40

ELSEVIER

Available online at www.sciencedirect.com

SCIENCE DIRECT

JNMU

Journal of Nanjing Medical University, 2009, 23(1):40–45

www.elsevier.com/locate/jnmu

Research paper

Association of Single Nucleotide Polymorphisms in *IRF6* and *TGFA* Genes With Nonsyndromic Cleft Lip With Or Without Cleft Palate in Chinese Patients ☆

Ya Shen[a,△], Yugui Cui[a], Weidong Wan[b], Xiaoping Zhou[a], Lu Cheng[c], Zuhong Lu[c], Jiayin Liu[a,*]

[a]*Center of Clinical Reproductive Medicine, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China*

[b]*The Plastic Surgery Department of Zhong Da Hospital, Southeast University, Nanjing 210009, China*

[c]*State Key Laboratory of Bioelectronics, Southeast University, Nanjing 210096, China*

Received 6 November 2008

Abstract

Objective: Nonsyndromic cleft lip with or without cleft palate(NSCL/P) is a common birth defect with unclear etiology. Both genetic and environmental factors may contribute to NSCL/P. Many genes have been identified as candidate genes associated with this disease. Interferon regulatory factor 6(*IRF6*) gene and transforming growth factor-a(*TGFA*) gene seem to be crucial in the predisposition of NSCL/P. Here we evaluated some single nucleotide polymorphisms(SNPs) loci of *TGFA* and *IRF6* genes in Chinese nuclear families consisting of fathers, mothers and affected offspring with NSCL/P. **Methods:**Fifty patients of NSCL/P were confirmed by the plastic surgeons. They and their parents were included in the study, all with the informed consents. SNPs loci of *TGFA* and *IRF6* genes were analyzed by microarray technology. Some PCR products were randomly chosen and sequenced to check microarray results. The distribution of gene type and allele frequency between patient group and parents group were compared. Then a Haplotype Relative Risk(HRR) and Transmission Disequilibrium Test(TDT) were performed. **Results:**The sequences of randomly selected PCR products were all consistent with the microarray results. All loci were in Hardy-Weinberg equilibrium. There were no significant differences in the distribution of genotypes and alleles between patients and their parents. Using HRR and TDT analyses the V274I of *IRF6* was associated with NSCL/P, while another SNP locus of *IRF6* was not. Strong evidence of linkage disequilibrium was found between the 2 SNP loci of *TGFA* and disease with the HRR analysis, but not with the TDT analysis. **Conclusion:**Our study confirms the contribution of *IRF6* in the etiology of NSCL/P in populations of Asian ancestry. The association of TGFA with NSCL/P requires further research.

Key words: Nonsyndromic cleft lip with or without cleft palate(NSCL/P); transforming growth factor-a(TGFA); Interferon regulatory factor 6(IRF6); single nucleotide polymorphisms(SNPs)

INTRODUCTION

Nonsyndromic cleft lip with or without cleft palate (NSCL/P) is a common congenital facial malformation without any other structural or developmental abnormalities. The prevalence of NSCL/P varies from 1 in 500 to 1 in 2 500, depending on the ethnicity and geographic location of the population[1,2]. The etiology of NSCL/P is complex and both genetic and environmental factors may contribute to NSCL/P. Maternal smoking, alcohol use and insufficient folic acid and multivitamins are thought to be environment factors associated with NSCL/P[3-6]. In addition, by applying a variety of genetic and epidemiologic methods investigators have identified several candidate genes and loci associated with NSCL/P. These genes are classified into several categories as follows: polarizing signals, growth

☆ This work was supported by the Medical Technology Development Foundation of Jiangsu Provincial Health Bureau of China (H200513), Changjiang Scholars and Innovative Research Team in University (IRT0631) and National 973 Program(2006CB944005).

△ Graduate student, now working in Nantong Maternal and Child Care Service Center

*Corresponding author.

E-mail address:jyliu_nj@126.com

factors and receptors, transcription factors, cell adhesion molecules, and extracellular matrix[7,8].

Interferon regulatory factor 6(*IRF6*) gene and transforming growth factor-*a* (*TGFA*) gene belong to transcription factors and growth factors respectively[9,10]. *IRF6* contains a helix-turn-helix DNA-binding motif and is thought to play an important role in NSCL/P as mutations have been identified in this gene in Van der Woude syndrome(VWS[MIM 119300]), which is a dominant disorder sharing some symptoms with NSCL/P[11,12]. However *IRF6* is not always associated with NSCL/P[13].

The protein encoded by *TGFA* is a secretary protein that binds to the epidermal growth factor(*EGF*) receptor[9]. It acts as a potent epithelial mitogen and may stimulate extracellular matrix biosynthesis[14]. Expressing *TGFA* in palatal tissue in culture and its presentation at high levels in epithelial tissue of the medial edge of the palatal shelves at the time of shelf fusion makes it a candidate gene of NSCL/P[9].

Because the relationship of *IRF6* gene and *TGFA* gene with NSCL/P remains unclear, especially in a Chinese population, we carried out the present study to evaluate the association of single nucleotide polymorphisms(SNPs) of the *IRF6* and *TGFA* genes with NSCL/P.

MATERIALS AND METHODS

Subjects

The subjects of this study were 50 patients of NSCL/P, aged from 5 months to 30 years, all of whom were Han people from southeast China. NSCL/P was confirmed by the plastic surgeons, excluding cleft-associated syndromes, such as DiGeorge syndrome, Stickler syndrome, Nager syndrome, Pierre Robin sequence, EEC syndrome, and Van der Woude syndrome. The parents of those NSCL/P patients were also included in this study. Blood samples(2 ml) were collected in EDTA Sterile Interior(BD Vacutainer).

All subjects were recruited in the First Affiliated Hospital of Nanjing Medical University and the Zhongda Hospital of Southeast University. This study was approved by the local ethical committee and all patients gave their informed consent.

DNA extraction and PCRs

Genomic DNA was extracted from blood samples using PureGene DNA extraction kit(Gentra System, USA). PCRs were performed in 30 μl volume containing 1×PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.4 μM common forward primer and 0.4 μM reverse primer modified with an acrylamide group at their 5' terminal, 0.5 units Taq polymerase, and 50-100 ng DNA. Thermocycling was carried out over 30-second steps at 94℃, the primer-specific annealing temperature, and 72℃, respectively, for 35 cycles. The 2 loci of *TGFA* shared the same primers since the distance between the 2 loci was only 24 bases. The primer information is given in ***Table 1***.

Microarray

After PCR amplification and gel electrophoresis, PCR products were processed by ethanol precipitation and evaporation. The acryl-modified slides were supplied by State Key Laboratory of Bioelectronics, Southeast University(Nanjing, China). Solutions containing acrylamide-modified PCR products, 3% acrylamide, 1% APS and 30% glycerol monomers were prepared[15], then spotted onto the acryl-modified slides. After spotting, the slides were placed in a humid sealed chamber in which a well containing tetramethylenediamine (TEMED) was deposited in advance. The pressure in the sealed chamber was reduced to about 1000 Pascal (Pa), and this pressure was maintained for at least 0.5 h at room temperature.

Following the attachment, dsDNA(double stranded DNA) on the slides was denatured by electrophoresis in 0.1mol/L NaOH for 10 min to obtain ssDNA(single stranded DNA) for hybridization analysis. Then the solution containing the labeled probes(data shown in ***Table 2***) was applied to the microarray and spread evenly by placing a coverslip on the top, and the hybridization was performed at room temperature for 4 h in a humid glass chamber sealed with a plastic film. After hybridization, the slides were subjected to electrophoresis under 5-30 V/cm for 5-12 min in 1 × Tris-borate-ethylene-diaminetetraacetic acid(EDTA)(TBE) buffer at room temperature. Then the slides were rinsed and dried. Images of the slides were captured by a scanner (Lux Scan-10K/A, Beijing, China) and analyzed with LuxSpot 3.0 software.

Sequencing

Some PCR products were randomly chosen and sequenced to check the result from the microarrays. The sequencing assay was performed by the China South Gene Company(Shanghai, China).

Statistical analysis

Hardy-Weinberg Equilibrium Test was performed and the distribution of gene type and allele frequency between the patient group and parents group were compared. Haplotype Relative Risk(HRR) analysis was used in all patient-parents triads, while Transmission Disequilibrium Test(TDT) was performed only in nuclear families with heterozygous parents. χ^2 analyses were performed, and $P < 0.05$ was considered significant. All of the statistical analyses were carried out using Stata9.0 software.

RESULTS

The sequences of randomly selected PCR products were all consistent with the results of microarrays. The distributions of genotypes and allele frequencies in nuclear families are shown in ***Table 3***. All loci were in Hardy-Weinberg equilibrium(data not shown). There were no statistically significant differences in gene type and allele frequency between two groups at each locus (***Table 4***).

HRR analysis

The C3827T, T3851C of *TGFA* and V274I but not rs2235375 of *IRF6* were associated with NSCL/P(***Table 5***).

TDT analysis

Only V274I of *IRF6* was associated with NSCL/P (χ^2=4.787, υ=1, P< 0.05). The V allele was found to be overtransmitted by parents to patients. The results were not significant for the association of other loci with NSCL/P(***Table 6***).

DISCUSSION

Craniofacial development is comprised of complex events, coordinated by a network of signaling molecules, transcription factors, and proteins conferring cell polarity, as well as interactions between cells[8]. Any gene playing a role in the network mentioned above could be a candidate gene for NSCL/P[5-7, 16].

Table 1 **Information about each primer**

Gene	Locus		Primers	Annealing Temperature
TGFA	C3827T	F	5'-GCCCAACACAGGAGATTTC-3'	60℃
	T3851C	R*	5'-acrylamide-GTGGCGATAGCTTGGGATG-3'	
IRF6	V274I	F	5'-GCTGCCGACTCTTCTATG-3'	55.3℃
		R*	5'-acrylamide-GACCAGTACACCTTGCAC-3'	
	rs2235375	F	5'-GTTTCTTTACTTCTTCCCTG-3'	57.7℃
		R*	5'-acrylamide-GCCCTTCTATAGCTCTCC-3'	

*primers modified with acrylamide group at their 5'-terminal.

Table 2 **Information about probes of each locus**

Gene	Locus		Probe sequence
TGFA	C3827T	CY3	5'-Cy3-cgagaccctcaac-3'
		CY5	5'-Cy5-cgagactctcaac-3'
	T3851C	CY3	5'-Cy3-cttccatggacac-3'
		CY5	5'-Cy5-cttccacggacac-3'
IRF6	V274I	CY3	5'-Cy3-GAGCAGGTCAAAT-3'
		CY5	5'-Cy5-GAGCAGATCAAAT-3'
	rs2235375	CY3	5'-Cy3-AAGACCCAGCAAG-3'
		CY5	5'-Cy5-AAGACCGAGCAAG-3'

CY3: probes labeled with Cy3 fluorescent dyes regarded as wild type probes;
CY5: probes labeled with Cy5 fluorescent dyes regarded as mutate probes.

Table 3 **Distribution of genotype and allele frequency in nuclear families**

Gene	Site	Group	Gene type(%)			Allele(%)	
			C1C1a	C1C2b	C2C2c	C1	C2
TGFA	C3827T	patients	14(28d)	28(56)	8(16)	56(56)	44(44)
		parents	42(42)	42(42)	16(16)	126(63)	74(37)
	T3851C	patients	47(94)	3(6)	0(0)	97(97)	3(3)
		parents	86(86)	14(14)	0(0)	186(93)	14(7)
IRF6	V274I	patients	28(56)	20(40)	2(4)	76(76)	24(24)
		parents	45(45)	48(48)	7(7)	138(69)	62(31)
	rs2235375	patients	8(16)	25(50)	17(34)	41(41)	59(59)
		parents	19(19)	53(53)	28(28)	91(45.5)	109(54.5)

C1: wild type base; C2: mutate base. a: wild type homozygote; b: heterozygote; c: mutate homozygote; d: distribution frequency.

Table 4 **Statistical analysis of gene type and allele frequency at each locus**

Gene	Site	Gene type		Allele	
		χ^2	*P*	χ^2	*P*
TGFA	C3827T	3.150 0	0.207	1.396 0	0.242
	T3851C	2.123 0	0.145	1.995 4	0.158
IRF6	V274I	1.799 4	0.407	1.597 5	0.206
	rs2235375	0.624 4	0.732	0.547 9	0.459

Table 5 **The HRR results of each locus**

Gene	Locus	T/NT	C1	C2	x^2	*P*
TGFA	C3827T	T	56	44	4.204 2	< 0.05
	T3851C	NT	70	30		
IRF6	V274I	T	97	3	4.915 5	< 0.05
		NT	89	11		
	rs2235375	T	76	24	4.581 6	< 0.05
		NT	62	38		
		T	41	59	1.633 2	> 0.05
		NT	50	50		

T: transmitted; NT: not transmitted; C1: wild type base; C2: mutate base.

Table 6 **The TDT results of each locus**

Gene	Locus	NT/T	C1	C2	x^2	*P*
TGFA	C3827T	C1	17	22	0.095	> 0.05
		C2	20	7		
IRF6	T3851C	C1	12	3	3.500	> 0.05
		C2	11	0		
	V274I	C1	19	17	4.787	< 0.05
		C2	31	5		
	rs2235375	C1	11	30	0.925	> 0.05
		C2	23	18		

T: transmitted; NT: not transmitted; C1: wild type base; C2: mutate base.

On the basis of its involvement in Van der Woude's syndrome, an autosomal dominant form of cleft lip and palate, investigators identified *IRF6* gene as a candidate gene of NSCL/P[17-19]. *IRF6* is expressed in the palate MEE prior to and during the fusion of the palate in the mouse. Zucchero *et al* studied 8003 patients belonging to 10 populations with ancestry in Asia, Europe, and South America[11], Jakobsen et al studied the suggestive linkage to a neighboring region of IRF6 in a cleft lip and palate multiplex family[20]. The linkage disequilibrium extended from 40 kbp in the 5' direction to 100 kbp 3' of *IRF6* and overtransmission of the valine(V) allele was found in the entire population data set. The V allele was over-transmitted to offspring from parents, and the results for some individual populations from South America and Asia were highly significant. In the current study the result of HRR at this locus was consistent with that of TDT, which showed a positive relationship between the V allele and NSCL/P at the V274I locus. The V allele was also overtransmitted to offspring from their parents in southeast China. The facts that I variant allele is rare in European populations and that the V allele is present in the Pan troglodytes indicate that the V allele is probably the ancestral allele in *Homo sapiens*[10,11]. During the *Homo sapiens* evolutionary process the variance from V allele to I allele may reduce the risk of NSCL/P. So the I allele might be a protective factor in NSCL/P. The positive result in TDT suggested that the linkage disequilibrium might exist between V274I and the susceptibility gene, and IRF6 was probably the susceptibility gene of NSCL/P. This finding strongly supports the involvement of IRF6 gene in populations of Asian ancestry. There were other SNPs loci found being overtransmitted in the study of Zucchero and colleagues[11]. One of them was rs2235375. Scapoli *et al* obtained strong evidence that linkage disequilibrium existed between rs2235375 and NSCL/P in an Italian population[12]. However the same evidence was not found between rs2235375 and NSCL/P in the current research. Since the etiology of NSCL/P is complex, the different results might be due to ethnic and/or environment variances between the Chinese population and Italian population, or the small sample size[18-21].

TGFA might be a modifier gene rather than a necessary and sufficient determinant related to NSCL/P since mice with a null mutation of the *TGFA* gene had abnormal skin, hair and eyes but did not have oral clefts[9, 22]. Also, the genetic correlation of human oral clefts with *TGFA* might be explained by the phenomenon that the incidence of oral cleft among mice with *EGF* receptor-negative/-negative was high[23]. If the ligand function of *TGFA* for the *EGF* receptor is altered, the proliferation and differentiation of palatal epithelial cells will be affected, which may result in an oral cleft, as is the case in mice with *EGF* receptor-negative/-negative.

Hayward *et al* first found the *TGFA* Taq I polymorphism with 4 missing bases in the fifth intron[24]. Since the first report of an association of *TGFA* with oral clefts a number of studies have been carried out[9, 23]. Other variants in *TGFA* include BamHI, RsaI, HinfI, C3296T, C3827T, and C3296T, C3827T and T3851C locate in the 3' untranslated region(3'-UTR) with unclear foundation[25]. Since most studies that were

aimed at showing that *TGFA* gene contributes to the formation of NSCL/P focused on the *TGFA* TaqI polymorphism[26], the SNPs loci in 3'-UTR were chosen in this study to see whether the *TGFA* gene played a role in the etiology of NSCL/P. A positive relationship between T3827C, T3851C, A3879G in *TGFA* K-primer region and CPO was found in the study of Shiang *et al*[27]. In a study comprised of 43 patients and 73 people as controls that was carried out by Tanabe *et al*[28], the distribution of genotypes at C3827T, T3851C in *TGFA* K-primer was statistically different between the patient group and control group, indicating that *TGFA* plays a role in the formation of NSCL/P. Schultz and colleagues confirmed that C3827T in the *TGFA* K-primer was associated with NSCL/P in Filipino families[29]. However two other reports did not support this conclusion[30]. In the present study the positive HRR results of C3827T, T3851C in the *TGFA* 3'-UTR suggested that these loci were associated with NSCL/P. The results in the TDT were negative at the same 2 loci, and we thought the reason might be that the TDT used only heterozygous parents and was less powerful than the HRR when there was no population stratification[31]. The HRR method, reducing but not eliminating potential population stratification bias, may lead to false positive result[32]. The different conclusions deduced from the HRR and the TDT analysis for both C3827T and T3851C may reflect the fact that the HRR contains more information than the TDT. However, the possibility of the potential population stratification bias leading to false positive result in HRR analysis can not be excluded[9,31,32]. It is also important to consider the possibility that the negative result in the TDT was probably due to the small sample size. It is of interest to note that there was a non-significant trend toward a positive association at the T3851C locus in the TDT. We feel that a larger sample size might provide adequate power to detect a positive association. More studies should be undertaken at these two loci.

To accelerate the discovery of disease-related genes, advanced technologies which can identify genetic polymorphisms rapidly and accurately are urgently needed. Among a number of newly described methods for detecting SNPs, DNA microarrays has been gradually achieving widespread use[15]. Microarray technology was used to evaluate the relationship between the SNPs of *TGFA* gene, *IRF6* gene and NSCL/P in this study. The advantage of microarray analysis consists of its automation, high throughput and great precision. Simultaneous analysis of a large amount of molecular information leads to a more rapid genotyping than traditional SSCP and RFLP methods[15].

The potential for spurious associations due to population stratification in the traditional patient-control design makes the design a target of criticism[31]. Some methods have been introduced to tackle the problem of population stratification such as the HRR and the TDT. When there is no stratification the HRR is more powerful than the TDT because the HRR uses not only homozygous parents but also heterozygous parents, while the TDT uses heterozygous parents only. The HRR reduces but does not eliminate potential population stratification bias while the TDT can eliminate it completely[32], so when there is stratification, the HRR is more likely to yield false positive results. The TDT has now become more widely used and it is the first choice when a test for association in the presence of linkage is needed. However, because the TDT uses heterozygous parents a larger number of subjects is needed to get positive result.

Although the sample size is not large enough to add to understanding the role of the IRF6 gene and the TGFA gene with NSCL/P, in a larger context this study provides evidence of the IRF6 gene contributing to the formation of NSCL/P in southeast China. Further study with advanced technology should be carried out to find out more loci in TGFA and IRF6 genes and new candidate genes in NSCL/P when a larger patient sample is available. Functional experiments are required to disclose the mechanism of the disturbed facial development. We hope that the results of the current study will help to clarify the etiology of this common congenital malformation.

References

[1] Murray JC. Gene/environment causes of cleft lip and/or palate. *Clin Genet* 2002;61: 248-56.

[2] Wyszynski DF, Wu T. Use of US birth certificate data to estimate the risk of maternal cigarette smoking for oral clefting. *Cleft Palate Craniofac* 2002; 39: 188-92.

[3] Shaw GM, Lammer EJ. Maternal periconceptional alcohol consumption and risk for orofacial clefts. *J Pediatr* 1999; 134: 298-303.

[4] Shaw GM, Nelson V, Carmichael SL, Lammer EJ, Finnell RH, Rosenquist TH. Maternal periconceptional vitamins: interactions with selected factors and congenital anomalies? *Epidemiology* 2002;13: 625-30.

[5] Ray JG, Meier C, Vermeulen MJ, Wyatt PR, Cole DE. Association between folic acid food fortification and congenital orofacial clefts. *J Pediatr* 2003;143: 805-7.

[6] Chiquet BT, Blanton SH, Burt A, Ma D, Stal S, Mulliken JB, Hecht JT. Variation in WNT genes is associated with nonsyndromic cleft lip with or without cleft palate. *Hum Mol Genet* 2008; 17: 2212-8.

[7] Zhang WG, Luo SJ, Zhu SF, Tang SM. Comparative genomic hybridization analysis of nonsyndromic cleft lip with palate. *Zhonghua Zheng Xing Wai Ke Za Zhi(in Chinese)* 2008;24: 123-5.

[8] Stanier P, Moore GE. Genetics of cleft lip and palate: syndromic genes contribute to the incidence of non-syndromic clefts. *Hum Mol Genet* 2004;13:R73-R81.

[9] Yuan KF, Lai QG, Zhou XH, Qin YF. Association of transforming growth factor-alpha gene polymorphism and environment factors with nonsyndromic cleft lip with or without cleft palate in Han nationality. *Hua Xi Kou Qiang Yi Xue Za Zhi(in Chinese)* 2007; 25: 285-8.

[10] Blanton SH, Cortez A, Stal S, Mulliken JB, Finnell RH, Hecht JT. Variation in IRF6 contributes to nonsyndromic cleft lip and palate. *Am J Med Genet A* 2005; 137A: 259-62.

[11] Zucchero TM, Cooper ME, Maher BS, Daack-Hirsch S, Nepomuceno B, Ribeiro L, *et al.* Interferon regulatory factor 6 (IRF6) gene variants and the risk of isolated cleft lip or palate. *N Engl J Med* 2004;351:769-80.

[12] Scapoli L, Palmieri A, Martinelli M, Pezzetti F, Carinci P, Tognon M, *et al.* Strong evidence of linkage disequilibrium between polymorphisms at the IRF6 locus and nonsyndromic cleft lip with or without cleft palate, in an Italian population. *Am J Hum Genet* 2005;76:180-3.

[13] Hering R, Grundmann K. The IRF6 p.274V polymorphism is not a risk factor for isolated cleft lip. *Genet Med* 2005; 7: 209-11.

[14] Dixon, M.J., Carette, M.J., Moser, B.B. Ferguson MW. Differentiation of isolated murine embryonic palatal epithelium in culture: exogenous transforming growth factor alpha modulates matrix biosynthesis in defined experimental conditions. *In Vitro Cell Dev. Biol* 1993; 29: 51-61.

[15] Xiao PF, Cheng L, Wan Y, Chen ZZ, Zhang SY, Zhang ZC, *et al.* An improved gel-based DNA microarray method for detecting single nucleotide mismatch. *Electrophoresis* 2006;27: 3904-15.

[16] Chiquet BT, Lidral AC, Stal S, Mulliken JB, Moreno LM, Arcos-Burgos M, *et al.* CRISPLD2: a novel NSCLP candidate gene. *Hum Mol Genet* 2007; 16: 2241-8.

[17] Gatta V, Scarciolla O, Cupaioli M, Palka C, Chiesa PL, Stuppia L. A novel mutation of the IRF6 gene in an Italian family with Van der Woude syndrome. *Mutat Res* 2004; 547: 49-53.

[18] Cobourne MT. The complex genetics of cleft lip and palate. *Eur J Orthod* 2004; 26: 7-16.

[19] Ghassibe M, Revencu N, Bayet B,Gillerot Y, Vanwijck R, Verellen-Dumoulin C. Six families with van der Woude and/or popliteal pterygium syndrome: all with a mutation in the IRF6 gene. *J Med Genet* 2004; 41: 15-9.

[20] Jakobsen LP, Ullmann R, Kjaer KW, Knudsen MA, Tommerup N, Eiberg H. Suggestive linkage to a neighboring region of IRF6 in a cleft lip and palate multiplex family. *Am J Med Genet A* 2007; 143A: 2716-21.

[21] Kondo S, Schutte BC, Richardson RJ, Bjork BC, Knight AS, Watanabe Y, *et al.* Mutations in IRF6 cause Van der Woude and popliteal pterygium syndromes. *Nat Genet* 2002; 32: 285-9.

[22] Mann GB, Fowler KJ, Gabriel A , Nice EC, Williams RL, Dunn AR. Mice with a null mutation of the TGFa gene have abnormal skin architecture, wavy hair, and curly whiskers and often develop corneal inflammation. *Cell* 1993; 73: 249-61.

[23] Miettinen PJ, Chin JR, Shum L, Slavkin HC, Shuler CF, Derynck R. Epidermal growth factor receptor function is necessary for normal craniofacial development and palate closure. *Nat Genet* 1999; 22: 69-73.

[24] Hayward NK, Nancarrow DJ, Bell GI. A TaqI polymorphism for the human transforming growth factor alpha gene(TGFA). *Nucleic Acids Res* 1987; 15: 5503-5.

[25] Machida J, Yoshiura K, Funkhauser CD, Nagato N, Kawai T, Murray JC. Transforming growth factor-a(TGFA): genomic structure, boundary sequences, and mutation analysis in nonsyndromic cleft lip/palate and cleft palate only. *Genomics* 1999; 61: 237-42.

[26] Alexandre R. Vieira. Association between the Transforming Growth Factor Alpha Gene and Nonsyndromic Oral Clefts: A HuGE Review.*Am J Epidemiol* 2006; 163: 790-810.

[27] Shiang R, Lidral AC, Ardinger HH, Buetow KH, Romitti PA, Munger RG, e*t al.* Association of transforming growth-factor alpha gene polymorphisms with nonsyndromic cleft palate only (CPO). *Am J Hum Genet* 1993; 53: 836-43.

[28] Tanabe A, Taketani S, Endo-Ichikawa Y, Tokunaga R, Ogawa Y, Hiramoto M. Analysis of the candidate genes responsible for non-syndromic cleft lip and palate in Japanese people. *Clin Sci (Lond)* 2000; 99: 105-11.

[29] Schultz RE, Cooper ME, Daack-Hirsch S, Shi M, Nepomucena B, Graf KA, *et al.* Targeted scan of fifteen regions for nonsyndromic cleft lip and palate in Filipino families. *Am J Med Genet* 2004; 125: 17-22.

[30] Lidral AC, Murray JC, Buetow KH, Basart AM, Schearer H, Shiang R. Studies of the candidate genes TGFB2, MSX1, TGFA, and TGFB3 in the etiology of cleft lip and palate in the Philippines. *Cleft Palate Craniofac J* 1997; 34: 1-6.

[31] Jorde LB. Linkage disequilibrium and the search for complex disease genes. *Genome Res* 2000;10: 1435-44.

[32] Spielman RS, Ewens WJ. The TDT and other family-based tests for linkage disequilibrium and association. *Am J Hum Genet* 1996; 59: 983-9.