

## ASSOCIATION STUDY OF FIVE MUTATION IN FGFR1 AND FGFR2 GENES IN INDIAN CHILDREN WITH CRANIOSYNOSTOSIS

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**Background:** Craniosynostosis is one the major genetic disorder in children and it occurs in 1 per 2,200 live births. It may be define as abnormal premature fusion of the cranial sutures bones in children. Several causes have been reported that may have a possible role in the development of the disorder. Fibrinogen growth Factor 1(FGFR1) & fibroblast growth factor receptor 2 (FGFR2) show a vital role in developing the craniosynostosis in western population's children but from India no report is available. The aim of this study was to investigate the association between mutation of FGFR1 and FGFR2 (IIIa and IIIb) genes with syndromic as well as non-syndromic craniosynostosis in Indian population.

**Methods:** Retrospective analysis of our records from January 2008 to December 2012 was done. A total of Sixty three children (along with their parents) with craniosynostosis and Fifty one children with No- craniosynostosis (healthy school going children) attending the Monday out Patient Door (OPD) facility of the department of Paediatric Surgery, All India Institute of Medical Sciences (AIIMSs), Delhi, India were considered for the study. A restriction fragment length polymorphism (RFLP) polymerase chain reaction (PCR) was carried out for genotyping Fibrinogen growth Factor 1 (FGFR1) & fibroblast growth factor receptor 2 (FGFR2) mutations in all the participants.

**Results:** There were 33 (80.4%) nonsyndromic cases of craniosynostosis while 8 (19.5%) were syndromic. Out of these 8 syndromic cases, 4 were Apert syndrome, 3 were Crouzon syndrome and 1 Pfeiffer syndrome. Phenotypically the most common nonsyndromic craniosynostosis was scaphocephaly (19, 57.7%) followed by plagiocephaly in (14, 42.3%). FGFR1 mutation (Pro252Arg)

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was seen in 1 (2.4%) case of nonsyndromic craniosynostosis while no association was noted either with FGFR1 or with FGFR2 (IIIa & IIIc) mutation in syndromic cases. None of the control group showed any mutation.

**Conclusion:** Our study provides the strongest evidence that association of mutation of FGFR1, FGFR2 (IIIa & IIIb) with syndromic as well as nonsyndromic craniosynostosis does not exist in Indian population as seen in western population.

*Key words:* Craniosynostosis, Fibrinogen growth Factor, Suture, Restriction fragment length polymorphism (RFLP)

## INTRODUCTION

All though population of not only the developing countries but also the developed countries is growing enormously but the children suffering from many genetic disorders remains same. craniosynostosis is one of them, an emerging epidemic, is responsible for great loss in the productive years of life in children. Craniosynostosis is premature bony fusion of sutures. Two subgroups of craniosynostosis are Syndromic and non-syndromic forms depend on the cranial suture anatomy at birth. A child suffering from genetic disorders are not only social liability but also impose an extra burden on the economy of developing countries like India, Pakistan and Sri Lanka. craniosynostosis is one of the major genetic disorder affecting the central nervous system in children with a reported incidence of 1 per 2,200 live births (REEFHUIS *et al.* 2003) First description of craniosynostosis was given by Otto in 1830 (OTTO *et al.* 1830). Since then multiple theories have been proposed to explain the pathogenesis, with recent studies focusing on genetic regulation (PERSING *et al.* 1989). Still the etiology of the disease is largely unknown however; the condition is related to abnormalities in the base of the skull and is frequently seen in association with osseous abnormalities of the face. Universally accepted hypothesis is an abnormal premature fusion of the cranial suture mainly because of an imbalance between proliferation and differentiation of cell. craniosynostosis can either be nonsyndromic or syndromic with the former more common than the latter. Because of the complexity of the disease the understanding of molecular pathology still remains dogmatic. The various authors of the western world have reported the role of Fibrinogen growth Factor 1 & 2 in craniosynostosis (HACKETT & ROWE 2006, WHITE *et al.* 2005, FARROW *et al.* 2006, MULLIKEN *et al.* 1999, ITO *et al.* 2005, MULLIKEN *et al.* 2004). Being a tertiary care center in the northern part of India serving a population of 40 million, we tried to assess the role of FGF1, FGF2 in Indian children suffering from this grave disease. Understanding the etiopathogenesis and genetic mutation not only help in providing the utmost care to the patients but also lay the foundation for future clinical research. Though more than 200 rare disease associated mutations affecting more than 20 different genes have already been identified, this is the preliminary report from India comparing pedigree of 41 Indian families suffering from craniosynostosis with the normal children. The majority of known genetic causes of craniosynostosis are mutations in the genes encoding fibroblast growth factor receptor types 1–3 (FGFR1, 2 and 3); other significant genes are TWIST1, EFNB1, MSX2 and RAB23. (PASSOS-BUENO *et al.* 2008). A major breakthrough in understanding the genetic background of craniosynostosis has been the identification of genetic defects in several syndromes, including the most common: Crouzon, & Apert's. In general more than 40 genes and several variants were reported in the literature as genetic risk factors for craniosynostosis and

only 60% heritability was explained. The presence of mutations in the group of genes coding for the fibroblast growth factor receptor (FGFR) in patients with Apert & Crouzon syndromes is now clearly established (VON GERNET *et al* 2000, HOLLWAY *et al* 1997, LAJEUNIE *et al* 2006). These genes (currently, 2 are identified) code for receptors on the cell surface, which mediate the effects of fibroblast growth factors (FGF). The effects of FGFs are not fully understood, but they are already clearly implicated in important cellular processes such as cell growth, differentiation, migration, and survival (YUN *et al.* 2010 and within references). Although the 4 different genes are located in different chromosomes, the receptor proteins they encode for being very similar structurally. No such information is available in the Indian population. A number of craniosynostotic disorders have recently been ascribed to mutations in genes coding for the fibroblast growth factor receptors (FGFR1, 2 & 3) (TARTAGLIA *et.al* 1999). The common features of these FGFR-associated conditions are the unilateral or bilateral premature ossification of the coronal suture. The present finding points out the importance, from both diagnostic and prognostic points of view, of early FGFR mutational screening in craniosynostotic conditions, even in forms that apparently do not involve closure of the coronal suture at birth. In view of the above, we aimed to evaluate the role of FGFR1 and FGFR2 mutation in the etiology of craniosynostosis in Indian population.

#### MATERIALS AND METHODS

**Selection of cases and controls:** A total of 63 children (along with their parents) with craniosynostosis and 51 children (healthy school going children) with No-craniosynostosis attending the Monday out Patient Door (OPD) facility of the department of Paediatric Surgery, All India Institute of Medical Sciences (AIIMS) Delhi, India were considered for the present study during the year 2008 to 2013.

**Inclusion criteria:** Craniosynostosis patients who have confirmed diagnosis on clinical examination and plain X-ray skull (anteroposterior, lateral and Towne's view) and non-contrast computed tomography with 3D reconstruction if required and Ages between 6 months to 12 years of age either preoperative or postoperative were included in the study. For control 51 healthy children's of a comparable age group (8, SD+ 3), belonging to the same geographical region were included in the study.

**Exclusion criteria:** Patients with primary microcephaly (secondary craniosynostosis), postural plagiocephaly, incomplete data and lost to follow up were not considered for the study.

**Sample collection:** Informed written consent was obtained from all participating individuals, and permission to proceed was granted by the Institute Ethical Committee. A team of expert Pediatric surgeon performed a detailed clinical examination and recorded the complete case history. Out of 63 registered cases, 41 satisfying the inclusion and exclusion criteria were taken for the study. The venous blood (3 ml) was collected from all the participants along with their parents (Mother & Father) for biochemical and molecular analysis and aliquoted in plain and EDTA-anticoagulated vacutainers. Serum and plasma was separated after centrifugation at 1300 rpm for 15 min.

**Determination of FGFR1 & FGFR2 mutation:** The genomic DNA was extracted from the samples by using phenol chloroform extraction method described by SAMBROOK *et al.* 1989, THANGARAJ *et al.* 2002 protocol with modifications. The isolated DNA was subjected to PCR amplification then followed by Restriction fragment length polymorphism (RFLP). Primers to diagnose the five common FGFR1 & FGFR2 mutation in this study are listed in Table 1.

Primers for FGFR1 & FGFR2 gene were designed as described by LIN *et.al*, 2012, and custom-synthesized primers (Sigma Aldrich Chemicals Pvt. Ltd, Bangalore, India). The optimized reaction conditions consisted of 20 ng of DNA, 2 to 5 pmole of each primer, 200 mM dinucleotide triphosphates, 10 X PCR buffer, 1.5 mM MgCl<sub>2</sub>, and 0.5 units of DyNAzyme II DNA Polymerase (Thermo Scientific) was performed in 0.2-mL, thin-walled tubes. The PCR reaction was carried out in a T-100 DNA Engine (Bio-Rad, Hercules, CA, USA) Thermal Cyclers under the following conditions: 95°C for 3 min, 35 cycles at 95°C for 30 sec, annealing temperature as in Table 1 for 30 s and 72°C C for 1 min/Kb and a final extension at 72°C for 7 min. Amplicons sized were verified by gel electrophoresis by running the PCR product on 2% Agarose gel with 100bp maker (ladder). After successful amplification, a small aliquot (5µl) of the FGFR1 reaction mixture was treated with 1 units of MnlI (NEB) and FGFR2\_IIIa is treated with BstUI, SfiI, BsaJI and FGFR\_IIIc is treated with BsaAI in different 0.2-mL, thin-walled tubes at 37°C for 3 hours details were in table 3, and subsequently analysed on a 3.0% agarose gel with 50bp Ladder (marker).

Table 1. Three primer pairs for amplification of the FGFR1 and FGFR2 gene used in present study.

FGFR2 Primers	Forward	Reverse	Amplicon size(bp)	Annealing temp (C°)
(IIIa)	GGTCTCTCATCTCCCATCCC	CCAACAGGAAATCAAAGAACC	325	62
(IIIc)	CCTCCACAATCATTCTGTGTC	ATAGCAGTCAACCAAGAAAAGGG	257	62
<b>FGFR1</b>	GGAATTCATCTCCACAGAGCGG	GGAATTCCTCAAGATCTGGACATAAGGCAG	216	64

## RESULTS

Children's with different phenotypes of craniosynostosis (Apert Syndrome, Crouzon & Pfeiffer Syndromes) and non-syndromic were included in our study, but no association with FGFR2 (IIIa & IIIc) mutation was detected in family but FGFR1 show mutation (Pro252Arg) in only one family. Our results suggest that the mutation is not associated with craniosynostosis in Indian children. There were 33 (80.4%) nonsyndromic while 8 (19.5%) syndromic cases. Out of these 8 syndromic cases, 4 were Apert, 3 were Crouzon and 1 Pfeiffer. Phenotypically the most common nonsyndromic craniosynostosis was scaphocephaly (19, 57.7%) followed by plagiocephaly in 14 (42.3%). FGFR1 mutation was seen in 1 (2.4%) case of nonsyndromic craniosynostosis while no association of mutation with either FGFR1 or FGFR2 (IIIa & IIIc) mutation was noted in syndromic cases. None of the control group showed any mutation. Our results suggest that the mutation is not associated with craniosynostosis with Indian children. Figure 1-3 showing the PCR product amplification of FGFR1 and FGFR2 (IIIa and IIIc) on 2% Agarose gel, after successful amplification the product was digested as mentioned in the Table 2 and visualized on 3% agarose gel (Figure 4-8). The result was successfully noted with the comparison of ladder size and product size as mentioned in Table 2.

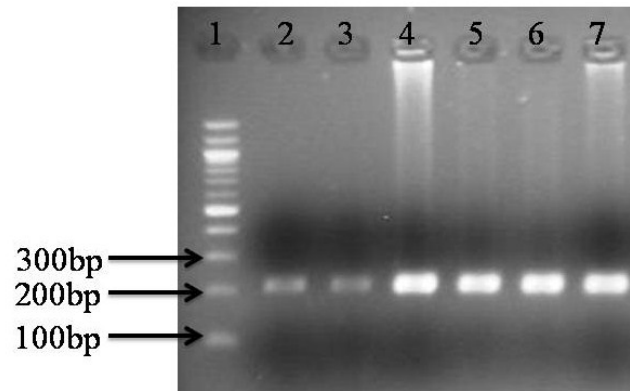


Fig1. PCR product of genomic DNA on 2% Agarose gel from Craniosynostosis and non-Craniosynostosis childrens which were analysis for FGFR1 amplification (216bp). 1) Molecular marker (100 bp); 2-7) PCR product of FGFR1 gene.

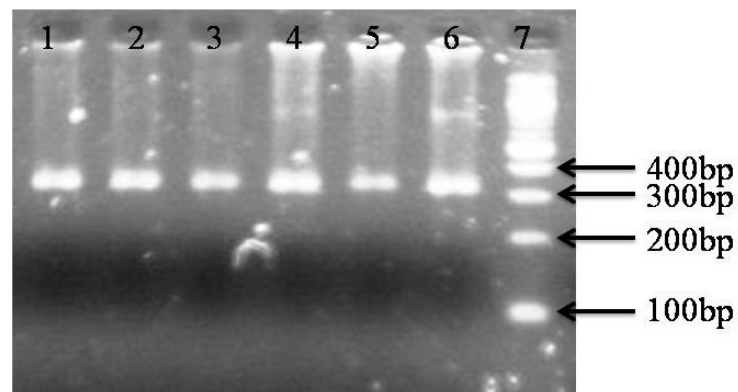


Fig2. PCR product of genomic DNA on 2% Agarose gel from Craniosynostosis and non-Craniosynostosis childrens which were analysis for FGFR2\_IIIa amplification (325 bp). 1-6) PCR product of FGFR2\_IIIa gene; 7). Molecular marker (100 bp).

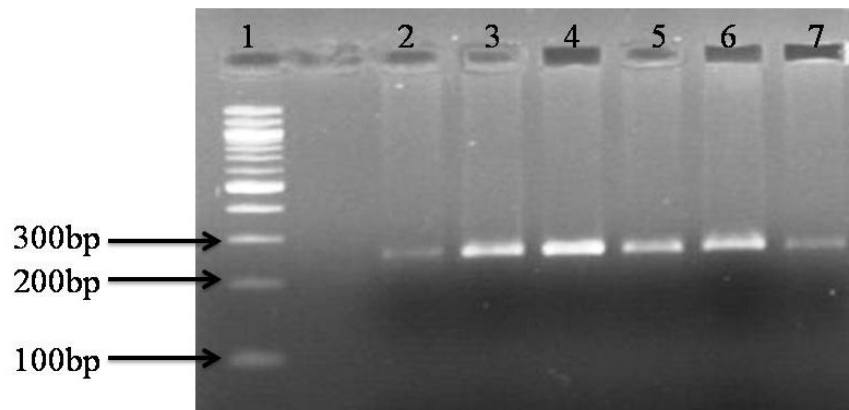


Fig3. PCR product of genomic DNA on 2% Agarose gel from Craniosynostosis and non-Craniosynostosis childrens which were analysis for FGFR2\_IIIc amplification (257 bp). 1) Molecular marker (100 bp); 2-7) PCR product of FGFR2\_IIIc gene.

Table 2. FGFR1 and FGFR2 amplicon Size (bp) after PCR amplification and Restriction Enzyme digestion

Gene	PCR product (bp)	Restriction enzymes	Type of mutation	CC	GG
FGFR1 (Pro252Arg)	216 bp	MnII	Missense Transversion	109 bp	136 bp
				69 bp	69 bp
				27 bp	11 bp
				11 bp	
FGFR2 IIIa (Arg253Pro)	325 bp	BstUI	Transversion	325 bp	266 bp 59bp
FGFR2 IIIa (S252W)	325 bp	SfiI	Transversion	325 bp	260 bp 65 bp
FGFR2 IIIa (Q289P)	325 bp	BsaJI		325 bp (AA)	157 bp (CC) 68 bp
FGFR2 IIIc (Arg342Cys)	257bp	BsaAI	Transition	257 bp (GG)	140 bp (AA) 117 bp

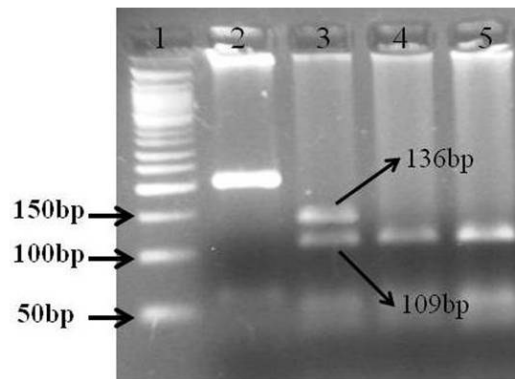


Fig 4. RFLP of PCR product of FGFR1 on 3% Agarose gel from Craniosynostosis and non-Craniosynostosis childrens which were analysis for MnlI digestion. 1) Molecular marker (50 bp); 2) PCR product for Craniosynostosis childrens; 3) Completely digested PCR product for Craniosynostosis childrens (GC) (136bp, 109bp); 4&5) Completely digested PCR product for non-Craniosynostosis childrens (CC) (109bp).

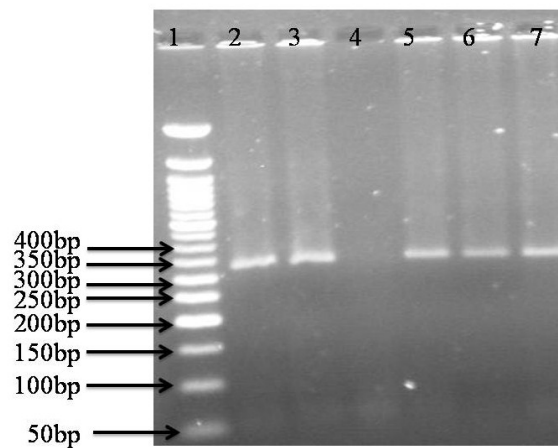


Fig 5. RFLP of PCR product of FGFR2\_IIIa on 3% Agarose gel from Craniosynostosis and non-Craniosynostosis childrens which were analysis for BstUI digestion. 1) Molecular marker (50 bp); 2,3,5-7) Completely digested PCR product for Craniosynostosis & Non-Craniosynostosis childrens.; 4) No product

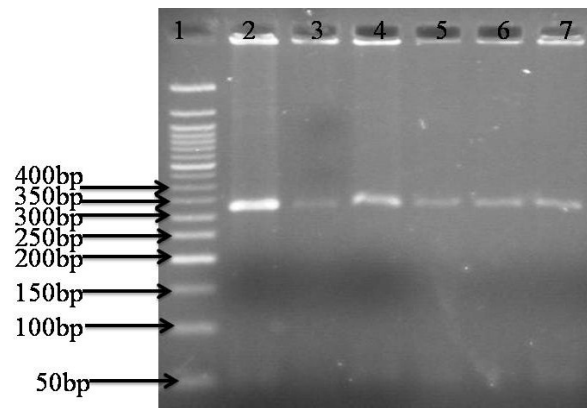


Fig 6 .RFLP of PCR product of FGFR2\_IIIa on 3% Agarose gel from Craniosynostosis and non-Craniosynostosis childrens which were analysis for SfiI digestion. 1) Molecular marker (50 bp); 2-7) Completely digested PCR product for Craniosynostosis & Non-Craniosynostosis childrens.

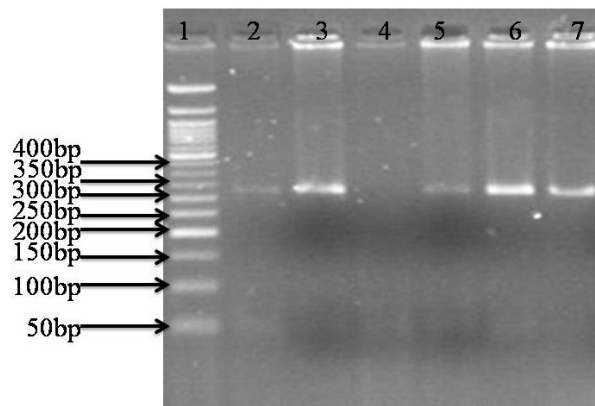


Fig 7. RFLP of PCR product of FGFR2\_IIIa on 3% Agarose gel from Craniosynostosis and non-Craniosynostosis childrens which were analysis for BsaJI digestion. 1) Molecular marker (50 bp); 2,3,5-7) Completely digested PCR product for Craniosynostosis & Non-Craniosynostosis childrens.; 4) No product



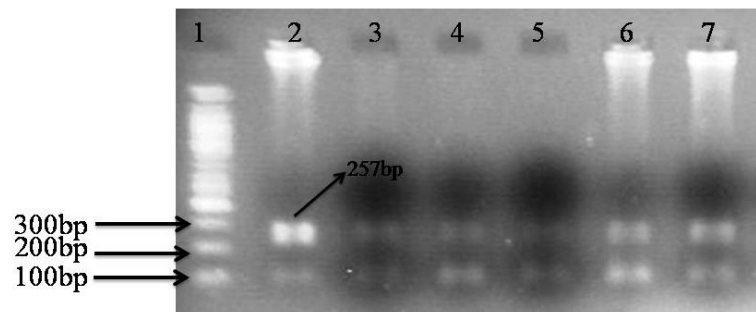


Fig 8. RFLP of PCR product of FGFR2\_IIIc on 3% Agarose gel from Craniosynostosis and non-Craniosynostosis childrens which were analysis for BsaAI digestion. 1) Molecular marker (100 bp); 2-7) Completely digested PCR product for Craniosynostosis & Non-Craniosynostosis childrens.

For figure 4, After digestion, all the fragments were resolved on 3% agarose gels. The homozygous normal allele (CC) produced 4 fragments of 109bp, 69bp, 27bp, 11bp size. The heterozygous (GC) produced 5 fragments of 109bp, 136bp, 69bp, 27bp, 11bp size whereas the homozygous mutant (GG) produced 3 fragments of 136bp, 69 and 11 bp. But due to 69bp, 27bp, 11bp are too small to be visualized so the normal, heterozygous and mutant allele were defined on the basis of bigger fragments i.e 109 and 136 bp. Lane 1 contains the PCR product of size 216bp, Lane 2 contains the heterozygous condition (GC) size 109 and 136 bp for Craniosynostosis children and lane 4, 5 shows digested PCR product of size 109 bp for non-Craniosynostosis childrens

#### DISCUSSION

To the best of our knowledge, none of the studies were published on FGFR1 & FGFR2 mutation study together to any aspect of craniosynostosis in Indian population. However, only few studies have been conducted in the west, but we cannot adopt that data, since, we are having different genetic makeup, life style and dietary habits, etc. Thus, it will be of its own kind of study in the country dealing with Indian genetic stuff, which is supposed to be one of the most conserved genome types. The present study would not only minimize the risks associated with craniosynostosis but will also provide an insight into the unexplored aspects of craniosynostosis. This is the first study on Indian children and sixth study worldwide addressing the association of the FGFR1 & FGFR2 mutation with craniosynostosis.

Our study is truly based on Indian population suffering from craniosynostosis. In English literature multiple reports are available regarding the role of FGFR1 and FGFR2 genes in craniosynostosis but similar data from the Indian subcontinent is still lacking. To the best of our knowledge, this is the first study of Indian children and sixth study worldwide addressing the association of the FGFR1 & FGFR2 mutation with craniosynostosis. In the present study we have screened the most common associated mutation of FGFR1 Pro252Arg (MUEKKE *et al.* 1994,

MEYERS *et al.* 1996) and S252W in FGFR2 (MOLONEY *et al.* 1996), Pro253Arg in FGFR2 (WILKIE *et al.* 1995, PARK *et al.* 1995a, SLANEY *et al.* 1996, HOLLWAY *et al.* 1997, FILKINS *et al.* 1997, PASSOS *et al.* 1997), Q289P (PICCIONE *et al.* 2009, FREITAS *et al.* 2006), Arg342Cys (Park *et al.* 1995b, Reardon *et al.* 1994, Rutland *et al.* 1995) in Indian population. Children's with both syndromic as well as nonsyndromic craniosynostosis (Apert Syndrome, Crouzon and Pfeiffer Syndromes) were included in this study. We did not find any association with FGFR2 (IIIa and IIIc) mutation in families of children suffering from craniosynostosis. Surprisingly in one family FGFR1 mutation was detected (Figure 4).

Our study provides evidence that the mutation is not associated with craniosynostosis in Indian children, although it has been shown to be associated with children in Australia and other western countries. Accordingly, we expect similar trends in other populations of India. Because India is known for its diversity and complexity of genome therefore, it is important to perform replicate studies of patients from diverse ethnic origins (different States of India) before either designating or excluding this mutation as a risk for craniosynostosis. This study will help in better understanding of the existing genetic mutations in Indian children suffering from this grave disease thereby providing an opportunity to the treating pediatric surgeons to reduce the agony and suffering of the children. The present study tries to establish a novel biomolecular marker for consideration and determination of craniosynostosis patients in India. Our study has its own limitation such as small sample size for arriving at any definitive conclusion. Further research is the need of the hour to curb the menace of this psychological disorder for proving a better future for future generation. In a recent study, WILKIE *et al.* 2013, has shown the prevalence and complications of a single gene and chromosomal disorders in craniosynostosis. They concluded that cytogenetic and molecular genetic testing, as a minimum for mutations in FGFR3 (P250R) and FGFR2 exons IIIa and IIIc, should be an integral part of management in children with bicoronal, unicoronal or multisuture synostosis. Research aimed at identifying new genes mutated in craniosynostosis requires careful choice of patients, as many with chromosomal abnormalities or other syndromes may have secondary causes. Other publications on craniosynostosis involving European ancestry (NHW) populations were important from the perspective of population specific understanding of the genetic causes. Justice *et al.* in their study of 130 nonsyndromic cases showed the susceptibility loci for nonsyndromic sagittal craniosynostosis near BMP2 and within BBS9 and was associated with familial (case-parent trios of European ancestry) craniosynostosis. It also represented the first major step toward deciphering the genetic etiology of nonsyndromic sagittal craniosynostosis (sins) (JUSTICE *et al.* 2012). YAGNIK *et al.* 2012 reported that ALX4 variants may have an impact on the genetic etiology of nonsyndromic craniosynostosis. SETO *et al.* 2007, in a series of 164 cases have shown that genetic testing of patients with isolated sagittal or coronal synostosis should include TWIST1 mutational analysis. Study of Asian continent through Korea by YU *et al.* 2009 showed the genotypic and phenotypic analyses of Korean patients with syndromic craniosynostosis. The references within latest meta-analysis conducted by JUSTICE *et al.* 2012 also concluded no association between above mentioned mutation in many ethnic craniosynostosis population.

#### CONCLUSION

In conclusion, the present study suggests that the FGFR1, FGFR2 (IIIa & IIIb) mutation is not a major genetic regulator in the etiology of craniosynostosis in the studied ethnic group. However,

larger sample size has to be analyzed to confirm the same. Our study will provide the necessary platform for further research to better understand the genomics of in Indian Population.

#### ACKNOWLEDGEMENT

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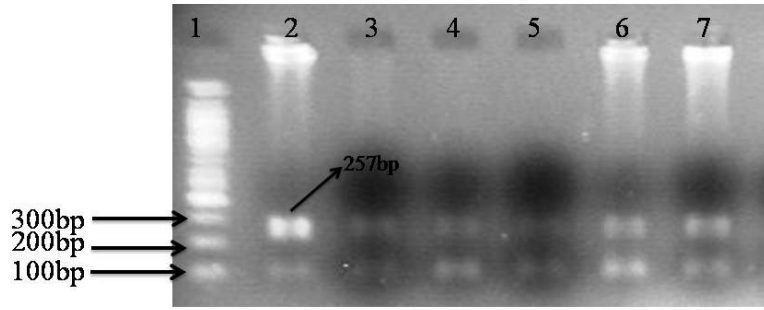
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**Figure8.**

**ASOCIJACIJA ISPITIVANJA PET MUTACIJA U FGFR1 I FGFR2 GENA KOD DECE  
U INDIJI SA GENETIČKIM POREMEĆAJEM KRANIOSINOSOSIS**

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**Izvod**

Kraniosintoza je jedan od glavnih genetičkih poremećaja kod dece i javlja se 1 slučaj na 2.000 živo rođene dece. Može da se definiše kao abnormalna rana fuzija kranialnih kostiju kod dece. Nekoliko uzroka pojave poremećaja je do sada navedeno da imaju moguću ulogu u razvoju poremećaja. Factor 1 rasta fibrinogena (FGFR1) i factor 2 receptora rasta fibroblasta (FGFR2) su pokazali vitalnu ulogu u razvoju poremećaja u zapadnim populacijama dece ali podaci iz Indije nisu dostupni. Cilj ovih ispitivanja je ispitivanje asocijacije između FGFR1 i FGFR2 (IIIa i IIIb) gena sa sindromnom i nesindromnom kraniosintozom u populaciji Indije.

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