

Study of deoxyribonucleic acid (DNA) damage in children with orofacial clefts.

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Abstract

Orofacial clefts are the commonest congenital structural anomalies of the lip and/or palate. Beyond cosmetic abnormality children often suffer from feeding difficulties, ear infections, hearing loss, speech and language delay, dental problems and multiple craniofacial/dental surgeries. Present study was conducted to assess DNA damage among children with orofacial clefts. One ml of peripheral blood was collected from children of isolated cleft lip, isolated cleft palate and cleft lip with cleft palate (n=80) and controls, subjected to single cell gel electrophoresis (Comet assay) for DNA damage. Comet parameters were measured. The mean percentage of undamaged DNA in the comet head was 83.9 ± 6.8 % in orofacial clefts and 95.4 ± 1.8 % among controls, ($p < 0.01$). The mean tail length in cases was 22.6 ± 15.5 μm and in controls 6.7 ± 2.7 μm ($p < 0.01$). There is significantly increased DNA damage in orofacial cleft cases than controls. The various stress factors due to comorbidities and complications in orofacial clefts could be the reasons for DNA damage in the present study.

Keywords: Cleft lip; Cleft palate; Comet Assay; Craniofacial anomalies; Single cell gel electrophoresis

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Introduction

Orofacial clefts are the most common craniofacial birth defect of the lip and/or palate in human and they arise as a failure of facial embryonic processes to completely fuse. It comprises a large fraction of all birth defects and is considered important for its significant lifelong morbidity and complex etiology [1]. The average prevalence of orofacial clefts is 1/700 live births [2]. Beyond the cosmetic appearance children often suffer from comorbidities like feeding difficulties, ear infections, hearing loss, speech and language delay. Dental problems and multiple craniofacial/dental surgeries may also be required [3]. Despite surgical interventions, associated comorbidities and social problems make them to lives challenging. Previous studies have identified the genes that harbour the cleft susceptibility loci on several chromosomes. Some of them are PTCH, STOM and FOXE1 on chromosome 9q in which missense mutations and polymorphic variants have been described as having a role in cleft lip/palate [1]. These facts clearly show that orofacial clefts have a strong genetic component. Numerous previous studies have proposed that many extrinsic factors might influence cleft formation. Thus orofacial clefts are considered to be genetically complex and multifactorial diseases. Several studies have identified copy number variants, polymor-

phisms of cleft susceptible genes at micro molecular level by linkage analysis, genome scans, candidate gene studies, FISH etc. But, the data on the topic of macromolecular DNA damage in children with cleft lip/palate is hardly available in the literature. So, aim of the study was to assess the level of DNA damage in children with cleft lip and palate by single cell gel electrophoresis (SCGE) or Comet assay. This is one of the easiest, rapid, cost effective and sensitive methods to analyse DNA damage [4]. Research in this area is likely to add to the existing knowledge and eventually be used for prevention, treatment, and prognosis for individuals affected by orofacial clefting.

Material and Methods

The study was approved by the Institute's Human ethics committee (JIPMER, Puducherry, India. Ref no - SEC/2011/4/1). A total number of eighty children (n=80) were included in the study. The patients were recruited from the Department of Paediatrics and Plastic Surgery. Informed consents were obtained from parents before data was collected from the age and sex matched cases and controls. Types of orofacial clefts, the presence of associated anomalies, infection, order of birth, maternal nutrition and drug history were also noted.

The comet assay is a simple & sensitive method to assess DNA damage in a single cell. The principle of the comet assay is based on migration of negatively charged damaged DNA fragments like single strand breaks, double strand breaks in alkali labile sites towards the anode during electrophoresis thereby forming a comet-like appearance.

By venipuncture, 1ml of blood was collected under strict aseptic conditions and subjected to centrifuging at 1500 rpm. After 30 minutes of centrifugation, the buffy coat containing lymphocytes was removed and layered with agarose gel. After the layer of gel was set, the slides were immersed in lysis solution for 1hr at 4⁰C. With this treatment the cell membrane and nuclear membrane were lysed and the majority of proteins were removed to expose the nucleoids. The slides were then placed in a horizontal gel electrophoresis tank. The slides were left in the high pH (>13) buffer for 30 mins to allow unwinding of DNA and expression of alkali labile sites and another 30 mins at 300 mA, 0.74V/cm for the movement of DNA fragments if any, towards anode through the gel. Staining was done with silver nitrate solution.

The stained slides were visualized using a 20x objective on a bright field light microscope and captured using CCD camera and they were scored using comet score software. Comet parameters like total length of comet (μm), tail length (μm), head diameter (μm), % of DNA in head, % of DNA in tail were evaluated in both cases and controls.

Statistical analysis

The data was presented as mean with SD (standard deviation) or median with range based on the distribution of data. Anova and Independent student t test was used for comparisons of continuous variables. All categorical data was presented as frequencies and percentages and was compared by using chi square. All statistical analysis at 95% confidence interval and P value < 0.05 was considered as significant.

Results

Forty children aged between 0-12 years with various types of orofacial clefts who attended Pediatrics and Plastic Surgery outpatient department (OPD) of JIPMER were the cases for the current study. Equal number of age and sex matched children were chosen as controls those who attended Pediatrics OPD for routine check-up. Among the orofacial clefts, twenty children had cleft lip with cleft palate, fourteen had only cleft lip and six were isolated cleft palate. All the cases were non-syndromic cleft lip and palate cases. At the time of blood collection patients were free from acute illness or distress.

The mean length of the comet in cases was $85.0 \pm 23.3 \mu\text{m}$ whereas in controls it was $77.1 \pm 10.1 \mu\text{m}$. The difference in total length of comet between cases and controls was not statistically significant. The mean head diameter in cases was $64.0 \pm 18.1 \mu\text{m}$ and in controls $70.3 \pm 9.9 \mu\text{m}$. The undamaged DNA in the nucleus was reduced in orofacial cleft than normal healthy children. But, the difference was not statistically significant, ($p > 0.05$) (Figure 1). But when we calculated the mean percentage of undamaged DNA was 83.9% and 95.4% in cases and controls respectively, the difference was statistically significant ($p < 0.05$) (Figure 2).

The mean comet tail length in cases $22.6 \pm 15.5 \mu\text{m}$ and controls $6.7 \pm 2.7 \mu\text{m}$ was statistically significant ($p < 0.01$). The mean percentage of DNA in comet head indicates the amount of undamaged DNA. In the present study, the mean percentage of DNA in comet head in cases was $83.9 \pm 6.8 \%$, and in controls $95.4 \pm 1.8 \%$ ($p < 0.01$).

The mean percentage of DNA in comet tail indicates the amount of damaged DNA. In the present study, the mean percentage of DNA in comet tail in cases was $16.1 \pm 6.8 \%$ and in controls $4.7 \pm 1.8 \%$ (Table1, Figure 2).

Table1 . Comparison of Comet parameters between the study groups

	Comet Length (μm)	Head Diameter (μm)	% of DNA in Head	Tail Length (μm)	% of DNA in tail
Cases	85.0 ± 23.3	64.0 ± 18.1	83.9 ± 6.8	22.6 ± 15.5	16.1 ± 6.8
Controls	77.1 ± 10.1	70.3 ± 9.9	95.4 ± 1.8	6.7 ± 2.7	4.7 ± 1.8
p value	$P > 0.05$	$P > 0.05$	$P < 0.01^*$	$P < 0.01^*$	$P < 0.01^*$

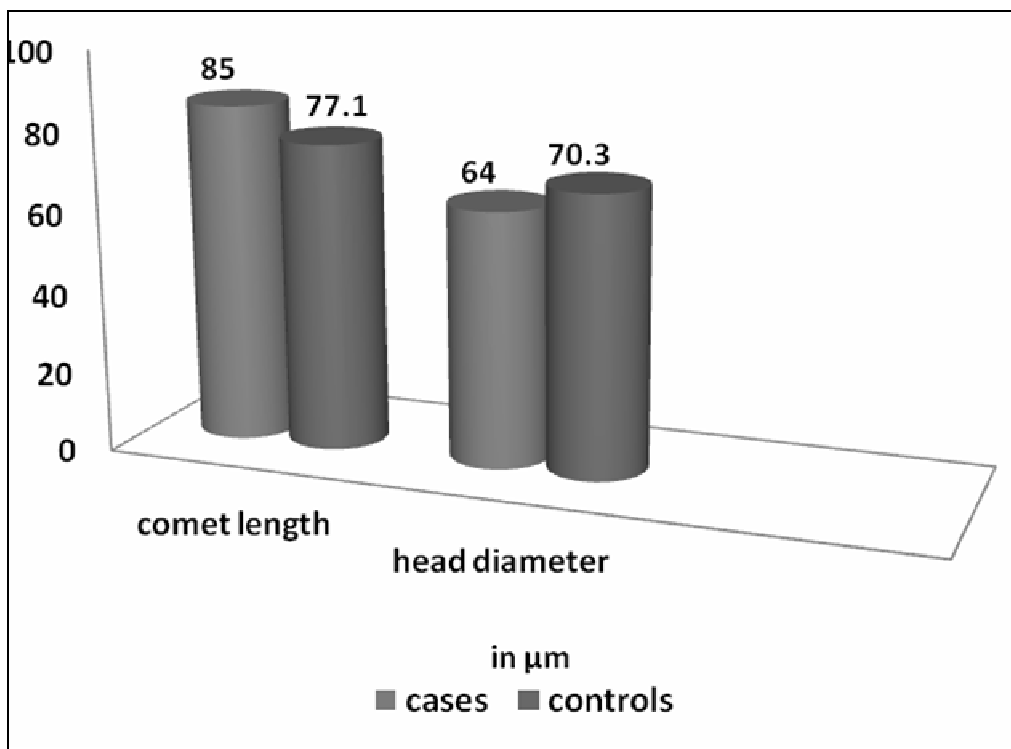


Figure 1. Comparison of Comet length and head diameter between the study groups ($P > 0.05$)

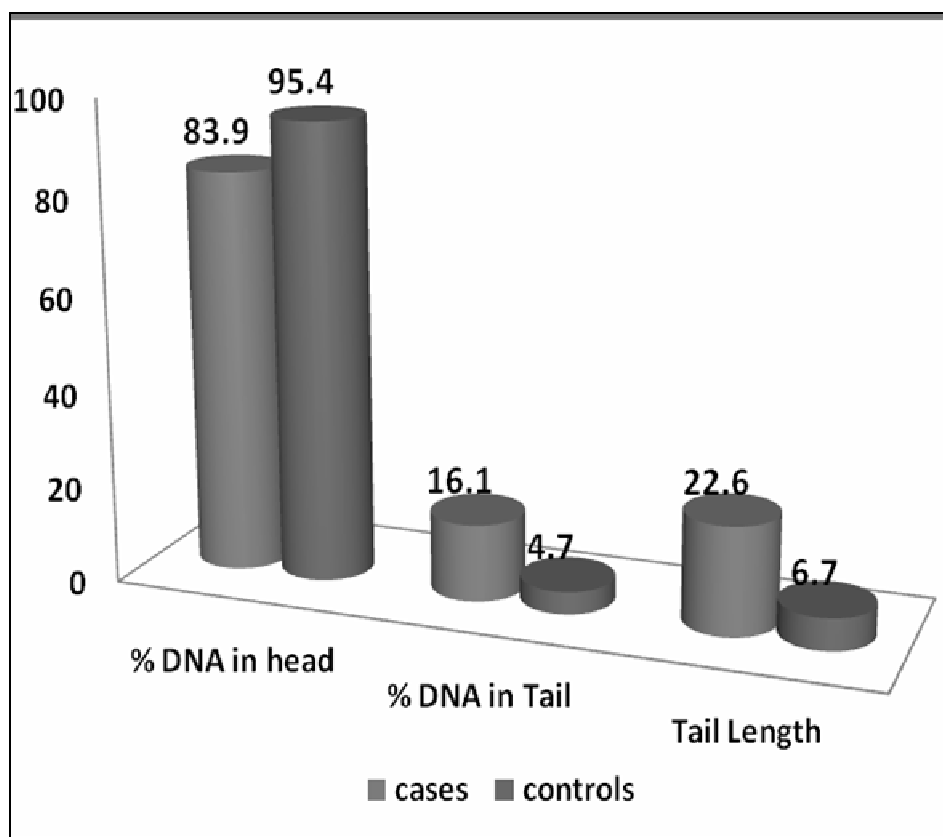


Figure 2. Comparison of % DNA in head, tail and tail length between the study groups ($P < 0.01^*$)

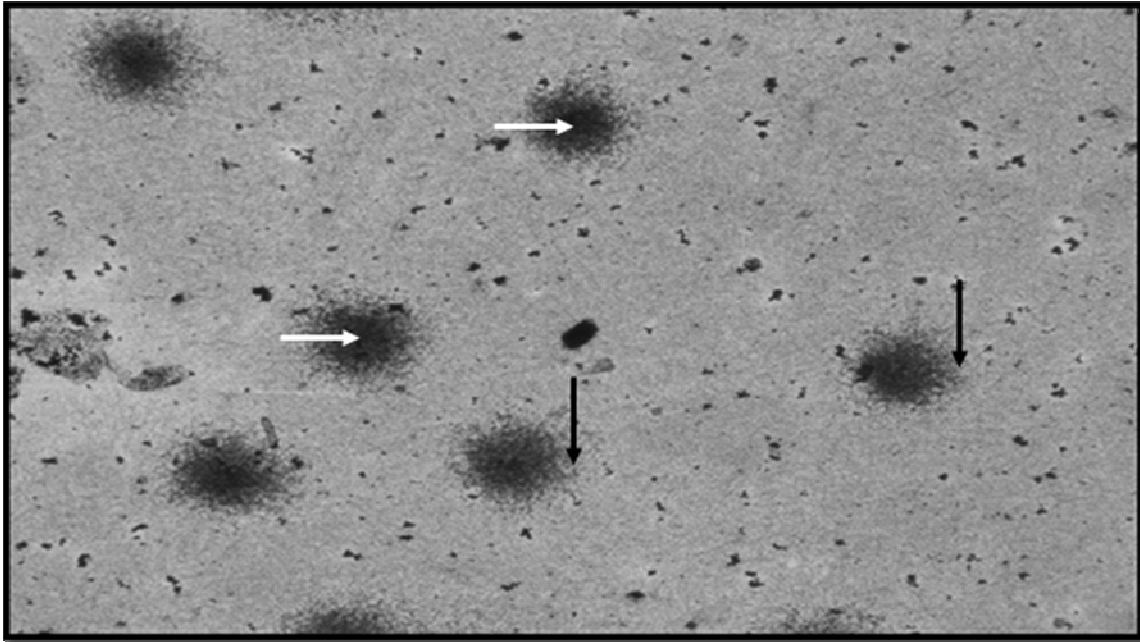


Figure 3. Comets observed in controls (20x). There was no classical tail formation. White arrows indicating the undamaged nucleus and black arrow indicates damage DNA which is very negligible

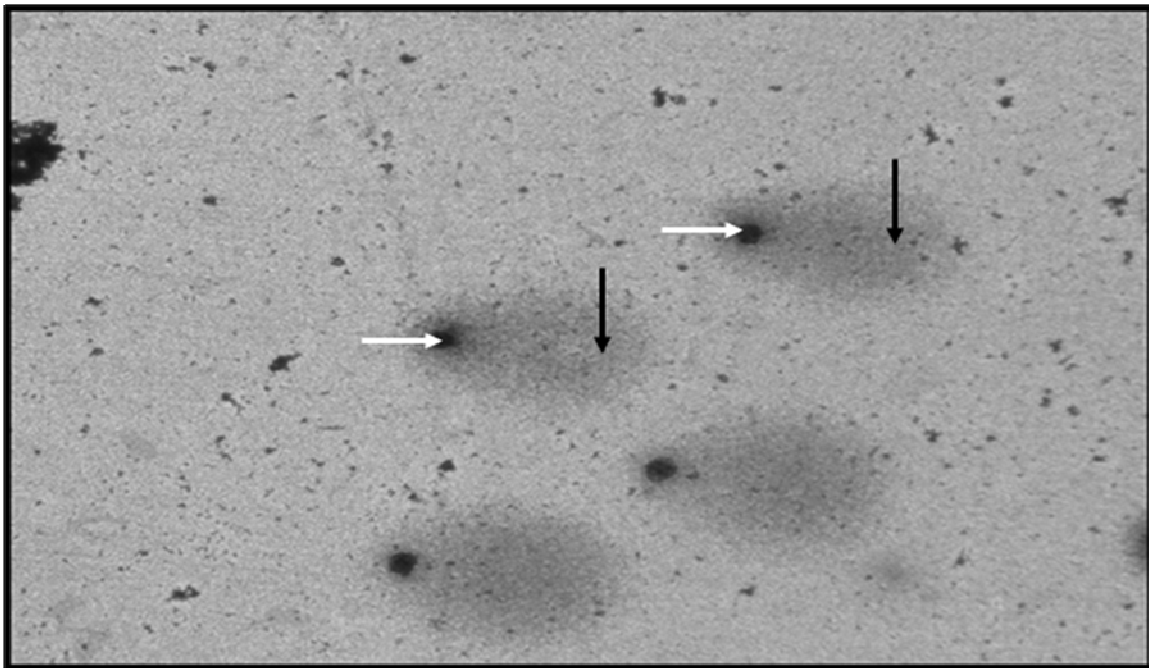


Figure 4. Comets observed in cases of orofacial clefts (20x). Classical comet is indicated by black arrow. White arrows indicate undamaged DNA in nucleus while black arrows indicate damaged DNA in tail of comets.

Discussion

Orofacial clefts are the most common craniofacial birth defects and the second most common congenital anomalies. In 40% to 60% of persons with birth defects, the eti-

ology is unknown. Genetic factors such as chromosomal abnormalities and mutant genes account for approximately 15%; environmental factors responsible in approximately 10%; combination of genetic and environmental influences (multifactorial inheritance) causes 20% to 25% [5]. On extensive literature search showed no data

on the assessment of DNA damage in orofacial clefts. The present study has been undertaken to assess the DNA damage and compare the level of DNA damage with age and sex matched normal healthy children.

In the current study the DNA damage was assessed by the SCGE (single cell gel electrophoresis) or comet assay which is considered as a simple and sensitive method to assess DNA damage in a single cell. The comet assay is based on the principle that damaged DNA moves towards anode forming a comet shaped image and the undamaged DNA remains within the cell (Figure 3, 4) [6-11].

Despite the detection of DNA damage in orofacial clefts in this study, the cause for the same remains unknown due to the complex interplay between genetic and environmental factors, hence opening channel for future research options. Genetic factors like mutation and its consequences could be hypothesized to be the causes of DNA damage in orofacial clefts.

Brasch Anderson et al. suggested that mutated genes by “dose gene effect, position effect” may cause 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 study [6]. However, the increases in the values of comet metrics are indicative of oxidative stress. Gene dosage effect and position effect lead to oxidative stress which results in production of various reactive oxygen species (ROS) [12-16]. The results revealed elevated number of DNA strand breaks and oxidized bases (purines and pyrimidines) in cases of orofacial clefts compared with controls. The elevated levels of DNA damage in cases with orofacial cleft observed in the current study can be due to the “Gene dosage effect and position effects”.

A study by shaw et al has shown mutation in MTHR gene can cause orofacial clefting. Mutation in MTHR gene causes diminished level of serum folic acid and increased serum homocysteine level in children with clefts [17,18]. This could be one of the reasons for DNA damage in orofacial clefts.

Apart from mutation and its consequences, children with orofacial clefts often suffer from comorbidities like feeding difficulties, ear infections, hearing loss, speech & language delay and dental problems [2]. In fact, individuals born with a cleft have increased incidence of mental health problems as well as higher mortality rates at all stages of life. Thus, orofacial clefts have a prolonged, adverse influence on the health over others. The stress due to above said factors may in turn cause DNA damage. A study by Flint et al found that excess production of sympathetic and other adrenal hormones like Epinephrine,

norepinephrine and cortisol during psychological stress may affect many cells directly. The consequences may be transient or long-lasting such as permanent DNA damage which may result in increased cell transformation and/or tumorigenicity [19].

DNA damage resulting from spontaneous or induced chromosome breakage/loss like single/double strand DNA breaks, alkali labile sites (apurinic/apyrimidinic sites), DNA cross links, base/base pair damages and apoptotic nuclei in the cells is detected as comet tails in peripheral lymphocytes. Most of them are cleared from circulation unless a stable mutation has occurred in the stem cells. If that has occurred, the mutation may accumulate over months or years and may end up being responsible for various health conditions in childhood or adulthood, in present or future generations. Camille et al revealed increased occurrence of breast cancer among females with cleft lip and/or cleft palate, primary brain cancer among females with cleft palate and primary lung cancer among males with both cleft lip and cleft palate [20].

Many Studies support an aetiological overlap between non syndromic orofacial clefts and cancer, and alterations in similar biological pathways may be associated with the occurrence of various types of cancer in breast, brain, and colon in individuals with a cleft as well as in their family members [21-23]. Kobayashi et al suggested DNA damage as a molecular mechanism for non-syndromic cleft lip and palate [22]. Collection of adequate exposure data (beginning from pregnancy and during childhood), considering the relative risks of genotoxic exposure to environmental pollution and a thorough look on clinical features will further facilitate our knowledge about DNA damage in children with orofacial clefts. It may help to predict the exact mechanism by which DNA damage causes cancer and other health conditions in such individuals.

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References

1. Letra A, Menezes R, Govil M, Fonseca RF, McHenry T, Granjeiro JM, et al. Follow-Up Association Studies of Chromosome Region 9q and Nonsyndromic Cleft

- Lip/Palate. *Am J Med Genet A*. 2010; 152A: 1701-1710.
2. Brito LA, Meira JGC, Kobayashi GS, Passos-Bueno MR. Genetics and management of the patient with orofacial cleft. *Plast Surg Int* 2012; e782821.
 3. Jugessur A, Farlie PG, Kilpatrick N. The genetics of isolated orofacial clefts: from genotypes to subphenotypes. *Oral Dis* 2009; 15: 437-453.
 4. Nandhakumar S, Parasuraman S, Shanmugam MM, Rao KR, Chand P, Bhat BV. Evaluation of DNA damage using single-cell gel electrophoresis (Comet Assay). *J Pharmacol Pharmacother* 2011; 2: 107-111.
 5. Sadler TW, Langman J. Langman's medical embryology. Philadelphia: Wolters Kluwer Health, India/Lippincott Williams & Wilkins; 2012; pp112: 117.
 6. Ahuja YR, Bhargava A, Sircar S, Rizwani W, Lima S, Devadas AH, et al. Comet assay to evaluate DNA damage caused by magnetic fields. *Proc Int Conf Electromagn Interf Compat* 97; 1997: 273-276.
 7. Faust F, Kassie F, Knasmüller S, Boedecker RH, Mann M, Mersch-Sundermann V. The use of the alkaline comet assay with lymphocytes in human biomonitoring studies. *Mutat Res* 2004; 566: 209-229.
 8. Ostling O, Johanson KJ. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochem Biophys Res Commun* 1984; 123: 291-298.
 9. 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.
 10. Tice R-R, Agurell E, Anderson D et al. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ Mol Mutagen* 2000; 35: 206-221.
 11. Valverde M, Ostrosky-Wegman P, Rojas E, et al. The application of single cell gelelectrophoresis or comet assay to human monitoring studies. *Salud Pública México* 1999; 41:S109-S113.
 12. Brasch-Andersen C, Christiansen L, Tan Q, Haagerup A, Vestbo J, Kruse TA. Possible gene dosage effect of glutathione-S-transferases on atopic asthma: using real-time PCR for quantification of GSTM1 and GSTT1 gene copy numbers. *Hum Mutat* 2004 ; 24: 208-214.
 13. Sreeramulu D, Reddy CVK, Chauhan A, Balakrishna N, Raghunath M. Natural Antioxidant Activity of Commonly Consumed Plant Foods in India: Effect of Domestic Processing. *Oxid Med Cell Longev* 2013; 12: 1474-1484.
 14. Zhang Q, Andersen ME. Dose Response Relationship in Anti-Stress Gene Regulatory Networks. 2007; 3: 3.
 15. Jayaprakash T, RamachandraRao K, Vishnu Bhat B, Parkash Chand NKS. DNA damage studies in cases of Trisomy 21 using Comet Assay. *Curr Pediatr Res* 2010; 14: 1-4.
 16. Robert L. Nussbaum, Roderick R. McInnes. Thompson & Thompson Genetics in Medicine: Philadelphia. Saunders; 2007; pp151-173.
 17. Shaw GM, Croen LA, Curry CJ. Isolated oral cleft malformations: associations with maternal and infant characteristics in a California population. *Teratology* 1991; 43: 225-228.
 18. Shaw GM, Rozen R, Finnell RH, Todoroff K, Lammer EJ. Infant C677T mutation in MTHFR, maternal periconceptional vitamin use, and cleft lip. *Am J Med Genet* 1998; 80: 196-198.
 19. Flint MS, Baum A, Chambers WH, Jenkins FJ. Induction of DNA damage, alteration of DNA repair and transcriptional activation by stress hormones. *Psychoneuroendocrinology* 2007; 32: 470-479.
 20. Bille C, Winther JF, Bautz A, Murray JC, Olsen J, Christensen K. Cancer Risk in Persons with Oral Cleft - A Population-based Study of 8,093 Cases. *Am J Epidemiol* 2005; 161: 1047-1055.
 21. Alexander Dietz DAP. Risk of breast cancer in families with cleft lip and palate. *Ann Epidemiol*. 2012;22:37-42.
 22. Kobayashi GS, Alvizi L, Sunaga DY, Francis-West P, Kuta A, Almada BVP, et al. Susceptibility to DNA Damage as a Molecular Mechanism for Non-Syndromic Cleft Lip and Palate. 2013; 8: e65677.
 23. Letra A, Bjork B, Cooper ME, Szabo-Rogers H, Deleyiannis FWB, Field LL, et al. Association of AXIN2 with Non-syndromic Oral Clefts in Multiple Populations. *J Dent Res* 2012; 91: 473-478.

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