Original Article

Genetic significance of muscle segment homeo box1 gene in South Indian population for cleft lip and palate

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BACKGROUND: Oral clefts having a prehistoric existence and the latest scientific technologies have shown new insights in identifying the cause and management. So this is a DNA/gene based study has been presented in this article which comprises the significance of MSX1 gene in cleft samples of major states of South India.
AIMS: To evaluate the significance of MSX1 gene in South Indian population having cleft lip and palate.
SETTINGS AND DESIGN: Four states of native population were set for the study. From each state renowned cleft operating center was selected with the prior ethical and suitable permission and patient consent was taken. Blood samples were collected from each effected sibling were studied and their details were coded. The collected blood samples were used for DNA isolation, PCR amplification and sequencing.
MATERIALS AND METHODS: Eighty patients with non-syndromic CL/CLP/CP from various cleft operating centers in southern states (Karnataka, Tamilnadu, Kerala and Andhra Pradesh) with different ethnic/cultural background were taken. Twenty samples (families) were collected from each state and sequenced and compared with earlier data.
RESULTS: Analysis of this study indicates that mutation of either G273A/C or C102G seems to cause cleft formation. In this analysis, we found a novel mutation (414G to T) which is submitted to NCBI Gene data bank (EF065625).
CONCLUSION: This study supports MSX1 gene leading to cleft lip and palate in the samples studied.

Key words: Cleft lip and palate, muscle segment homeo box1, polymerase chain reaction, sequencing

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Introduction

Oral facial clefts are congenital structural anomalies of the lip or palate that affects 1/1OOO's lives. The prevalence of orofacial clefts varies from 1/500 to 1/2500 births depending upon geographical origin, facial, ethnic backgrounds and socioeconomic status. These individuals after undergoing surgical repairs undergo speech, dental and sociological therapies. The aetiology of orofacial clefts is complex including multiple genetic and environmental factors. Thus the complex aetiology of clefts offers access to find the main cause such as genetic and environmental interactions, which alters the normal craniofacial development.

Facial Development

Normal facial development begins with migrating mesial crest combining with mesoderm cells. These mesenchymal cells are being responsible for matured face and head. These cells are activated at spectrum of singling molecules, transcriptions and growth factors. Recently a group of genes like TGFA 1 (transforming growth factor Alpha 1), RARA (Retinoic Acid Receptor Alpha), TGFB (transforming growth factor Beta), MSX1 (Muscle segment homeo box 1), PAX1 (paired box 1), TGFA2 (transforming growth factor Alpha 2), PVRL 1 (Polio virus related 1) have been held responsible in the role of development of head particularly in development of lips and palate.[1-3]

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Environmental Factors

Warkany (1943) 1st reported that various nutritional deficiencies were responsible for cleft lip/palate.[4] He recognized that teratogens such as Phenytoin, Valproic acid and Thalidomide were responsible for orofacial clefts.[4] The other causes encountered are maternal alcohol drinking/cigarette smoking, involvement of pesticides such as Dioxin.[5] Epidemiological studies for cleft lip/palate conducted till today in various parts of world indicated the involvement of environmental factors for clefts, especially in low economic status people. In Philippines a comparative study between people of high and low socio-economic status showed 1% of cleft lip/palate more in low socio economic group than in high socio group.[6] Like-wise, frequencies of incidence were similar in other Asian group with same socio-economic status.[7] A further increase in these geographical areas occurred whenever there was a history of maternal infections, smoking, alcohol consumptions and vitamin deficiencies during pregnancy.[8,9]

Genetics Vitamins and Congenital Factors

Orofacial clefts provide a unique genetic model for detail study. The genetic factors in clefting can be analyzed by various techniques including segregation analysis, additional genetic linkage etc.

Genetic linkage studies cleft lip/palate led to the specification of certain candidate genes situated in chromosomes 2, 4, 6, 17 and 19.[10,11] Studies from Denmark and Italy reported that loci on 6p have consistently shown linkage to cleft lip/palate.[12] But such study has not been conducted in Indian race till recently. An epidemiological survey was been conducted for cleft lip/palate in and around Chennai population in 1962 and also in Calcutta in 2004. Finally, recent studies from different parts of world have shown the genes TGFA, 84S192, MSX1, TGFB3 and RARA as the most possible candidate gens for orofacial clefts.[6,7,8,13-17]

In lieu the above evidences some studies have reported that vitamin deficiency, particularly vitamin A, leads to cleft lip/palate formation. Earlier, it was thought that consangual marriages might lead to cleft lip/palate formation. But a report in 2002 which was a comparative study of clefts with consangual and non-consangual families showed no significant difference in the incidence of clefts but, the low socio-economic status families of either group had more incidence of clefts.[1,9-11,17]

Animal Studies and Reports

Several animal studies have indicated similar features of gene expression as that of in humans. MSX1 gene is proved to be responsible for growth and development for earthworm.[18] Recent studies on chimpanzee reported similar functions of TGF, MSX1, BMP genes as in human beings.[18] Seven types of MSX1 gene found to be responsible for shape and development in Drosophila. Out of these, HOX/MSX7 are similar in function with respect to MSX1 of humans.[19,20]

Gene Environment Interaction

Studies exploring the effect of environment factors on gene function leading to cleft lip/palate are relatively rare. Variation of TGFA expression due to smoking during pregnancy leading to cleft has been reported.[17,21] Vitamin A and its congeners Acutane are known to induce craniofacial anomalies possibly altering TGFB3 expression. Genetic involvement of cleft lip/palate formation due to folate deficiency remains controversial.[18,22]

Diagnosis and Prevention

Even though the knowledge of interactions of gene and environmental factors are relatively less but still the detail history related to the interaction may be useful for better diagnosis and prevention of clefts.[23-25] Studies related to reduction in smoking and consumption of alcohol during pregnancy has shown to lower the risk of having the facial abnormality child.[26] Drugs like anticonvulsant medications have been evaluated carefully and balanced against the risk of withdrawal for a mother affected with seizure disorder during pregnancy as the anticonvulsants are set to be teratogens leading to birth defects (cleft associate defect).[27-33] With respect to genetic diagnosis, a thorough counselling of any evidences like hypodontia/

tooth agenesis and lip pits in pregnant mother should be advised to evaluate MSX1 gene mutations or to rule out any other known gene mutations and syndromes related to the findings. Finally, nutritional supplements such as folic acids, vitamin B6 and micronutrients provided to pregnant mother have proved to be responsible in good morphodifferentiation of the cells and their function provided the gene status is good.[34-37] In future, gene therapy may be the treatment of choice with proper evaluation, planning and with proper vectors and with target zones/genes in respect to treat the facial defects.[38-40]

In lieu the above literature, the present genetic study had been conducted to find out the possible alteration (mutation) of the MSX1 gene causing cleft/palate in South Indian population.

Materials and Methods

Eighty patients with non-syndromic CL/CLP/CP from various cleft operating centers in southern states (Karnataka, Tamilnadu, Kerala and Andhra Pradesh) with different ethnic/cultural background and 20 samples were collected from each state. The data collection was performed in accordance with individual ethical clearance from their cleft operating institute with the informed consent form signed by the patient and it was institutional based research. The DNA was extracted from the blood soaked blotting papers of the patients. Separate numbering was given for each collection zone.

DNA was extracted by standard Phenol/chloroform extraction protocol or disposable DNA extraction kits. The primers designed for Exon1 and Exon2 excluding the intron and UTR (untranslated region) were used for PCR amplification and then the amplified DNA samples were sequenced. To amplify protein binding sites the primers were designed with some modifications of previously published ones. The primer design as follows:

EXON1: Forward primer: 5'-CAT GCC CGG CGG CTG GCC AGT GCT-3'

Reverse primer: 5'-CTG CAG GTA CGC AGG CGC CGG AG-3'

EXON2: Forward primer: 5'-CGG CAC CGA GGC ACT TGG CGG CAC T-3'

Reverse primer: 5'-GAG CAC GTC CGG GGG TAC AGC ACC A-3'

Gene Information: GENE Muscle Segment Homeo Box1 [Figure 1]

Gene sequence: Complete coding sequence (894 bp)

ATGACTTCTTTGCCACTCGGTGTCAAAGTGGAG
GACTCCGCCTTCGGCAAGCCGGCGGGGGGAGG
CGCGGGCCAGGCCCCCAGCGCCGCCGCGGCCAC
GGCAGCCGCCATGGGCGCGGACGAGGAGGGGGC
CAAGCCCAAAGTGTCCCCTTCGCTCCTGCCCTTC
AGCGTGGAGGCGCTCATGGCCGACCACAGGAAG
CCGGGGGCCAAGGAGAGCGCCCTGGCGCCCTC
CGAGGGCGTGCAGGCGGCGGGTGGCTCGGCGCA
GCCACTGGGCGTCCCGCCGGGGTCGCTGGGA
GCCCCGGACGCGCCCTCTTCGCCGCGGCCGCT
CGGCCATTTCTCGGTGGGGGGACTCCTCAAGC
TGCCAGAAGATGCGCTCGTCAAAGCCGAGAGC
CCCGAGAAGCCCGAGAGGACCCCGTGGATGCA
GAGCCCCCGCTTCTCCCCGCCGCCGGCCAGGC
GGCTGAGCCCCCCAGCCTGCACCCTCCGCAAA
CACAAGACGAACCGTAAGCCGCGGACGCCCTTC
ACCACCGCGCAGCTGCTGGCGCTGGAGCGCAAG
TTCCGCCAGAAGCAGTACCTGTCCATCGCCGAGC
GCGCGGAGTTCTCCAGCTCGCTCAGCCTCACTG
AGACGCAGGTGAAGATATGGTTCCAGAACCGCC
GCGCCAAGGCAAAGAGACTACAAGAGGCAG
AGCTGGAGAAGCTGAAGATGGCCGCCAAGCCCAT
GCTGCCACCGGCTGCCTTCGGCCTCTCCTTC
CCTCTCGGCGGCCCCGAGCTGTAGCGGCCGCGGC
GGGTGCCTCGCTCTACGGTGCCTCTGGCCC
C C A G C G C G C C G C G C T G C C T G T G G
CGCCCGTGGGAC TCTACACGGCCCATGTGGG
CTACAGCATGTA CCACCTGACATAG

Protein amino acid sequence for msx-I gene: (297 Amino acids)

MTSLPLGVKVEDSAFGKPAGGGAGQAPSAA
AATAAAMGADEEGAKPKVSPSLLPFSVEALM
ADHRKPGAKESALAPSEGVQAAGGSAQPLG

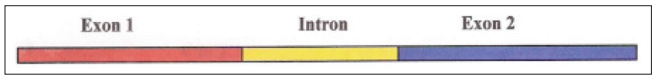

Figure 1: GENE MSX-I (Muscle segment homeo box gene 1)

VPPGSLGSLGAPDAPSSPRPLGHFSVGGLLKLPE
DALVKAESPEKPERTPWMQSPRFSPPPARRLS
PPACTLRKHKTNRKPRTPFTTAQLLALERKF
RQYLSIAERAEFSSSLSLTETQVKIWFQN
RRAKAKRLQEAELEKLKMAAKPMLPPAA
FGLSFGLSFPLGGPAAVAAAAGASLYGA
SGPFQRAALPVAPVGLYTAHVGYSMYHLT.

Exon 1 reference sequence:

(451 bp)ATGACTTCTTTGCCACTCGGTGTCA
AAGTGGAGGACTCCGCCTTCGGCAAGCCGCG
G G G G A G G C G C G G G C C A G G C
CCCCAGCGCCGCCGCGGCCACGGCAGCCGCCA
T G G G C G C G G A C G A G G A G G G G G C
CAAGCCCAAAGTGTCCCCTTCGCTCCTGCCCTCA
G C G T G G A G G C G C T C A T G G C C G A C
CACAGGAAGCCGGGGqCCAAGGAGAGCGCCC
T G G C G C C C T C C G A G G G C G T G C A G
GCGGCGGGTGGCTCGGCGCAGCCACTGGGCGT
C C C G C C G G G G T C G C T G G G A G C C C
CGGACGCGCCCTCTTCGCCGCGGCCGCCGGC
C A T T T C T C G G T G G G G G G A C T C C T C
AAGCTGCCAGAAGATGCGCTCGTCAAGCCGA
G A G C C C C G A G A A G C C C G A G A G G A
CCCCGTGGATGCAGAGCCCCCGCTTCTCCCCG
CCGCCGGCCA

Exon 2 reference sequence

(443 bp) GGCGGCTGAGCCCCCCAG
C C T G C A C C C T C C G C A A A C A C A A G A
C G A A C C G T A A G C C G C G G A C G C C C
T T C A C C A C C G C G C A G C T G C T G
G C G C T G G A G C G C A A G T T C C G C A G A A
G C A G T A C C T G T C C A T C G C C G A G C G C G C G
G A G T T C T C C A G C T C G C T C A G C C C
A C T G A G A C G C A G G T G A A G A T A T G G T T C C A G A A
C C G C C G C G C C A A G G C A A A G A G A
C T A C A A G A G G C A G A G C T G G A G A A G C T G A A G A T
G G C C G C C A A G C C C C A T G C T G C C A
C C G G C T G C C T T C G G C C T C T C C T T C C C T C T C G
G C G G C C C C G C A G C T G T A G C G G C C
G C G G C G G G T G C C T C G C T C T A C G G T G C C T C T G
G C C C C T T C C A G C G C G C C G C G C T G C C T G T G G
C G C C C G T G G G A C T C T A C A C G G C C C A T G T G G G
C T A C A G C A T G T A C C A C C T G A C A T A G

Intron sequence (2326 bp)

GTGAGTAGCCAGAACCCAGGCGCAGAGGGAG
G G G G C C G G G T G G G G G C C G G G
T G G G G T G T G G G A C C C G A G G G C T C C T G G T G
G C C T C C G G C G C C T G C G T A C C T G C A G C
C G G T G C T A G G G A G C C G T GGGCTGCAAGGCCGG
G T C T T G C G C C T C C C T C C A C T C C C
A C C C A G G A A G A A G GTTCCAGACCTCCTCGCCT
T G G C C C A G A G A C G C T G C G G G T G G
G A G T T A A C G G A T A G G A C A C C G A T G T C T G G G C
A C C C T G T C C T C C T G C C C C C A C C A A
A C G A C C T C A G G G G T CCATGATCCCTCATCTGA
T C C C A A A C T C T G T T T C A T C G G C T T
C A C C C C A G C G G A T G A A T G T G T G G T G C G G
T A T C T T C C C T G C A C C C G G A G T T T C A
C T T T C T C G C A G T A G G A G C T G G T G T C C C C C
A G C C C C T C T T C C C T T T C A A G T A C C T C T
T T G C C T A G A G G T T C C G A A G C T C C T A C A G A A T
T C T A C C T C C C C A T G C C C T T T G A G T T
T G A G G C A G A T A G T T G G TGCTTTGGGCGGAT
G G A T G A T T C A G G G G T G G G G A C A T T C
A G G T T C C A G T G G A G G G G G C G G G G C A C C
A A G T C A A T T A G G G G A A G G CGCCCCCGC
T A A T C C T A T G G G A A GCTCCCAAACGTCTA
G G A C T G A G C C A T T A A A G T G G A C T C C A
G G T G C C C A A G G C G G T T C G C T C C A A G G C C
T C A C G G C C C C C T G G C T G C T C T A C T C A G
A G A A C A C G C T C G G A G A T ATTTCAGGAGCA
C G G G A A A T T C C C A A G T T T T C C T C G T T
T C C T C C G A T T A T T T T G C T C G G C A T A A
T A G C A G C C A G A T T T C A A T G GCGTGATGCTG
A G G A A T G A T T T TTATCTGGGGATTAAACGTCTTT
G A A A G G C C A G T C C C T C C C T A A
G C C T A A T G G C C GGAGAAGGTGGCCCCGCTC
T G G G T T G T C G C C G C T G A A G G G A G T G
A C G T T T C T C T C G G C G C C C G C C C C T C G G G C G G C C
C G G C G G A A A G C T A G T T G G G G G C
C A A G C G C T T C C C G G A C T C C C G G T G G C C T C C
A G C A G G G A A G A A G C G G G G T G T T A A
C A C G A G A T T T C G T T T G A C T C A C A T C C T
G G T G G T C T G A A A G T C C A A AGGATCGTTG
T G T T T T C T T T G T T T T G T T T T G T T T T T T C T G
T T T G T T T G T G G T T G T T T T T T A G A G A G G T
G T G A A A A A A T G C A T A C T T A G G C A A A A C C C
G C G T G G T G A A A C A T C T T C G A T T T G A A T T

CACTTTCTGCCGGGAAAGCTGCTGCA
TAGGCAAAGTGTCCTTTCCAACGCTTAG
GGCCTTGGGCCCCAAGACCCCGAAGTCAA
AGCGATCCCGGCTGTGTTGGGATAATTGA
GGCAGATAGTTGGTGCTTTGGGCG
GATGGATGATTCAGGGGTGGGGACATTC
AGGTTCCAGTGGAGGGGGCGGGGCACC
AAGTCAATTAGGGGAAGGCGCCCCCGC
TAATCCTATGGGAAGCTCCCAAACGTCT
AGGACTGAGCCATTAAAGTGGACTCCA
GGTGCCCAAGGCGGTTCGCTCCAAGGCCTCAC
GGCCCCCTGGC TGCTCTACTCAG AGAACACGC
TCGGAGATATTTCAGGAGCA CGGGAAATTCCCAA
GTTTTCCTCGTTTCCTCCGATTATTTTGC
TCGGCATAATAGCAGCCAGATTTCAAT
GGCGTGATGCTGAGGAATGATTTT
TATCTGGGGATTAAACGTCTTTGAAAGGC
CAGTCCCTCCCTAA GCCTAATGGCCGGA
GAAGGTGGCCCCGCTCTGGGTTG
TCGCCGCTGAAGGGAGTGACGTTTCTCTCG
GCGCCCGCCCCTCGGGCGGCCCGGCG
GAAAGCTAGTTGGGGGCCAAGCGCTTCCCGG
ACTCCCGGTGGCCTCCAGCAGGGAA
GAAGCGGGGTGTTAACACGAGATTTC
GTTTGACTCACATCCTGGTGGTCTGAA
AGTCCAAAGGATCGTTGTGTTTTCTTTGTTTT
GTTTTGTTTTTTCTGTTTGTTTGTGG
TTGTTTTTTAGAGAGGTGTGAAAAAAT
GCATACTTAGGCAAAACCCGCGTGGTGAA
ACATCTTCGATTTGAATTCACTTTCTGCCG
GGAAAGCTGCTGCATAGGCAAAGTGTC
CTTTCCAACGCTTAGGGCCTTGGGCC
CCAAGACCCCGAAGTCAAAGCGATCCCGG
CTGTGTTGGGATAAT

PCR

The PCR was performed for 35 cycles in 100 μl and the mater mix composition is as follows:

DNA: 100 ng

dNTP mix (2.5 mM each): 4.0 μl

Forward Primer: 400 ng

Reverse Primer: 400 ng

Hot-start Taq polymerase assay buffer (10X): 1X

Hot-start Taq polymerase Enzyme: 5U

Glass distilled water was added to make up the volume to 100 μl

Sequencing

The PCR product was detected on a 1.5% agarose gel and then extracted from agarose by DNA extraction kit for sequencing.

ABI applied Bio-system incorporated sequencing kit was used for sequencing reaction and data was analyzed on genetic analyzer model no ABI3100.

The results were compared to the published sequence Nr. AF 426432 in NCBI database [Table 1].

Results and Discussion

Twenty cleft samples from the natives of each state where been studied for genetic analysis. Analysis of exon 1 of MSX1 of samples from different states reveals that except for Andhra Pradesh, more than one single point mutation was found in other states. Moreover, out of these mutations noticed, one of them was a silent mutation (C330T). Finally the result indicated that the cleft samples so collected from Andhra Pradesh had only one

Table 1: Muscle segment homeo box1 gene analysis for exon 1

Sample collection zone	Total no. of sample collection	Single nucleotide polymorphism sites	Sequence variation/alleles	Allelic frequency
Karnataka	20	G273A/C, (Gly91, Asp/Arg)	3/40	0.075
		C102G, (Ala34Gly)	4/40	0.1
		C330T, (Gly110Gly)	3/40	0.075
		G414T	1/40	0.025
Andhra Pradesh	20	G273A/C (Gly91, Asp/Arg)	4/40	0.1
Tamil Nadu	20	C102G, (Ala34Gly)	7/40	0.17
		C330T, (Gly110Gly)	8/40	0.2
Kerala	20	G273A/C, (Gly91, Asp/Arg)	12/40	0.3
		C102G, (Ala34Gly)	10/40	0.25
		C330T, (Gly110Gly)	9/40	0.225

G: Guanine; T: Thymine; A: Adenine; C: Cytosine: Ala: Alanine; Gly: Glycine; Asp: Aspartic acid; Arg: Arginine

point mutation of G to A/C at 273 positions. The importance of this mutation site is further substantiated by the fact that all the samples obtained from the states of Karnataka and Kerala also had the same. However this mutation was not noticed in the samples from Tamilnadu instead a silent mutation at C330T, and point mutation at C102G was noticed. Interestingly, this mutation was also noticed in the samples of Karnataka and Kerala. Therefore, analysis of Table 1 indicates that mutation of either G273A/C or C102G seems to be quite important for cleft formation and the mutation of G273A/C and C102G as lead to the change of either an acidic amino acid aspartic acid or a bulky, basic amino acid, arginine form a neutral, small amino acid, glycine is clear. So change in amino acid from a small, neutral one to a bulky, charged would have lead to a significant change in the conformation of the protein resulting in a functionally different protein.

In this analysis we also found a novel mutation (414G to T) which is submitted to NCBI Gene data bank (EF065625). We found less data in relation to Andhra zone for MSX1 whereas a good data was available in Kerala. We also noticed some samples produced insignificant sequence due to rich GC content of the gene.

Summary: In this analysis, we also found a novel mutation (414G to T) which is submitted to NCBI Gene data bank (EF065625). We found less data in relation to Andhra zone for MSX1 whereas a good data was available in Kerala. We also noticed some samples produced insignificant sequence due to rich GC content of the gene.

In case of exon 2, though some studies have shown change in amino acid from arginine to serine in position 151 and silent mutations at positions 181 and 275 but in our study we had no significant findings in exon 2.[29]

Conclusion

In comparison to the previous gene sequence Nr. AF426432 data (NCBI) this research article projects similar reports and positively reflects the genetic implications in South Indian population for MSX1 gene. In spite of multiple cultural/ethnic backgrounds genetic variations were similar in comparison to previous reports. We feel that this study may be the stepping stone for prenatal investigations/counseling and genetic correction through genetic engineering in future days to come for oral clefts.

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Besides we are also grateful to NGO organizations of all South Indian states that came forward in providing details of patients' nativity and also providing the basic historical ethnic, cultural and community backgrounds which were required for our study, which helped us while collecting samples.

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This research report was the part of the main research program which was registered for Rajiv Gandhi University of Health Sciences Bangalore, Karnataka, India, and it was an Institutional fundamental research and we are still extensively working on it. The detail research program started from 2004-2008 and it was started in A. B. Shetty Dental College which was the first PhD registered dental school for Rajiv Gandhi University of Health sciences and was awarded PhD in Sept 2008.

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