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Genetic Analysis of *TGFA, MTHFR,* and *IFR6* in Korean Patients Affected by Nonsyndromic Cleft Lip with or without Cleft Palate (CL/P)

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Abstract

Nonsyndromic cleft lip with or without cleft palate (CL/P) is a common craniofacial birth defect that is the result of a mixture of genetic and environmental factors. While studies have identified a number of different candidate genes and loci for the etiology of CL/P, the results have not been consistent among different ethnic groups. To study the genetic association of the candidate genes in Korean patients affected by CL/P, we genotyped 97 nonsyndromic CL/P patients and 100 control individuals using single nucleotide polymorphic markers at the *MTHFR*, *TGFA*, and *IRF6* genes. We report that the T3827C marker at *TGFA* showed significant association with nonsyndromic CL/P, but all the other markers tested were not significantly associated with nonsyndromic CL/P in Korean patients.

Keywords: cleft lip and cleft palate, genetic analysis, *IRF6, MTHFR, TGFA*

Introduction

Cleft lip with or without cleft palate (CL/P) is the most common craniofacial birth defect with an average prevalence of 1 in 500 to 1 in 2,500 births (Tolarova *et al*., 1998). The incidence of CL/P significantly varies with different ethnic groups, geographic locations and socioeconomic status. African Americans exhibit the lowest prevalence of 0.3 in 1,000 births, followed by Caucasians with prevalence of 1 in 1000 births (Wyszynski *et al*., 1996). In contrast, Asians and Native Americans show the highest prevalence of 2.1 and 3.6 in 1,000 births, respectively (Wyszynski *et al*., 1996). CL/P is epidemiologically and etiologically distinct from isolated cleft palate (CP) which shows an ethnic heterogeneity of incidence that is distinct from CL/P (Jones, 1993). Orofacial clefting also occurs as a characteristic clinical feature in syndromic cases, such as Van der Woude syndrome, cleft palate with ankyloglossia, and Treacher-Collins syndrome (Stainier *et al*., 2004). However, most cases of orofacial clefting are nonsyndromic, showing no association with other structural abnormalities.

Nonsyndromic CL/P is a genetically and developmentally complex disorder, with a mutifactorial etiology that encompasses genetic and environmental components. Palatal developmental is an elaborately controlled process involving a number of different factors including growth factors, transcription factors, receptors, cell adhesion proteins, extracellular matrix proteins, and matrix metalloproteinases (Stainier *et al*., 2004). A number of genes, including *TGFA* (transforming growth factor alpha), *TGFB2* (transforming growth factor beta 2), *TGFB3* (transforming growth factor beta 3), *IRF6* (interferon regulatory factor 6), *MSX1* (muscle segment homeobox Drosophila homolog 1), and *MTHFR* (methylenetetrahydrofolate reductase), have been reported in association studies with nonsyndromic CL/P (Cobourne, 2004; Jugessur *et al*., 2005). The epigenetic influence of environmental factors such as folate deficiency, steroid and statin use, retinoid exposure, maternal smoking, and maternal alcohol use are also associated with the incidence of CL/P (Cobourne, 2004; Jugessur *et al*., 2005).

In an attempt to re-examine association of *MTHFR*, *TGFA*, and *IRF6* with nonsyndromic CL/P in Korean patients, we genotyped 97 nonsyndromic CL/P patients with the *MTHFR*-C677T, *TGFA*-C3296T, *TGFA*-T3827C and *IRF6*-G820A markers. In these studies, *TGFA*-T3827C showed significant association with nonsyndromic CL/P, but the other markers were not significantly associated with nonsyndromic CL/P in Korean patients.

Materials and Methods

Patients and DNA samples

The study group consisted of 97 Korean patients with

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nonsyndromic CL/P and 100 non-related healthy individuals. The CL/P phenotype spectrum included unilateral or bilateral cleft lip with or without cleft palate. Some of the patients also showed bifid uvula. After informed consent was obtained from patients and control individuals, blood samples were taken to be used in the study. Genomic DNA samples of the participating individuals were extracted from whole blood using a Puregene DNA Isolation Kit according to the manufacturer's protocol (Gentra Systems).

Genotype analysis

The genomic regions encompassing *MTHFR*-C677T, *TGFA*-C3296T, *TGFA*-T3827C, and *IRF6*-G820A were PCR-amplified using primers derived from the flanking sequences of each marker. The sequences of the primers are listed in Table 1. Thermocycling was performed using *Taq* polymerase (Promega) according to the manufacturer's instructions. For restriction fragment length polymorphism (RFLP) analysis of *MTHFR*-C677T, *TGFA*-C3296T, and *IRF6*-G820A, the PCR-amplicons were digested with *Hinf*I, *Dra*I, or *Dpn*II, respectively. After digestion with the appropriate restriction enzyme, the DNA fragments were analyzed by electrophoresis in 10% non-denaturing polyacrylamide gels or 2.5% agarose gels. For the analysis of *TGFA*-T3827C which does not have an informative restriction site, genotyping was performed by sequencing the PCR-amplified fragments using the Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Sequence scanning was carried out using an ABI 3100 genetic analyzer (Applied Biosystems). Allele frequencies in CL/P patients and controls were determined by counting alleles and calculating sample proportions. The Hardy-Weinberg equilibrium was evaluated for all markers. Fisher's exact probability test was utilized to assess *P* and χ^2 values. *P*-values less than 0.05 were considered to indicate statistical significance.

Table 1. The PCR primer sequences used for genotyping analysis

Gene	Marker		Primer sequence
MTHFR	C677T	Forward	5'-CAAAGGCCACCCCGAAGC-3'
		Backward	5'-AGGACGGTGCGGTGAGAGTG-3'
TGFA	C3296T	Forward	5'-CTTATTTTCCCAACGTGGCC -3'
		Backward	5'-CTGGCCAAACTCCTCCTCTGGGCTCTTTA-3'
	T3827C	Forward	5'-TCACATGAAGACCCTAGCTT-3'
		Backward	5'-AGGCCAGACATTTCTAATCA-3'
IRF6	G820A	Forward	5'-TGGCTTGATCTTAATGGACT-3'
		Backward	5'-GCTAGTGAACAGCTTCTGCT-3'

Results

The genotypes at *MTHFR*-C677T, *TGFA*-C3296T, and *IRF6*-G820A were determined by RFLP analysis. A nucleotide change from C to T at position 667 of *MTHFR* creates a *Hinf*I restriction site resulting in fragments of 175 bp and 71 bp following *Hinf*I digestion of the 246 bp DNA. The 246 bp fragment represents the C allele while the 175 bp fragment represents the T allele (Fig. 1A). Similarly, a C to T change at *TGF*α-C3296T creates a *Dra*I restriction site that leads to digestion of the 98 bp fragments into 70 bp and 28 bp fragments (Fig. 1B). In the case of *IRF6*-G820A, a G to A change introduces a *Dpn*II restriction site leading to digestion of the 298 bp fragments into 234 bp and 64 bp fragments (Fig. 1C). Genotypes at *TGFA*-T3827C were assessed by DNA sequencing analysis of the PCR-amplified fragments.

Table 2. Genotype frequencies of the markers used for the association studies with CL/P

Marker	Genotype	CL/P (n= 97)	Control (n=100)	χ^2	*P* value
MTHFR C677T	CC	23.7%	22.0%	0.93	0.628
	CT	49.5%	56.0%		
	TT	26.8%	22.0%		
TGFA C3296T	CC	40.2%	46.0%	0.69	0.798
	CT	53.6%	48.0%		
	TT	6.2%	6.0%		
TGFA T3827C	CC	34.0%	32.0%	11.0	0.004
	CT	28.9%	49.0%		
	TT	37.1%	19.0%		
IRF6 G820A	GG	48.5%	34.0%	5.07	0.079
	GA	41.2%	57.0%		
	AA	10.3%`	9.0%		

Table 3. Allele frequencies of the markers used for the association studies with CL/P.

Marker	Allele	CL/P (n= 97)	Control (n=100)	χ^2	*P* value
MTHFR C677T	C	48.5%	50.0%	0.09	0.764
	T	51.5%	50.0%		
TGFA C3296T	C	67.0%	70.0%	0.41	0.522
	T	33.0%	30.0%		
TGFA T3827C	C	48.5%	56.5%	2.56	0.110
	T	51.5%	43.5 %		
IRF6 G820A	G	69.1%	62.5%	1.89	0.169
	A	30.9%`	37.5%		

Genotype frequencies of *MTHFR*-C677T, *TGFA*-C3296T, *TGFA*-T3827C, and *IRF6*-G820A in CL/P patients and normal controls are summarized in Table 2. At *MTHFR*-C677T, *TGFA*-C3296T, and *IRF6*-G820A, there were no statistically significant differences in the genotype frequencies between CL/P patients and controls. In contrast, the genotype frequencies of *TGFA*-T3827C were significantly different between the patient and control groups with a *P*-value of 0.004 (Table. 2), indicating that the TT polymorphism is significantly associated with CL/P in the patient group tested. However, there was no statistically significant

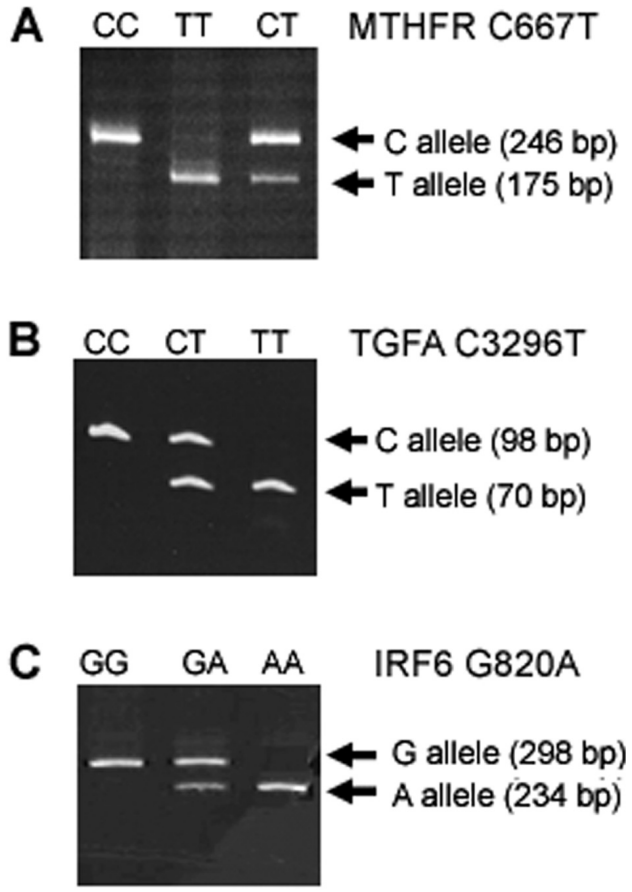

Fig. 1. RFLP analysis at *MTHFR*-C667T, TGFA-C3296T, and *IRF6*-G820A. **A.** At *MTHFR*-C6667T, the 246 bp and 175 bp DNA fragments represent the C and T alleles, respectively. **B.** At TGFA-C3296T, the 98 bp and 70 bp DNA fragments represent the C and T alleles, respectively. **C.** At *IRF6*-G820A, the 298 bp and 234 bp DNA fragments represent the G and A alleles, respectively.

difference in the allele frequencies of C and T between CL/P patients and controls (Table. 3).

Discussion

MTHFR plays a key role in the metabolism of folate. The C677T change in *MTHFR* leads to the substitution of a highly conserved alanine residue into a valine residue in the catalytic domain of MTHFR. The reduced MTHFR activity resulting from the C677T mutation leads to an increase in the plasma homocysteine level and a decrease in the plasma folate level (Blom, 1998). Given that the intake of folate reduces the probability of women giving birth to a child affected by a neural tube defect (NTD), this enzyme was suggested as an attractive candidate for the etiology of CL/P (MRC Vitamin Study Research Group, 1991). Genotype analysis of several different ethnic groups has revealed higher frequencies of 677TT homozygosity in NTD patients than in controls (de Franchis *et al*. 1995; Kirke *et al*., 1996; Mills *et al*., 1995). However, some studies have not shown a significant association between the C667T allele and NTD in different ethnicities (Koch *et al*., 1998; Mornet *et al*., 1997; Papapetrou *et al*., 1996). In concurrence with previous studies, the study presented here did not show a statistically significant association between *MTHFR*-C677T and CL/P. We detected approximately similar frequencies of 677TT homozygosity in both CL/P and control groups.

Van der Woude syndrome (VWS; OMIM 119300) is an autosomal dominant disorder characterized by congenital lip pits with CL/P. VWS is the most common form of syndromic cleft lip and palate, accounting for approximately 2 % of all cleft lip and palate cases. It is distinguished from nonsyndromic cleft lip and palate by the presence of lower lip pits (Burdick *et al*., 1985). By direct sequence analysis of the genomic VWS region of a pair of monozygotic twins discordant for the VWS phenotype, *IRF6* was identified as the gene mutated in this orofacial malformation, suggesting a possibility that *IRF6* may also play a role in nonsyndromic CL/P (Kondo *et al*., 2002). Recently, several studies have reported that a single nucleotide polymorphism, G820A in *IRF6*, is associated with CL/P in several different ethnic groups (Scapoli *et al*., 2005; Zucchero *et al*., 2004). The G820A polymorphism results in a substitution from valine to isoleucine at codon 274, which is located in a putative Smad-interferon regulatory factor-binding domain of IRF6. The Smad proteins are a family of transcription factors known to transduce signals from the TGF-β superfamily (Brivanlou *et al*., 2002). However, in our study the G820A was not significantly associated with nonsyndromic CL/P, but the A allele was less frequently found in CL/P than in controls. These contradictory results may reflect genetic heterogeneity, incomplete penetrance, or limited sample sizes.

During craniofacial development, TGFα, a well characterized mammalian growth factor, is expressed in the medial edge of the epithelium of fusing palatal shelves, and promotes synthesis of extracellular matrix and mesenchymal cell migration into the palates (Dixon *et al*., 1991). A number of studies have reported genotype analysis of CL/P patients with genetic markers in *TGFA* since the first finding that polymorphisms in *TGFA* were associated with nonsyndromic CL/P (Ardinger *et al*., 1989). In the genotyping studies presented here, *TGFA*-T3827C showed a statistically significant association with CL/P, with a higher frequency of the TT genotype in CL/P patients than in controls, while *TGFA*-C3296T displayed no statistical

significance in its association with CL/P. Several previous studies also reported the association of *TGFA*-C3827T with nonsyndromic CL/P in different ethnic backgrounds including Caucasians and Filipinos (Prescott *et al*, 2000; Schultz *et al*., 2004).

In summary, our genotype analyses show a significant association of *TGFA*-T3827C with nonsyndromic CL/P. However, *TGFA*-C3296T, *MTHFR*-C677T and *IRF6*-G820A showed no statistical significance in their association with CL/P. These contradictory results are probably due to genetic heterogeneity, incomplete penetrance, limited sample sizes and different study designs. A meta-analysis in a wider range of patient groups with more complete clinical descriptions of affected individuals may be required to more completely characterize the association between genetic markers and nonsyndromic CL/P.

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References

Ardinger, H.H., Buetow, K.H., Bell, G.I., Bardach, J., VanDemark, D.R., and Murray, J.C. (1989). Association of genetic variation of the transforming growth factor-alpha gene with cleft lip and palate. *Am. J. Hum. Genet.* 45, 348-353.

Blom, H.J. (1998). Mutated 5,10-methylenetetrahydrofolate reductase and moderate hyperhomocysteinaemia. *Eur. J. Pediatr.* 157, 131-134.

Brivanlou, A.H. and Darnell, J.E. (2002). Signal transduction and the control of gene expression. *Science* 295, 813-818.

Burdick, A.B., Bixler, D., and Puckett, C.L. (1985). Genetic Analysis in families with Van der Woude syndrome. *J. Craniofac. Genet. Dev. Biol.* 5, 181-208.

Cobourne, M.T. (2004). The complex genetics of cleft lip and palate. *Eur. J. Orthod.* 26, 7-16.

de Franchis, R., Sebastio, G., Mandato, C., Andria, G., and Mastroiacovo, P. (1995). Spina bifida, 677 C-T mutation, and role of folate. *Lancet* 346, 1703.

Dixon, M.J., Garner, J., and Ferguson, M.W.J. (1991). Immunolocalization of epidermal growth factor (EGF), EGF receptor and transforming growth factor alpha (TGF α) during murine palatogenesis in vivo and in vitro. *Anat. Embryol*. 184, 83-91.

Jones, M.C. (1993). Facial clefting. Etiology and developmental pathogenesis. *Clin. Plast. Surg.* 20, 599-606.

Jugessur, A. and Murray, J.C. (2005). Orofacial clefting: recent insights into a complex trait. *Curr. Opin. Genet. Dev.* 15, 270-278.

Kirke, P.N., Mills, J.L., Whitehead, A.S., Molloy, A., and Scott, J.M. (1996). Methylenetetrahydrofolate reductase mutation and neural tube defects. *Lancet* 348, 1037-1038.

Koch, M.C., Stegmann, K., Ziegler, A., Schroter, B., and Ermert, A. (1998). Evaluation of the MTHFR C677T allele and the MTHFR gene locus in a German spina bifida population. *Eur. J. Pediatr.* 157, 487-492.

Kondo, S., Schutte, B.C., Richardson, R.J., Bjork, B.C., Knight, A.S., Watanabe, Y., Howard, E., de Lima, R.L., Daack-Hirsch, S., Sander, A., McDonald-McGinn, D.M., Zackai, E.H., Lammer, E.J., Aylsworth, A.S., Ardinger, H.H., Lidral, A.C., Pober, B.R., Moreno, L., Arcos-Burgos, M., Valencia, C., Houdayer, C., Bahuau, M., Moretti-Ferreira, D., Richieri-Costa, A., Dixon, M.J., and Murray, J.C. (2002). Mutations in IRF6 cause Van der Woude and popliteal pterygium syndromes. *Nat. Genet.* 32, 285-289.

Mills, J.L., McPartlin, J.M., Kirke, P.N., Lee, Y.J., Conley, M.R., Weir, D.G., and Scott, J.M. (1995). Homocysteine metabolism in pregnancies complicated by neuraltube defects. *Lancet* 345, 149-151.

Mornet, E., Muller, F., Lenvoise-Furet, A., Delezoide, A.L., Col, J.Y., Simon-Bouy, B., and Serre, J.L. (1997). Screening of the C677T mutation on the methylenetetrahydrofolate reductase gene in French patients with neural tube defects. *Hum. Genet.* 100, 512-514.

MRC Vitamin Study Research Group. (1991). Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. *Lancet* 338, 131-137.

Papapetrou, C,, Lynch, S.A., Burn, J., and Edwards, Y.H. (1996). Methylenetetrahydrofolate reductase and neural tube defects. *Lancet* 348, 58.

Prescott, N.J., Lees, M.M., Winter, R.M., and Malcolm, S. (2000). Identification of susceptibility loci for nonsyndromic cleft lip with or without cleft palate in a two stage genome scan of affected sib-pairs. *Hum. Genet.* 106, 345-350.

Scapoli, L., Palmieri, A., Martinelli, M., Pezzetti, F., Carinci, P., Tognon, M., and Carinci, F. (2005). 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.* 76, 180-183.

Schultz, R.E., Cooper, M.E., Daack-Hirsch, S., Shi, M., Nepomucena, B., Graf, K.A., O'Brien, E.K., O'Brien, S.E., Marazita, M.L., and Murray, J.C. (2004). Targeted scan of fifteen regions for nonsyndromic cleft lip and palate in Filipino families. *Am. J. Med. Genet. A.* 125, 17-22.

Stanier, P. and Moore, G.E. (2004). Genetics of cleft lip and palate: syndromic genes contribute to the incidence of non-syndromic clefts. *Hum. Mol. Genet.* 13, R73-R81.

Tolarova, M.M. and Cervenka, J. (1998). Classification and

birth prevalence of orofacial clefts. *Am. J. Med. Genet.* 75, 126-137.

Vanderas, A.P. (1987). Incidence of cleft lip, cleft palate, and cleft lip and palate among races: A review. *Cleft Palate J.* 24, 216-225.

Wyszynski, D.F., Duffy, D.L., and Beaty, T.H. (1997). Maternal cigarette smoking and oral clefts: A meta-analysis. *Cleft Palate Craniofac. J.* 34, 206-210.

Zucchero, T.M., Cooper, M.E., Maher, B.S., Daack-Hirsch, S., Nepomuceno, B., Ribeiro, L., Caprau, D., Christensen, K., Suzuki, Y., Machida, J., Natsume, N., Yoshiura, K., Vieira, A.R., Orioli, I.M., Castilla, E.E., Moreno, L., Arcos-Burgos, M., Lidral, A.C., Field, L.L., Liu, Y.E., Ray, A., Goldstein, T.H., Schultz, R.E., Shi, M., Johnson, M.K., Kondo, S., Schutte, B.C., Marazita, M.L., and Murray J.C. (2004). Interferon regulatory factor 6 (IRF6) gene variants and the risk of isolated cleft lip or palate. *N. Engl. J. Med.* 351, 769-780.