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WNT10A rs147680216 G>A mutation indicates a higher risk for non-syndromic oral cleft in a northeastern Chinese population

C. Feng [a], Q. Wang [a], Z. Cao [a], H. Guan [a], Z.-F. Xu [b,*]

[a] *2nd Department of Orthodontics, Hospital of Stamotology, China Medical University, Liaoning, China*

[b] *Department of Oromaxillofacial – Head and Neck Surgery, Hospital of Stamotology, China Medical University, Liaoning, China*

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Abstract

Non-syndromic oral cleft lip and palate is a heterogeneous group of congenital malformations that consist of cleft palate, and cleft lip with or without cleft palate. The members of the wingless type mouse mammary tumour virus (MMTV) integration site family (Wnts) regulate various developmental processes including craniofacial development, and have a role in that of cleft lip and palate. We aimed to identify the potential polymorphisms in the Wnt10a gene, and to explore the association between the variations in the gene and the risk of development of cleft palate. A total of 198 affected patients (cleft lip, n = 67; cleft palate, n = 48; and both, n = 83) together with 187 healthy controls were enrolled (all from the Chinese Han population in NE China). A fragment of 316 bp was amplified from the blood genome of each participant by polymerase chain reaction (PCR) using specific primers that targeted the Homo sapiens Wnt10a gene. By using the restriction enzyme AluI, the population analysed were classified into three genotypes, GG (316 bp), GA (316 bp, 117bp, 199bp) and AA (117bp, 199bp) based on the rs147680216 G/A polymorphism (Gly>Ser mutation at position 213 of Wnt10a protein) of theWnt10a gene. The frequency of allele A in the affected group was significantly higher (14.1% compared with 3.2% in the control group). The allele G with an odds ratio (OR) of 0.201 and 95% CI of 0.445 to 0.091 was not a risk factor for the condition in the affected group. However, the distribution of the genotype did affect its occurrence in the affected group ($p < 0.001$), but not the classification of types ($p = 0.901$). In conclusion we found an rs147680216 G>A mutation that was associated with non-syndromic cleft lip and palate in the Wnt10a gene.

Keywords: Non-syndromic oral cleft; Wnt10a; polymorphism; polymerase chain reaction; restriction fragment length polymorphism

Introduction

Oral disease is a major global health burden, which has a huge impact on people's socioeconomic lives. Non-syndromic oral clefts are generally classified into three groups - cleft palate, cleft lip without cleft palate, and cleft lip with cleft palate – which are heterogeneous groups of congenital malformations. Reportedly one of every 700 live births has such a defect.[1] The residual malformation and morbidity still have an appreciable effect on some infants, though the quality of care has improved.[2] Although the exact cause of oral clefts is still not clear, environmental and genetic risk factors (including maternal metabolic disorders, smoking, and cleft-related genetic loci) are the aetiological factors.[2]

The development of normal craniofacial structures is a process that is exquisitely orchestrated by genes that are involved in human clefting.[3] There is an association between genetic mutations in several genes, such as interferon regulatory factor 6,[4] methionine synthase,[5] and bone morphogenetic protein 4,[6] and the occurrence of oral clefts. Wingless type mouse mammary tumour virus (MMTV) inte-

* Corresponding author at: No. 117. Nanjing North Street, Heping District, Shenyang, Liaoning 110002, China. Tel.: +86 13478286595; Fax: +86 24 86602310.

E-mail address: xzf7090@hotmail.com (Z.-F. Xu).

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gration site family members (Wnts) are lipoglycoproteins that activate the cell surface receptors Frizzleds to mediate and influence multiple developmental processes.[7] The Wnt genes have a regulatory role during development of the midface and fusion of the upper lip, and have therefore been considered as aetiological candidates for non-syndromic cleft lip and palate.[8] Chiquet et al investigated potential single nucleotide and polymorphism sites in seven Wnt genes, and showed that those sites in Wnt3a, Wnt5a, and Wnt11 are associated with the aetiology.[8] Menezes et al also interrogated Wnt genes, and found that subjects who carry variant alleles in the Wnt3a gene have an increased risk of developing an oral cleft.[9] These studies suggested that the variations in the Wnt gene may upset craniofacial development.

As documented in the National Center for Biotechnology Information (NCBI) database, the Wnt10a gene is mapped in chromosome 2q35, which encodes 417 amino acids. An earlier study showed that a singular nucleotide polymorphism (rs147680216 G/A) in the Wnt10a gene was a risk factor for non-syndromic hypodontia.[10] This study also suggested that rs147680216 in the Wnt10a gene is associated with craniofacial abnormality in humans. Non-syndromic cleft lip and palate is also a craniofacial abnormality, and this locus has not been reported in it yet. Our aim, therefore, was to expand our knowledge of genetic variations of Wnt10a gene in that condition.

In an effort to identify additional genetic candidate loci, we used polymerase chain reaction-restriction (PCR)\fragment length polymorphism (RFLP) to seek potential polymorphisms of the Wnt10a gene at rs147680216 loci.

Material and methods

Subjects

A total of 198 patients with non-syndromic cleft lip or palate (or both) who were admitted to the Department of Oral and Maxillofacial Surgery of the Affiliated Stomatological Hospital of China Medical University (Shenyang, Liaoning, China) from 2008 to 2011 were included. Healthy subjects (n = 187) with no congenital malformations of the body and no family history of genetic disease were selected as controls. The control subjects were recruited from the same region and had a similar sex distribution as the patients. All subjects were members of the Chinese Han population. Signed consent forms were obtained from all participants or their guardians.

Detection of polymorphisms within the Wnt10a gene and genotyping

We used PCR-RFLP to detect the polymorphism within the Wnt10a gene at rs147680216 loci. Briefly, the blood genome was isolated from peripheral blood using a DNA isolation kit (Tiangen Biotech, Beijing, China). A pair of primers (Forward: 5' GCACCGCTTACAACTGGAT 3'; Reversed: 5' CCTCAGAAGAGAGGTAGGCC 3') was designed based on the reference sequence for the human Wnt10a gene (NCBI Reference Sequence: NG_012179.1) published in the NCBI database on the Primer Premier 5.0 software (Premier Biosoft International).

Table 1
Clinical characteristics of patients with cleft lip and palate.

Type of cleft	Male	Female	Total
Cleft lip:			
Unilateral	40	19	59
Bilateral	6	2	8
Cleft palate:			
Incomplete	20	28	48
Complete	0	0	0
Cleft lip and palate:			
Unilateral	42	15	57
Bilateral	18	8	26
Total	126	72	198

The PCR assay was done in a 20-μL system containing DNA templates1 μL, 2 × Power Taq PCR MasterMix 10 μL (BioTeke), 1 μL of each primer, and double-distilled water 7 μL. The steps of the amplification reaction were: an initial denaturation at 95 °C for five minutes; denaturation at 95 °C for 30 seconds, annealing at 52 °C for 30 seconds and extension at 72 °C for 45 seconds (30 cycles); and a final extension stage at 25 °C for two minutes. Then PCR products 10 μL were digested with AuI 1 (NEB) 1 μL for one hour at 37 °C. To distinguish the different genotype in all the participants, the digested mix was further separated by electrophoresis in 1.5% agarose gel containing GoldView I solution (Solarbio).

Statistical methods

The genotypes and alleles were counted. Statistical analysis was done with the help of IBM SPSS Statistics for Windows (version 20.0, IBM Corp). The Hardy-Weinberg equilibriums of genotypes were analysed in cases and controls. Probabilities of less than 0.05 were accepted as significant. An odds ratio (OR) of > 1 designated an increased risk of disease, and OR < 1 was the opposite.

Results

Analysis of polymorphisms within the Wnt10a gene in the analysed groups

A total of 198 patients were enrolled in the study. The male:female ratio was 1.8:1 (Table 1), and the sex ratio in the control group (n = 187) was similar (data not shown). PCR was done using primers as described, and a 316-bp product was amplified (Fig. 1). Thirty DNA-amplified products for the Wnt10a gene were randomly selected for sequencing. The results showed an rs147680216 G/A single nucleotide polymorphism in the Wnt10a gene (Fig. 2). After we had

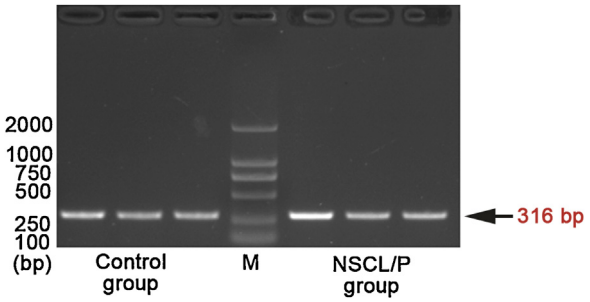

Fig. 1. Electrophoresis patterns for the products of polymerase chain reaction (PCR).
A fragment of 316 bp was amplified from the blood genome of each participant by PCR using specific primers targeting Homo Sapiens Wnt10a gene. M indicated the DNA marker (DL2000, BioTeke).

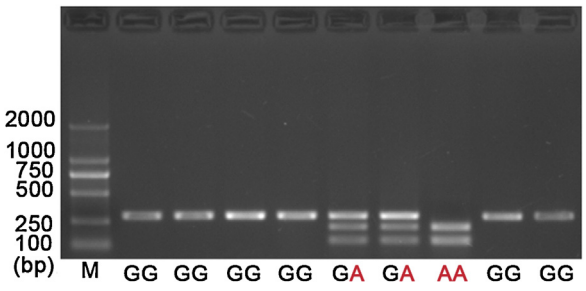

Fig. 3. Electrophoresis patterns of reaction-restriction\fragment length polymorphism analysis for Wnt10a rs147680216 G/A single nucleotide polymorphisms.
M indicates the DNA marker (DL2000). Genotype AA = 117bp and 199bp fragments, genotype GA = 316 bp, 117bp and 199bp fragments, and genotype GG = 316 bp fragments.

analysed the sequence information using the BioXM.2.6 software, we found one restriction enzyme cutting site for AluI (ag/ct) within the PCR products when the rs147680216 alleles of the Wnt10a gene were GA or AA. The PCR-RFLP was therefore done for the rest of the samples. As indicated in Fig. 3, the amplified products from the AA genotype were

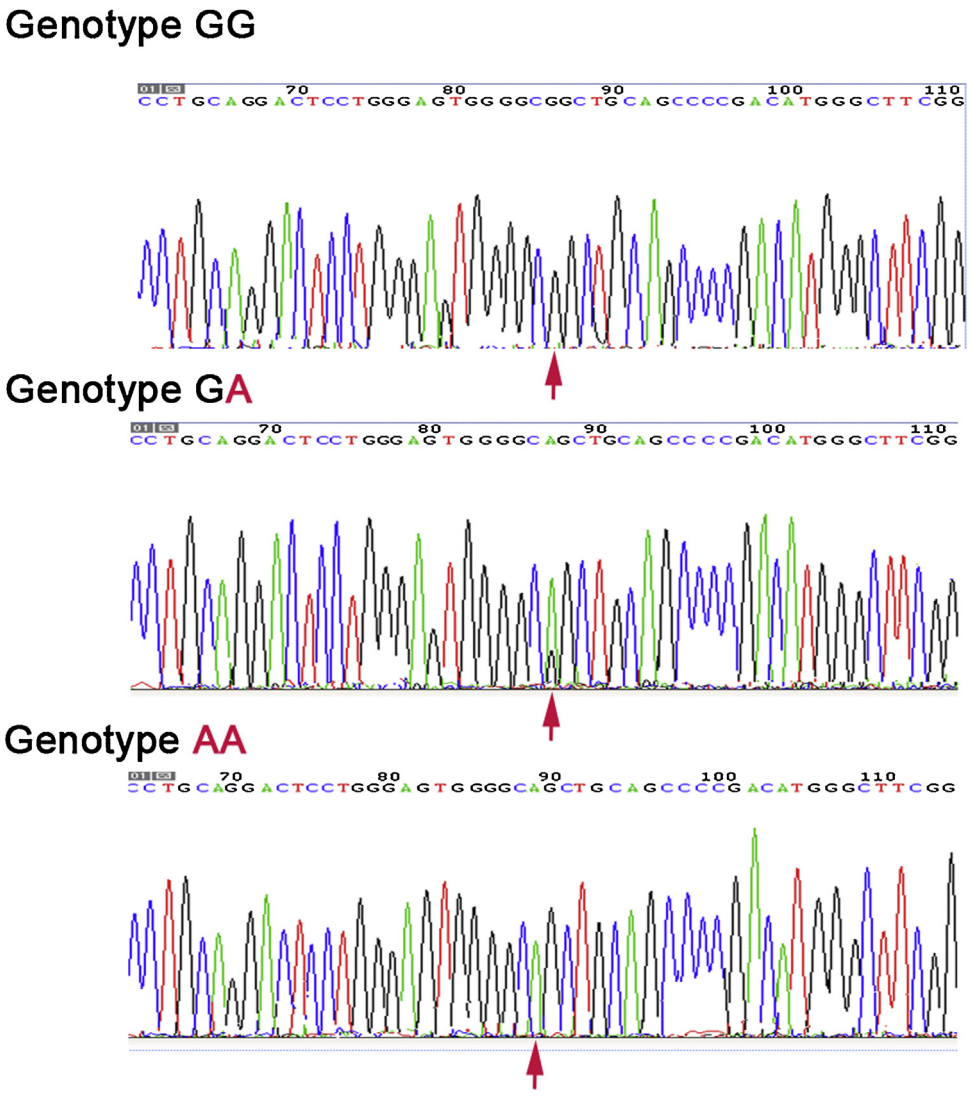

Fig. 2. Sequencing results of the rs147680216 G/A single nucleotide polymorphisms within the Wnt10a gene. Red arrows = rs147680216 G/A.

Table 3
Genotype frequencies for rs147680216 G/A SNP of *Wnt10a* gene in subgroups of patients with non-syndromic oral clefts and healthy controls.

Groups	Genotypes			Chi squared	p value
	GG	GA	AA		
Control	175	12	0	25.46	0.000003
NSCL/P	149	42	7		
CL	50	15	2	1.06	> 0.05
CP	38	9	1		
CLP	61	18	4		

NSCL/P = Non-syndromic oral clefts; CP = cleft palate; CL = cleft lip without cleft palate; CLP = cleft lip with cleft palate.

digested into two fragments (117bp, 199bp), while those from the GG genotype could not be digested (316 bp). Three fragments (316 bp, 117bp, and199bp) therefore indicated the GA genotype (Fig. 3).

Genotypic distribution of Wnt10a rs147680216 SNP in the analysed group

The PCR-RFLP assay was applied to identify the genotypic distribution in patients and controls based on the rs147680216 SNP. As shown in Table 2, 175 of the 187 controls were GG, and the rest were GA, genotype. In the cleft group, 149 subjects were type GG, 42 were type GA, and seven were type AA. Genotype GG was predominant within the group analysed, with a higher frequency of allele G. Nonetheless, the frequency of allele A was significantly higher in the group of patients (14.1% compared with 3.2% in the control group). The data showed that the allele G with an OR of 0.201 and a 95% CI of 0.445 to 0.091 was not a risk factor for non-syndromic cleft lip and palate in the group analysed. The distribution of genotypes in the site within the control group ($p = 0.650$) and the three subgroups of patients ($p = 0.075$) agreed with the Hardy-Weinberg equilibrium. The chi squared test confirmed that the genotypes GA and AA affected the occurrence of cleft in the group analysed ($p < 0.001$), but did not affect the classification of types of cleft ($p = 0.901$) (Table 3).

Discussion

It has been reported that the risk of orofacial cleft is increased with specific detoxification-gene variants.[11] In previous work we have shown that a 392 C>T variant in the Wnt10a gene is associated with the development of non-syndromic cleft lip and palate.[12] In the present study, a new single nucleotide polymorphism (G/A) in the rs147680216 site of the Wnt10a gene was identified, which lead to a mis-sense mutation Gly>Ser at amino acid position 213 of Wnt10a. After we had analysed the sequence information of the amplified Wnt10a fragments, we found that rs147680216 allele A provided a recognition site for restriction enzyme AluI. Based on the PCR-RFLP results, therefore, the studied population was classified into three genotypes, GG, GA, and AA. To the best of our knowledge, our study is the first to show this single nucleotide polymorphism in patients with non-syndromic clefts.

Non-syndromic cleft lip and palate is a heterogeneous group of conditions that affect the lips and oral cavity, the causes of which are poorly understood. Affected children require multidisciplinary care from birth until adulthood, and they have a high risk of poor psychological health.[13] A series of exquisitely orchestrated events, including migration, growth, and differentiation of cells are involved in the development of the lip and palate.[1] It is well known that the neural crest cells migrate into the developing craniofacial area, where they participate in the formation of the frontonasal prominence and the paired maxilla and mandible.[1] Of note is that the neural crest probably signals to the extreme anterior domain (a region where the ectoderm and endoderm are directly juxtaposed) through the Wnt/planar cell polarity pathway.[14,15] Additionally, Liu and Millar have correlated the dynamic activation of the Wnt/β-catenin signalling pathway with the development of oral clefts.[16] These studies emphasised the vital role of Wnt genes in the development of the lips and oral cavity. The rs147680216 single nucleotide polymorphism has led to a mis-sense mutation from Gly to Ser in the Wnt10a protein. A change from the non-polar amino acid (Gly) into the polar amino acid (Ser) may result in change in the structure of the protein, and eventually affect its function. Such a change may also affect the canonical and non-canonical signalling transduction that is mediated by Wnt10a.

Gene variants in members of the Wnt family have been reported to be risk factors for many human diseases, such as malignant tumours,[17,18] prevalent skeletal disorders,[19,20] cardiovascular diseases,[21] and diabetes,[22] among others. Human Wnt10a is a protein (417 aa) encoded by the Wnt10a gene. It has been reported that mutations within this gene are linked to ectodermal development or developmental diseases. For example, Kimura et al found that the common polymorphisms (such as rs7349332 and rs10177996) in the Wnt10a gene affect the morphology of teeth.[23] Arzoo et al showed that Wnt10a mutations account for 25% of population-based isolated oligodontia, and have phenotypic correlations.[24] The Wnt10a gene is also clustered with the Wnt6 gene in the chromosome 2q35 region, and this cluster has been suggested to be associated with the incidence of oral clefts.[25] Based on the rs147680216 SNP site of the Wnt10a gene, we identified three genotypes (GG, GA, and AA) in patients with non-syndromic cleft lip and palate, and only two genotypes (GG and GA) in healthy controls. Interestingly, although the allele G was the predominant allele for the Wnt10a gene at this site, it was relatively less common in patients than in healthy controls (OR 0.201, 95% CI 0.445 to 0.091). These results suggest that allele A, not allele G, was the risk factor for this condition. A previous study has reported this single nucleotide polymorphism in

Table 2
Genotype frequencies for rs147680216 G/A SNP of Wnt10a gene in patients with non-syndromic oral clefts and healthy control.

Groups	Genotype			Total	Allele		Chi squared (HWE)	p value (HWE)	OR (95% CI)
	GG	GA	AA		G	A			
CL	50	15	2	67	115	19	0.430	0.512	0.201 (0.445 to 0.091)
CP	38	9	1	48	85	11	0.277	0.599	0.256 (0.503 to 0.130)
CLP	61	18	4	83	140	26	2.663	0.103	0.178 (0.419 to 0.076)
NSCL/P	149	42	7	198	340 (85.9%)	56 (14.1%)	3.167	0.075	0.201 (0.445 to 0.091)
Control	175	12	0	187	362 (96.8%)	12 (3.2%)	0.205	0.650	–

CP = cleft palate; CL = cleft lip without cleft palate; CLP = cleft lip with cleft palate; NSCL/P = non-syndromic oral clefts; HWE = Hardy-Weinberg equilibrium; OR = odds ratio.

non-syndromic hypodontia, which also illustrates allele A at this site as a risk factor.[10] In addition, patients who carry allele A in Wnt10a gene (genotypes GA and AA) were more susceptible to non-syndromic cleft lip and palate in the subjects analysed.

As well as rs147680216, we also detected other potential sites, rs121908120 and rs121908118, in the group analysed, but found no mutation (data not shown). One limitation of our present study was the relatively small sample size, and the fact that only two genotypes were identified in the healthy control group may be the result of this. The Wnt10a gene has three exons encoding a 417 amino acid product. Our present work investigated only one single nucleotide polymorphism in its third exon that may not be enough to show its role in oral clefts. Further analysis to investigate Wnt10a gene polymorphisms in a larger group or in other areas of China is needed.

In summary, we identified a novel single mucleotide polymorphism (G/A) in rs147680216 loci of the Wnt10a gene in a northeastern Chinese population with and without non-syndromic cleft lip and palate. The G>A mutation of Wnt10a leads to a mis-sense mutation of Gly>Ser in its protein, and allele A may be a risk factor for this condition.

Conflict of interest

We have no conflicts of interest.

Ethics statement/confirmation of patients' permission

The study protocol was approved by the Ethics committees of the Hospital of Stomatology. All the subjects signed informed consent.

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