and 17 ± 0.4 respectively in an earlier study indicating an enormous deviation between the affected and the control [16]. The current study also showed statistically significant values with reference to tail length between cases and controls with values being $18.35357 \pm 8.9 \mu\text{m}$ and $2.614701 \pm 1.1 \mu\text{m}$ respectively which is consistent with the findings of the earlier study [16].

The amounts of hydrogen peroxide induced and residual oxidized purines were significantly higher in DS children compared to the controls in an earlier study. DNA damage in DS children was greater than that of DS adults [15]. The current study was restricted to children below 5 years of age. Age wise comparison didn't show any variation among the different age groups.

The extent of oxidative damage recognized by NTH1 and Fpg in DS children was significantly higher than in controls. DNA damage in lymphocytes exposed to hydrogen peroxide at the beginning and at 120 min of repair incubation showed reduced DNA damage at 120 min in the control group, whereas that of DS group showed increased DNA damage levels with time. The observations showed that lymphocytes of DS patients had no ability to repair DNA damage by hydrogen peroxide and MNNG. The extent of DNA damage increased with time, suggesting that children with DS may be characterized by impaired cellular reaction to DNA damage which increases the probability of occurrence of cancer in these children [17]. DNA damage in DS individuals observed in the current study can be attributed to findings observed in the above study.

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Curr Pediatr Res 2010; 14 (1): 1-4