National Journal of Basic Medical Sciences | ORIGINAL ARTICLE | Volume - IV, Issue-1

ESTIMATION OF DNA DAMAGE IN NON SYNDROMIC CONGENITAL SKELETAL MALFORMATIONS USING COMET ASSAY

Rijied Thompson Swer[1], Mary Hydrina D'Silva[2], Dyutimoy Datta[3]

ABSTRACT

Background and Objectives: Chromosomal aberrations, gene defects and environmental interactions between them are known to cause 20% to 40% of birth defects. Genome instability in the form of chromosomal breaks, deletions and translocations has been observed in the children with these anomalies. This study was attempted on non syndromic congenital skeletal malformations to correlate the phenotype with the extent of DNA damage. *Materials and Methods*: A total of 20 children were studied. 10 of them, from newborns to 5 years of age presenting with various skeletal malformations not attributed to any syndrome, in the absence of other system anomalies, formed the case material. A proportionate number of them matched for age and sex formed the controls. Peripheral lymphocytes from both, cases and controls were subjected to the standard Comet Assay. This very sensitive assay to measure DNA damage is a single cell gel electrophoretic technique where damaged DNA moves out of the cell towards the anode forming a comet, the length of the comet tail being a measure of DNA damage. *Results*: The skeletal deformities observed were Congenital Dysplasia Hip, Congenital Talipes Equino Varus and Arthrogryposis Multiplex Congenita. The mean tail length in cases was 21.55 µm and in controls was 1.992 µm with very high statistical correlation (P value <0.0001). *Conclusion*: Gene mutations have been widely quoted in errors of osteogenesis and ossification, particularly related to Fibroblast Growth Factor (FGF) FGF1 and FGF2 mutations. Thus the comets of the cases reflect either the chromosomal origin or the gene/s alteration of the skeletal malformations.

BACKGROUND AND OBJECTIVES

Congenital malformations or birth defects, as they are commonly called, are a leading cause of infant mortality, accounting for about 21% of the same. 40% to 60% of the defects are attributed to unknown aetiology. Gene defects in the form of breaks, deletions and translocations account for the remaining congenital anomalies. Birth defects are described in the form of malformations, disruptions and deformations. The risk of having a major anomaly with a single minor anomaly is 3%; the risk of major anomaly increases to 10% with two minor anomalies and to 20% with three or more minor anomalies. Thus minor anomalies play a role in alerting to the possibility of underlying major anomalies[1].

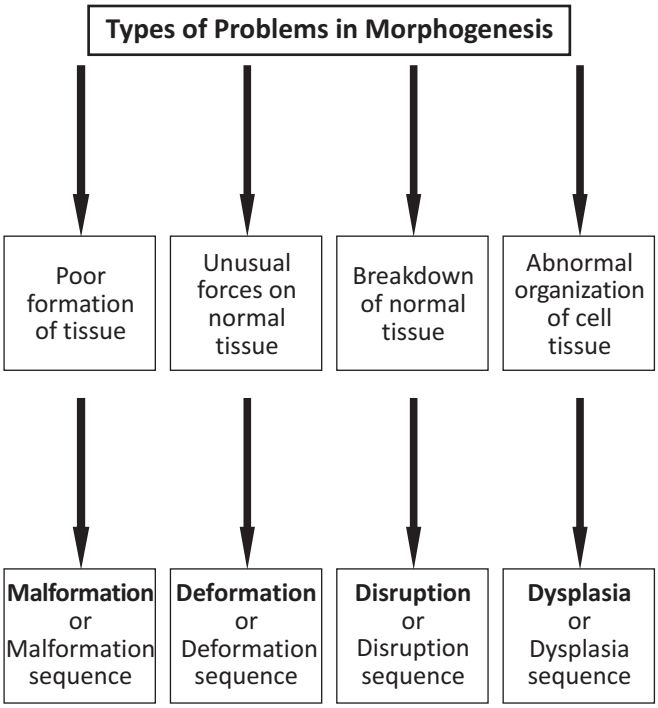
Malformations are due to poor formation of tissues and the defect may be single and localized or multiple. Morphological alterations of already formed structures lead to destructive process and breakdown of normal tissues leading to disruptions. Unusual forces that mould a part of the fetus for prolonged duration give rise to deformations. Abnormal organization of tissues gives rise to dysplasias[1]. Predominant limb deformities have been observed in children with genomic instability[2,3]. Skeletal abnormalities are the commonest limb deformities particularly syndactly, polydactyly and clinodactly with telangiectasia[4].

Congenital malformations may be major or minor. Major malformations mean a structural physical deformity of cosmetic and functional significance, and account for 4% to 6%; half of these are observed at childbirth and the other half by the time the child attains 5 years of age[5]. The less severe forms which are not life threatening and not requiring medical or surgical intervention for survival are regarded as minor anomalies; they are found in approximately 10% of all newborns[6]. Major malformations as compared to minor ones were found to be more frequent in some studies[7,8]. However some studies have shown that the incidence of minor malformations was higher than the major ones[9,10].

[1,2]Assistant Professor, Department of Anatomy, MGMC & RI, Puducherry,
[3]Assistant Professor, Department of Anatomy, Shri Sathya Sai Medical College & Research Institute, Kancheepuram.

All structural defects may be referred to as sequences and are divided into four categories according to developmental pathology as shown in fig. 1[11].

Table 1: Sequence of structural defects



Malformations occur in varying grades of severity with a recurrence risk, in subsequent pregnancies, of 1% to 5%. Such anomalies include the commonest birth defects like cleft lip, club foot, congenital pyloric stenosis, congenital dysplasia of hip and cardiac defects[12]. Knowledge of normal morphogenesis is important and assists study of structural defects and vice versa.

The genetic information of an individual is wholly contained in the zygote, and after cell divisions, differentiation begins to take place through activation or inactivation of particular genes, allowing cells to assume diverse roles. The entire process is programmed in a timely and sequential manner allowing little room for error, especially in early morphogenesis[11,13].

The common denominator of these mutations or other contributory factors being DNA damage, we attempted to study the inherent DNA damage in the genome of the individual presenting with skeletal anomalies. The Comet Assay is a very sensitive, relatively inexpensive, faster and reproducible single layer gel electrophoretic technique, where the damaged DNA moves towards the anode[14].

MATERIALS AND METHODS

The study was approved by the Institute Research Council and ethics Committee, JIPMER. 20 children below 5 years age, presenting to the Out Patient Departments (OPDs) of Paediatrics and Orthopaedics of our hospital from 2007 to 2009, were included in this study according to the following criteria.

Inclusion Criteria:

1. Children with non syndromic skeletal congenital malformations below 5 years age formed the cases - Total 10 children.
2. Normal Children with no malformations matched for age and sex with the cases formed the controls - Total 10 children.

Exclusion Criteria:

Children with syndromic malformations were excluded from the study.

Comet Assay was carried out in the Cytogenetics Lab in Department of Anatomy, JIPMER. 2ml of peripheral venous blood was collected from each of the cases and controls. Lymphocytes from the samples were subjected to the assay according to the protocol established by Singh NP et al[14]. The low current used for electrophoresis prevents the normal DNA from moving out of the cell and only the damaged DNA moves towards the anode forming a tail behind a head giving rise to the appearance of a comet. The tail length is a measure of the extent of DNA damage.

By altering the conditions at which the study is carried out, particularly the pH, making it more alkaline, the technique acquires more sensitivity in detecting very low levels of DNA damage, particularly those of single strand breaks[15]. The whole procedure was carried out in dim light to prevent artificial DNA damage.

For scoring of the comets, a bright field transmission light binocular research microscope with photomicrograph attachment was used. 50 cells were randomly chosen in each slide under 20X magnification. Comet metrics were measured for each cell using an ocular micrometer fitted to the eyepiece. Images were captured and transferred to

the system and photographs were printed on glossy grade photography paper. Extent of DNA damage was calculated from the comet metrics as below.

Comet Tail Length (µm) = Total Length of the comet – The Head Diameter of the comet.

The mean comet tail lengths of 1000 comets, 500 from controls and 500 from cases were compared.

OBSERVATION AND RESULTS

The cases were in the age groups from 45 days to 5 years and the number of children in each group is shown in the table 1. The number of male children was 5 with equal number female children as cases. The skeletal malformations observed were mainly Clubfoot otherwise known as Congenital Talipes Equino Varus (CTEV), followed by Congenital Dysplasia of Hip (CDH), congenital dislocation of knee and synpolydactly with one child having multiple skeletal deformities. One child had a combination of CTEV, CDH and Arthrogryposis Multiplex Congenita (AMC). The skeletal deformities according to age and gender are shown in table 2.

Table 2: No. of cases in each age group

Sl. No	Age Group	Total Cases
1	< 6 months	4
2	6 months - 2 years	2
3	> 2 years	4
Total		10

Table 3: Skeletal deformities according to age and gender

Sl. No.	Gender	Age (yrs)	Skeletal Deformities
1	Male	45/365	Congenital dislocation of knee
2	Male	5/12	Right CTEV
3	Male	5/12	AMC, B/L CTEV, B/L CDH
4	Female	5/12	Right CTEV with hypoplastic deformity
5	Female	9/12	Right toes synpolydactly
6	Male	2	Right CTEV
7	Male	4	B/L CTEV
8	Female	4	B/L CDH
9	Female	4	B/L CDH
10	Female	5	Multiple skeletal deformities

The controls were 10 randomly chosen normal healthy children who presented to the Paediatrics OPD for immunization and with other minor ailments but without any congenital malformation and they were matched for age and gender with the cases.

The peripheral lymphocytes from these 20 children were subjected to the comet assay. The mean comet tail length among individual cases is shown in table 3. The longest tail was seen in a 5 months old male child followed by a 5 years old female child; both of them having multiple skeletal deformities. The mean tail length in the skeletal malformation group ie cases was 21.55 µm compared to the controls whose mean tail length was 1.992 µm, with a very high statistical correlation between them (P value <0.0001). This is shown in table 4.

Table 4: Mean comet tail length among individual cases

Sl. No.	Gender	Age (yrs)	Skeletal Deformities	Tail Length (µm)
1	Male	45/365	Congenital dislocation of knee	18.77
2	Male	5/12	Right CTEV	14.64
3	Male	5/12	AMC, B/L CTEV, B/L CDH	34.03
4	Female	5/12	Right CTEV with hypoplastic deformity	20.54
5	Female	9/12	Right toes synpolydactly	16.1
6	Male	2	Right CTEV	24.44
7	Male	4	B/L CTEV	15.03
8	Female	4	B/L CDH	22.75
9	Female	4	B/L CDH	16.01
10	Female	5	Multiple skeletal deformities	33.12

P value <0.0001- significant

Table 5: Comparison of mean tail lengths between cases and controls

	Mean Comet Total Length in µm
Skeletal malformations group	21.55
Normal Controls	1.992

P value <0.0001- significant

DISCUSSION

Lupski JR reported the association of chromosomal aberrations, gene mutation and involvement of multiple loci in the aetiology of congenital malformations[16].

Vissers et al reported deletion, duplication and inversions as a cause of genomic rearrangements leading to silent oncogene activation resulting in undesirable gene expression[17]. Since karyotyping was not performed in our study, the correlation with chromosomal aberrations cannot be commented on.

Congenital anomalies frequently arise sporadically making it difficult to determine whether or not they are of genetic aetiology. Nevertheless, rapid progress in molecular techniques has been made over the recent years in localization and identification of specific gene mutations, chromosomal factors and environmental contributions in specific anomalies. The list comprises 139 loci, including 65 specifically identified genes[18].

Some of the commonly involved genes in the maldevelopment of the skeletal system, with their loci in the chromosome, and the loss of such fragments causing deleterious skeletal defects in the form of a clinical profile, are shown in a tabular form in fig 2[1].

Table 6: Tabular form showing gene loci abnormality with the phenotype

Gene	Chromosome	Abnormality	Phenotype
FGFR1	8p12	Pfeiffer syndrome	Craniosynostosis, broad great toes and thumbs, cloverleaf skull, underdeveloped face
FGFR2	10q26	Pfeiffer syndrome	Same
		Apert syndrome	Craniosynostosis, underdeveloped face, symmetric syndactyly of hands and feet
		Jackson-Weiss syndrome	Craniosynostosis, underdeveloped face, foot anomalies, hands usually spared
		Crouzon syndrome	Craniosynostosis, underdeveloped face, no foot or hand defects
FGFR3	4p16	Achondroplasia	Short-limb dwarfism, underdeveloped face
		Thanatophoric dysplasia (type I)	Curved short femurs, with or without cloverleaf skull
		Thanatophoric dysplasia (type II)	Relatively long femurs, severe cloverleaf skull
		Hypochondroplasia	Milder form of achondroplasia with normal craniofacial features
MSX2	5q35	Boston-type craniosynostosis	Craniosynostosis
TWIST	7p21	Saethre-Chotzen syndrome	Craniosynostosis, midfacial hypoplasia, cleft palate, vertebral anomalies, hand and foot abnormalities
HOXA13		Hand-foot-genital syndrome	Small, short digits, divided uterus, hypospadius
HOXD13	2q31	Synpolydactyly	Fused, multiple digits

In our study skeletal anomalies in the form of CTEV, CDH, AMC, synpolydactly and multiple skeletal deformities reported had significantly elevated comet tail lengths emphasizing DNA damage due to gene defects, which is comparable to the reported literature. In the current series all the cases were of major malformation category as per the assignment of degree of malformations by Kumar et al[5]. A child with CTEV is shown in Fig 3.

Fig 1. Child with CTEV

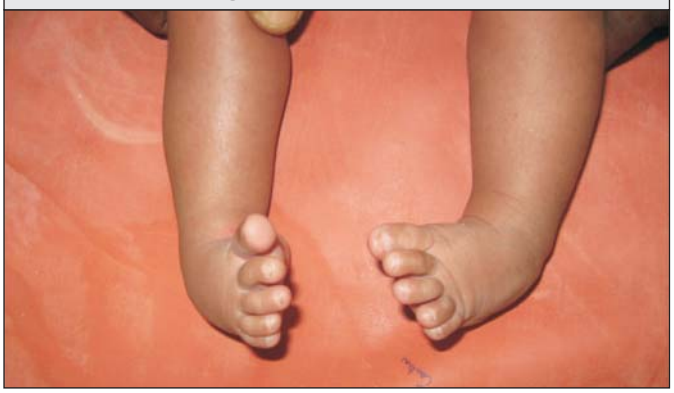
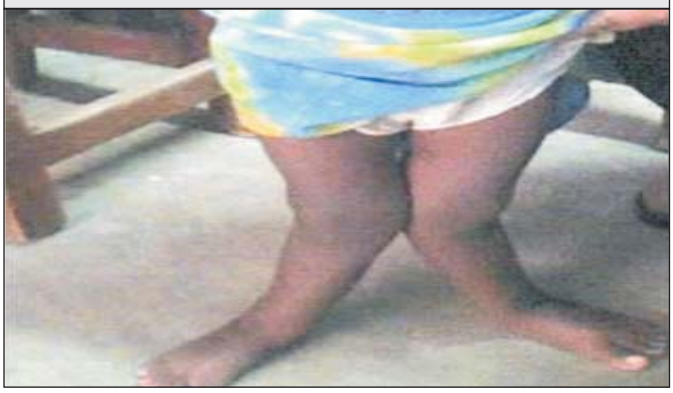


Fig 2. Child with Multiple Skeletal deformities



Among the cases the comparison of mean tail lengths was significant and in our study it can be seen that the two children with multiple skeletal deformities have longer comet tails than the others thus again proving that the amount of DNA damage correlates with the abnormal phenotype. It is evident that genomic instability was seen in the form of comet formation in all the cases, the comets reflecting the damaged DNA. Lymphocytes by virtue of being rapidly dividing cells represent the broad genomic instability in them and as they are easily accessible through peripheral blood collection, they are

used to show the formation of comets in a single gel layer through electrophoresis. The formation of comet is conventionally described as migration of damaged DNA with its negative charge towards the anode. The migration starts at the alkali labile spots of the DNA strands. The extent of migration directly reflects the amount of damaged DNA in the nucleus of a particular cell. Fig. 4 shows a cell from a control with no DNA migration. Fig. 5 shows a cell from a case showing DNA migration from the nucleus.

Fig. 3: No migration of DNA in controls

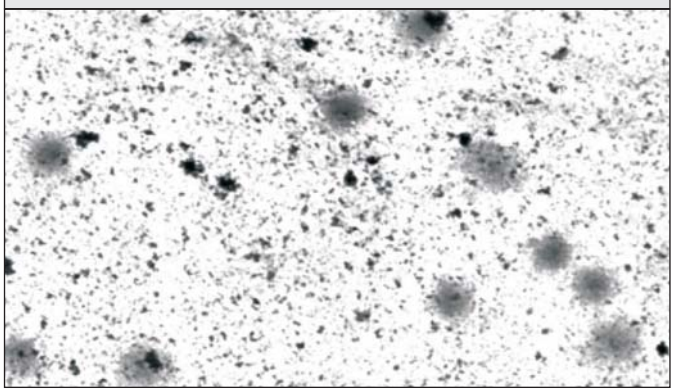
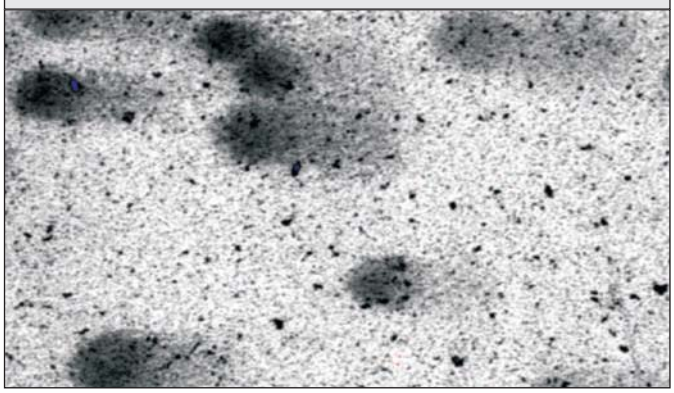


Fig. 4: DNA migration in a case of Congenital malformations showing Comets



The damaged DNA seen as a comet is commonly ascribed to either single strand or double strand breaks. Since the comet assay done by us was using under alkaline conditions, the damaged DNA seen in the migrated form could be an alkali labile site of the DNA strands mainly that of a single strand break. It is claimed that base excision repair, nucleotide excision repair and mismatched repair take place as an alternative mechanism for the repair process in the case of single strand breaks and this mismatched repair results in damaged DNA[6]. Resynthesis of DNA polymerase and DNA ligases are necessary for the final nick sealing stage in the removal of damaged DNA. Defects related to the above would lead to accumulation of such damaged DNA. Single base damage is generally stated to be caused by oxidation, alkylation, hydrolysis and deamination[6]. Perhaps it is placental insufficiency during the late embryonic or early fetal period that could be the triggering factor for DNA damage resulting in malformations. It may also be that amniotic bands or oligohydramnios exert a pressure effect on the limbs resulting in deformities.

SUMMARY AND CONCLUSION

The current investigation of DNA damage in different congenital skeletal malformations showed significant changes through comet study.

1. Mean comet tail length in the 10 children (cases) between 45 days to 5 years presenting with congenital skeletal malformations was significantly higher than the mean comet tail length of the 10 healthy children (controls) matched for age and sex with the above group of children.
2. Among the children with skeletal anomalies, the mean comet tail length was definitely longer in the cases with multiple deformities.

Thus the presence of DNA damage appears to be causing the congenital anomalies. A correlation with karyotyping in future studies will help in identifying the site of the chromosomes affected.

ACKNOWLEDGMENT

We express our sincere thanks to Dr Parkash Chand, Prof. Department of Anatomy, Dr Vishnu Bhat, Prof. Department of Pediatrics and Dr Ramachandra Rao Head of the department of Anatomy, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Pondicherry for their timely advice and guidance to undertake this study.

REFERENCES

1. Sadler TW. Langman's Medical Embryology Lippincott Williams and Wilkins. Published by Wolters Kluwer Health (India) Pvt. Ltd, New Delhi, 2006;10:111
2. Boles RG, Pober BR, Gibson LH, Willis CR, McGrath J, Roberts DJ, Yang-Feng TL. Deletion of chromosome 2q24-q31 causes characteristic digital anomalies: case report and review. Am J Med Genet. 1995; 55:155–160.
3. Maas SM, Hoovers JM, van Seggelen ME, Menzel DM, Hennekam RC, Interstitial deletion of the long arm of chromosome 2: a clinically recognisable microdeletion syndrome? Clin Dysmorphology. 2000; 9:47–53.
4. David WS, Kenneth LJ. Recognizable patterns of human malformation Sounders, W.B. company, Philadelphia. Volume VII in the series major problem in clinical pediatrics. 1982;3(7).
5. Kumar, Abbas and Fausto, eds. Robbins and Cotran's Pathologic Basis of Disease, 2000; 7:70.
6. Turnpenny P, Ellard S. Emery's elements of medical genetics. Edinburg: Churchill Livingstone Elsevier. 2007;13:.237.
7. Saifullah S, Chandra RK, Pathak IC, Dha p.ll GI. Congenital malformations in new born – A prospective longitudinal study. Indian Pediatr. 1968; 4: 251-261.
8. Khrouf N, Spang R, Podgorna T, Miled SB, Moussaoni M, Chibani M. Malformations in 10,000 consecutive birth in twins. Acta Pediatr Scand 1986; 75: 534-539.
9. Ghosh S, Bali I. Congenital malformations in newborn. Indian J Child Health 1963; 12: 448-457.
10. Khanna KK, Prasad LN. Congenital malformations in newborn. Indian J Pediatr 1967; 34: 63-67, 4: 251.
11. Smith's Dysmorphology Kenneth LJ. Recognizable patterns of human malformation. Elsevier Sounders, W.B. company, Philadelphia. 2006;6:2.
12. Jon M Aase. Diagnostic Dysmorphology, University of New Mexico School of Medicine, Albuquerque, New Mexico 1990;11.
13. Epstein CJ, Erickson RP, Wynshaw-Boris A. Inborn errors of development. The molecular Basis of Clinical Disorders of Morphogenesis. New York. Oxford University Press, 2004.
14. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 1988; 175: 184–191.
15. Singh NP, Stephens RE, Schneider EL. Modifications of alkaline microgel electrophoresis for sensitive detection of DNA damage. Int J Radiat Biol. 1994; 66 (1): 23- 8.
16. Lupski JR, Stankiewicz P. (eds) Genomic disorders: the genomic basis of disease. Humana Press, Totowa. 2006; 1-427.
17. Vissers LELM, Stankiewicz P, Yatsenko SA, Crawford E, Creswick H. Complex chromosome 17p rearrangements associated with low-copy repeats in two patients with congenital anomalies.Hum Genet, 2007; 121:697–709.
18. Andrew OM, Wilkie Joanna, Amberger S, Victor A, McKusick I. A gene map of congenital malformations. Med Genet 1994;31:507-517.

MANUSCRIPT SUBMISSION

1. Author should Courier / Post four hard copies and one soft copy (CD) addressed to
   Dr. Milind V. Bhutkar
   Editor-in-Chief, NJBMS
   Professor and HOD, Department of Physiology,
   VMKVMC, Chinna Seeragapadi, Salem - 636 308.
   Tamilnadu, India.
2. **For publication the 1st Author should be a life member of Society for Basic Medical Sciences**
3. Life Membership Number must be quoted for any communication