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Association of *TGFβ3* variants with Nonsyndromic Cleft Lip and Palate in Guatemalan Population

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Abstract

Introduction: Nonsyndromic cleft lip with or without cleft palate (*NCL/P*) is a common orofacial anomaly with a multifactorial etiology. The genetic component of *NCL/P* etiology is complex with multiple genes involved. The transforming growth factor beta 3 (*TGFβ3*) is among the strong susceptibility genes that have been shown to be involved in morphogenesis of lip and palate.

Materials and Methods: Case-control study design was used in this study. Cases (n=237) were patients affected with *NCL/P* identified at the Roosevelt Hospital in Guatemala City, Guatemala. Controls (n=168) were individuals with no history of *NCL/P* from the same hospital. Genotypes were established by PCR and PAGE for *TGFβ3* rs2300607 A/T SNP and by PCR and sequencing for *TGFβ3* rs2268625 C/T SNP.

Results: For rs2300607A/T, a proportion of TT homozygotes was significantly higher (p=0.029) in cases than in controls (13.92% vs 5.36%). T allele frequency was 0.340 in cases and 0.274 in controls. Also for rs2268625 C/T, a significantly higher proportion of TT homozygotes was found among cases than among controls (p=0.041; 54.0% vs. 48.98%). Frequency of mutated T allele was 0.744 in cases and 0.694 in controls.

Conclusion: The results of our study showed for the first time the association between *TGFβ3* rs2268625 C/T polymorphism and *NCL/P* and confirmed for Guatemalan population the association between *TGFβ3* rs2300607 A/T polymorphism and *NCL/P* found by Ichikawa in Japanese by Ichikawa et al. in 2006. Thus, our findings suggest that rs2268625 C/T and rs2300607 A/T mutations in the *TGFβ3* gene are associated with *NCL/P* in Guatemalan population.

Keywords Nonsyndromic cleft lip and palate *TGFβ3*; *TGFβ3* rs2300607 A/T; *TGFβ3* rs2268625; C/T Guatemala; Case-control study

Introduction

Orofacial clefts (OFC) are among the most common craniofacial malformations in humans. The prevalence rates vary between 0.5 and 3 per 1000 total births, with considerable variations between populations, genders and geographic regions [1].

In general, Asian and Amerindian populations have the highest frequencies of OFC, often at 1/500, and African-derived populations have the lowest frequencies at 1/2500 [2]. Tolarova and Cervenka [3] analyzed the occurrence of OFC in an ethnically diverse population in California. The non-Hispanic whites had the highest birth prevalence of isolated clefts, followed by Asians and blacks who had the lowest values.

In the USA, twenty infants are born with an orofacial cleft on an average day, or 7500 every year. Children who have an OFC require several surgical procedures and complex medical treatments and, together with their families, often suffer serious psychological problems. The estimated lifetime medical cost for each child with an OFC is \$100,000, amounting to \$750 million for all children with OFC born each year in the USA [4].

Recently, over 30 potential candidate loci and candidate genes throughout the human genome were identified as strong susceptibility genes for OFC. The *MSX1* (4p16.1), *TGFα* (2p13), *TGFβ1* (19q13.1), *TGFβ2* (1q41), *TGFβ3* (14q24), *RARA* (17q12), *MTHFR* (1p36.3) genes are among the strongest candidates [5-7].

The *TGFβ3* was identified as a strong candidate for clefting in humans based on a mouse model. Generally, palatogenesis in mice parallels that of humans and shows that comparable genes are involved [8]. Kaartinen et al. [9] demonstrated that mice lacking the *TGFβ3* peptide exhibit cleft palate. In addition, it was shown that the exogenous *TGFβ3* peptide can induce palatal fusion in chicken embryos, although the cleft palate is a normal feature in chickens [10].

In humans, association studies between the *TGFβ3* gene and *NCL/P* have shown conflicting results. Lidral et al. [11] reported failure to observe an association of a new allelic variant of *TGFβ3* with *NCL/P* in a case-control study of the Philippines' population. Another study by Tanabe et al. [12] analysed DNA

samples from 43 Japanese patients and compared results with those from 73 control subjects with respect to 4 candidate genes including *TGF β 3*. No significant differences in variants of *TGF β 3* between case and control populations were observed.

On the other hand, more recent case-control association studies, family based studies, and genome scans have supported a role of *TGF β 3* in cleft development. Beaty et al. [13] examined markers in five candidate genes on 269 case-parent trios ascertained through a child with non-syndromic OFC. Eighty five percent of the probands in the study were Caucasian. Markers at two of the five candidate genes (*TGF β 3* and *MSX1*) showed consistent evidence of linkage and disequilibrium due to linkage. Similarly, Vieira et al. [14] attempted to detect transmission distortion of *MSX1* and *TGF β 3* in 217 South American children from their respective mothers. A joint analysis of *MSX1* and *TGF β 3* suggested that there might be an interaction between these two genes increasing cleft susceptibility. These results suggest that *MSX1* and *TGF β 3* mutations make a contribution to clefts in South American populations.

In a study of the Korean population, Kim et al. [15] reported that the G allele at the SfaN1 polymorphism of *TGF β 3* is associated with an increased risk of *NCL/P*. The population study consisted of 28 *NCL/P* patients and 41 healthy controls.

In 2004, Marazita et al. [5] performed a meta-analysis of 13 genome scans of 388 extended multiplex families with *NCL/P*. The families came from seven diverse populations including 2,551 genotyped individuals. The meta-analysis revealed multiple genes in 6 chromosomal regions including the region containing *TGF β 3* (14q24).

In the Japanese population, blood samples from twenty families with *NCL/P* patients have been analyzed using *TGF β 3* CA repeat polymorphic marker. Based on the results of the study, the investigators concluded that either the *TGF β 3* gene itself or an adjacent DNA sequence may contribute to the development of cleft lip and palate [16].

Recently, another study by Ichikawa and colleagues [17], investigated the relationship between *NCL/P* and seven candidate genes (*TGF β 3*, *DLX3*, *PAX9*, *CLPTM1*, *TBX10*, *PVRL1*, *TBX22*), in a Japanese population. The sample consisted of 112 *NCL/P* cases with their parents and 192 controls. Both population based case-control analysis and family based transmission disequilibrium test (TDT) were used. The results showed significant associations of SNPs in *TGF β 3* and *NCL/P*, especially IVS+5321(rs2300607) with a p value of 0.0016. Although IVS-1572 (rs2268625) alone did not show a significant difference between cases and controls, the haplotype "A/A" for rs2300607-rs2268625 showed significant association. The author concluded that the results demonstrated positive association of *TGF β 3* with *NCL/P* in the Japanese.

To explore a possible role of *TGF β 3* polymorphism in the etiology of *NCL/P* in Guatemala, we investigated case and control samples from Guatemala City.

Materials and Methods

TGF β 3 gene characteristics

The *TGF β 3* gene is mapped on chromosome 14, at 14q24 (Figure 1). It consists of 7 exons and 6 introns. It covers 23.91 Kb [18]. The rs2268625 C/T SNP of *TGF β 3* is located in intron 1 of the gene.

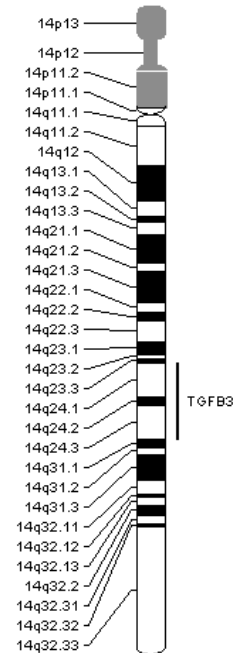


Figure 1 Location of the *TGF β 3* gene on the chromosome 14.

Beta-type transforming growth factors form a group of polypeptides that control proliferation and differentiation of multiple cell types. *TGF β 3* plays a significant role in normal fusion of palatal shelves during embryonic development (OMIM database). It is especially expressed in epithelial cells lining a medial edge of palatal shelves. It is essential for adhesion of the medial edge epithelium and elimination of the midline seam, hence providing for normal fusion of the palatal shelves. It functions similarly during fusion of facial processes forming lips. It controls angiogenesis and proliferation of mesenchymal cells [19].

Sample characteristics

Cases and controls were identified in the Roosevelt Hospital in Guatemala City, Guatemala, in 2001, 2002, 2003, and 2005. The group of cases with *NCL/P* consisted of 237 subjects. They were selected from a group of individuals who came to the hospital seeking treatment for various craniofacial malformations. For this study, only patients with non-syndromic cleft lip or cleft lip and palate (*NCL/P*) were included in the case group.

The control sample consisted of 168 individuals with no history of birth defects who had their blood drawn at the same hospital for various follow-ups during the time of collection of cases and their specimens.

Physical and dental exams were performed for cases and controls. Personal interviews were conducted with the patients, if their age permitted, or most often with their mothers or guardians. Information on family and medical history, demographics, and digital photographs of individuals were taken. Written informed consent was obtained from individuals in both case and control groups.

Location characteristics

The Republic of Guatemala has a unique location within Central America. It is considered the geographic center of the continent. It is bordered by Mexico on the North and West, by El Salvador and Honduras on the Southeast, by Belize and the Caribbean Sea on the Northeast, and by the Pacific Ocean on the South. Guatemala occupies a total area of 108,890 sq km which is slightly smaller than Tennessee. It has a population of 12.7 million with more than half of Guatemalans being descendants of indigenous Mayan people. Spanish as well as 23 Amerindian languages are officially recognized. About 56% of the population lives below the poverty line. Coffee is the mainstay of the economy, but tourism has become the second most important source of foreign revenue in recent years. However, agriculture is still the most important part of economy. The country produces and exports sugar, bananas, fruits, vegetables, flowers, cardamom and macadamia nuts. Additionally, Guatemala has developed excellent industries devoted to the assembly of clothes and electronic products, as well as the manufacture of furniture and canned goods. Also its petroleum industry is rapidly growing [20].

Collection of specimens

Intravenous blood, mainly from the cubital veins, was collected from all individuals in vacutainers with EDTA solution and was refrigerated until ready for use. Within 10 days of drawing the samples, blood was spotted on 6 cm by 10 cm sheets of filter paper using disposable transfer pipettes. A new pipette was used for each sample. Six-to-twelve blood spots were made per sheet of filter paper and two sheets were used for each sample. Blood spots were allowed to dry for 24-72 hours before being stored in individual envelopes.

DNA analysis

DNA isolation: DNA was extracted from dry blood spots on filter paper using the technique described by Polski et al. [21] for each specimen; an individual blood spot was cut from the filter paper using sterile scissors. Each spot was cut into squares approximately 1 mm × 1 mm and dropped into a sterile 1.5 mL plastic tube. One mL of sterile DNase- and RNase- free water was added to each tube and the mixtures were incubated at room temperature for 30 minutes. Then, each tube was spun at 10,000 rpm for 3 minutes and the supernatant was removed and discarded. Two-hundred µL of

Chelex-100 was added and the tubes were then incubated at 56°C for 30 minutes. The tubes were then vortexed at high speed and incubated at 95°C for 8 minutes. The tubes were again vortexed, spun at 10,000 rpm for 3 minutes, and then stored at 4°C until ready for DNA amplification by Polymerase Chain Reaction (PCR).

Polymerase Chain Reaction (PCR): The fragment of DNA containing the rs2300607 A/T SNP region or the rs2268625 C/T SNP of the *TGFβ3* gene was amplified by polymerase chain reaction (PCR). A reaction mixture containing 52.5% sterile H₂O, 20% 5X Taq Master, 10% 10X Taq Buffer, 2% 10 mM dNTP's, 2.5% forward primer specific for either the rs2300607 A/T SNP or the *TGFβ3* rs2268625 C/T SNP, 2.5% reverse primer specific for either the rs2300607 A/T SNP or the *TGFβ3* rs2268625 C/T SNP, and 0.5% Taq DNA polymerase (Qiagen) was prepared. Primer sequences are shown in **Table 1** and **Table 2**.

Table 1 Primer sequences used to amplify the rs2300607 A/T SNP region of the *TGFβ3* gene.

Primer Sequences for the <i>TGFβ3</i> rs2300607 A/T SNP	
Forward primer	5'-CGATGATTCATTCCACAGATT-3'
Reverse primer	5'-CCTGGACAACATAGGGAGGA-3'

Table 2 The primer sequences used to amplify the rs2268625 C/T SNP region of the *TGFβ3* gene.

Primer Sequences for the <i>TGFβ3</i> rs2268625 C/T SNP	
Forward primer	5'-TGAATGGTTTAGGGAATCGT-3'
Reverse primer	5'-AAGCTCATTCCAGCCATGTC-3'

Forty-five µL of this preparation was mixed with 5 µL of DNA isolated by the aforementioned technique in 200 µL vials. This solution underwent a specific program of temperature cycling, using a programmed Mastercycler (Eppendorf). Each cycle started at 95°C for 15 seconds, switched to 61°C for 30 seconds, and finished at 72°C for 15 seconds. This cyclic treatment was repeated 40 times.

Following PCR, a presence of the amplified DNA was confirmed using agarose gel electrophoresis. To test for contamination, a negative control sample containing 5 µL of sterile H₂O in place of 5 µL of isolated DNA was mixed with the PCR mixture and run on the Mastercycler along with each batch. Following PCR, each negative was run on an agarose gel along with its corresponding batch of samples. If the agarose gel indicated the presence of DNA in the negative control, then PCR was repeated for that entire batch of samples until a true negative was achieved.

Polyacrylamide Gel Electrophoresis (PAGE): Specific patterns for AA, TT, and AT genotypes associated with rs2300607 A/T *TGFβ3* gene polymorphism (**Figure 2**) were identified in our laboratory through Single-Stranded Conformation Polymorphism (SSCP) analysis by using a polyacrylamide gel electrophoresis [22]. Polyacrylamide gel solution was prepared by mixing 10 mL MDE (Mutation

Detection Enhancement) gel monomer solution (Lonza, Inc., Rockland, ME), 2.4 mL 10X TBE buffer, 27.4 mL distilled water, 0.25 mL 10% ammonium persulfate, and 25 μ L TEMED. A sample loading buffer was prepared by mixing 2.1 mL formamide, 25 μ L M NaOH, 100 μ L 0.5 M EDTA, 25 μ L 0.02% bromophenol blue/0.02% xylene cyanol dye mixture, and 250 μ L distilled water. Three μ L of the loading buffer were mixed with 3 μ L of each sample of amplified DNA in a 0.2 mL plastic tube. The sample/loading buffer mixture was heated to 95°C for 2-3 minutes. Four μ L were then loaded into a well in the polyacrylamide gel using a fine tip pipette. A new sterile pipette tip was used for each individual sample. A negative control was run with each batch of samples to reconfirm the absence of foreign DNA contaminants in the amplified DNA. Electrophoresis was carried out at a constant power of 30 W for approximately 4 hours until the bromophenol blue dye band reached the 32 cm distance from the start wells. The gel was developed by soaking it first in 0.1% silver nitrate solution for 10 minutes, followed by soaking in a mixture of 1.5% NaOH, 0.01% sodium borohydride, and 0.15% formaldehyde for 20-30 minutes. Finally, the gel was soaked in 0.75% sodium carbonate for 3 minutes and washed in distilled water. The *TGF β 3* rs2300607 A/T genotypes were determined based on a specific configuration of the stained DNA bands.

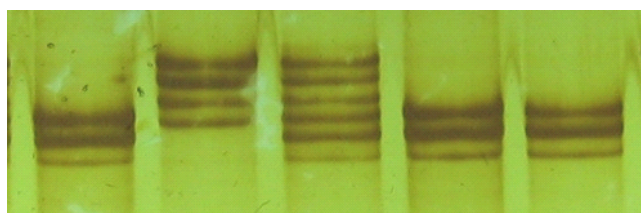


Figure 2 *TGF β 3* rs2300607 A/T genotypes on PAGE. A=Adenine, T=Thymine, AA=wild type homozygote, AT=heterozygote with one mutated (T) allele and wild (A) allele, TT=homozygote with both alleles mutated. Genotypes from left to right: AA, TT, AT, AA, AA.

To confirm the location of bands corresponding to the *TGF β 3* rs2300607 A/T polymorphism on the polyacrylamide gel, a complete sequencing of the amplified gene segment was performed for several samples. Once the genotypes were established, the samples were run on a polyacrylamide gel to display locations and patterns specific for AA homozygotes, AT heterozygotes, and TT homozygotes. These positive control samples were always run on the polyacrylamide gels along with the unknown samples to be available for comparison when genotyping was performed.

DNA purification and sequencing: We have found no specific pattern for rs2268625 C/T polymorphism using polyacrylamide gel electrophoresis (PAGE). Therefore, sequencing, preceded by purification of amplified DNA products, was conducted to determine genotypes for this single nucleotide polymorphism. QIAquick purification kit (Qiagen) was used to purify the amplified DNA products from primers, nucleotides, Taq polymerase and salts following the manufacturer's purification protocol. The purified DNA was

eluted by 40 μ L of PCR water and stored at -20°C until ready for sequencing.

The rs2268625 C/T of *TGF β 3* gene is located on the minus (complementary) strand. The sequenogram, on the other hand, is shown as a plus strand (**Figures 3A-3C**). Therefore, the genotypes were determined as the complementary bases of those bases presented by the sequenogram (G complements with C, A complements with T, C complements with G, and T complements with A). For example, GA on the sequenogram was indicative of CT genotype of the rs2268625 C/T polymorphism.

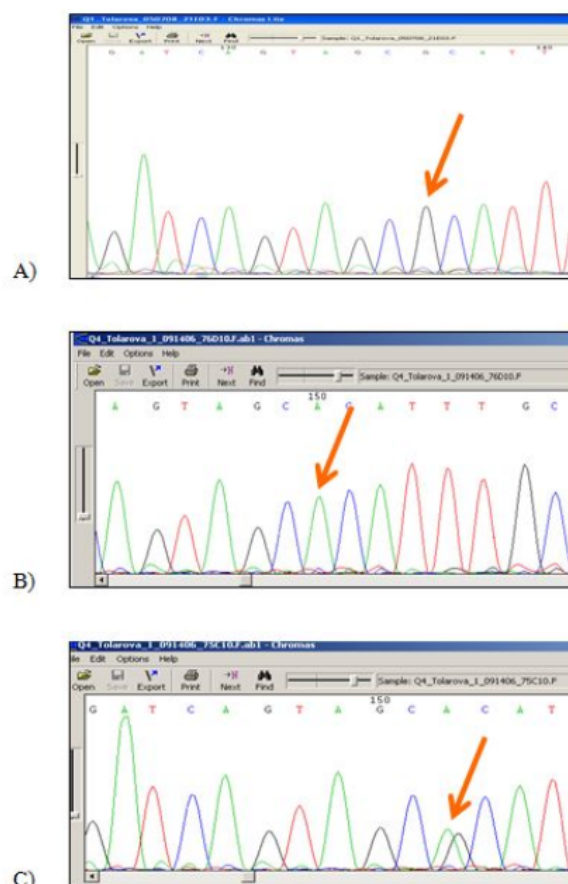


Figure 3A, B, C. Sequenograms of CC, TT, and CT genotypes. CC = homozygote with wild type alleles (shown as GG on the sequenogram). TT = homozygote with mutated alleles (shown as AA on the sequenogram). CT = heterozygote with one mutated (T) and one wild (C) allele (shown as GA on the sequenogram). A= Adenine, C = Cytosine, G = Guanine, T = Thymine.

Results

The *TGF β 3* rs2300607 A/T SNP (IVS+5321)

Altogether, the total of 237 cases (individuals affected with *NCL/P*) and 168 controls were analyzed for the *TGF β 3* rs2300607 A/T polymorphism.

Distribution of *TGFβ3* rs2300607 A/T genotypes in cases and controls

In the case sample, 109 (45.99%) individuals were homozygous for the wild type allele (AA), 95 (40.08%) individuals were heterozygous (AT) and 33 (13.93%) were homozygous for the mutated allele (TT).

In the control sample of 168 subjects, 50.59% (n=85) of control individuals were homozygotes for the wild type allele AA, 44.05% (n=74) were heterozygotes AT and only 5.36% (n=9) were homozygotes TT for the mutated allele (**Table 3**) (**Figure 4**).

Table 3 Distribution of *TGFβ3* rs2300607 A/T genotypes in cases and controls.

Genotypes–No (%)				
Sample type	AA	AT	TT	Total
Cases	109 (45.99)	95 (40.08)	33 (13.93)	237 (100.00)
Controls	85 (50.59)	74 (44.05)	9 (5.36)	168 (100.00)
$\chi^2=7.626$; $p=0.0206$				

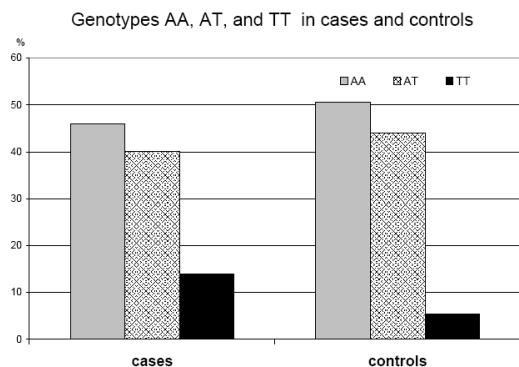


Figure 4 Comparison of proportions of *TGFβ3* rs2300607 A/T genotypes in cases and controls.

The χ^2 test revealed a statistically significant difference between distributions of genotypes in cases and controls ($p=0.0206$; $\chi^2=0.7626$).

Proportions of A and T alleles of the *TGFβ3* rs2300607

The frequency of the wild type A allele was 0.6603 in cases and 0.7262 in controls. The frequency of the T mutated allele was 0.3397 in cases and 0.2738 in the control group. Thus, there was a higher frequency of the mutated allele T in the case group as compared to the controls (**Table 4**) (**Figure 5**).

The difference was on the margin of statistical significance stated for this study ($p=0.0554$; $\chi^2=3.6692$).

Table 4 A and T allele frequencies of the *TGFβ3* rs2300607 in cases and controls.

Alleles–No (%)			
Sample type	A	T	Total
Cases	313 (66.03)	161 (33.97)	474 (100.00)
Controls	244 (72.62)	92 (27.38)	336 (100.00)
$\chi^2=3.6692$; $p=0.0554$			

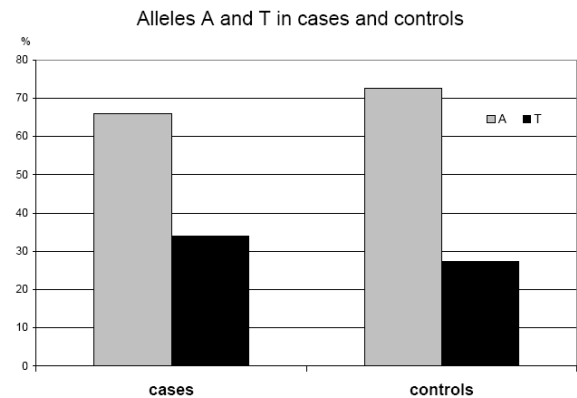


Figure 5 the *TGFβ3* rs2300607 A/T in cases and controls.

The *TGFβ3* rs2268625 C/T SNP (IVS-1572)

For the *TGFβ3* rs2268625 C/T polymorphism, 237 cases and 98 controls were analyzed.

Distribution of *TGFβ3* rs2268625 C/T genotypes in cases and controls: **Table 5** and **Figure 6** present the distribution of *TGFβ3* rs2268625 C/T genotypes in cases and controls.

Table 5 Distribution of *TGFβ3* rs2268625 C/T genotypes in cases and controls.

Genotypes–No (%)				
Sample	CC	CT	TT	Total
Cases	8 (3.38)	101 (42.62)	128 (54.00)	237 (100.00)
Controls	10 (10.20)	40 (40.80)	48 (49.00)	98 (100.00)
$\chi^2=6.4038$; $p=0.0407$				

In cases, 8 (3.38%) were homozygous for the wild type allele (CC), 101 (42.62%) were heterozygous (CT), and 128 (54.00%) were homozygous for the mutated T allele (TT). In controls, 10 (10.20%) were homozygous for the wild type allele (CC), 40 (40.80%) were heterozygous (CT), and 48 (49.00%) were homozygous for the mutated T allele (TT).

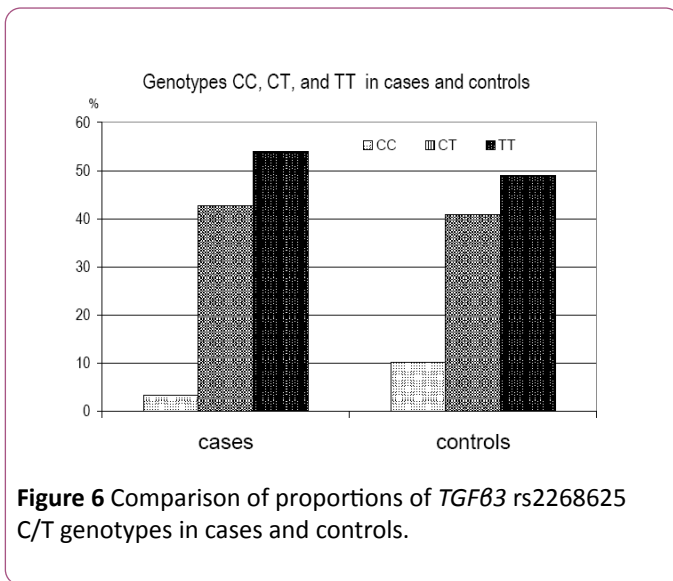


Figure 6 Comparison of proportions of *TGFβ3* rs2268625 C/T genotypes in cases and controls.

The distribution of *TGFβ3* rs2268625 C/T genotypes significantly differs between cases and controls ($p=0.041$). In addition, the proportion of homozygotes for wild type allele (CC) genotype was significantly lower in cases ($p=0.024$) compared to controls, while proportion of mutated TT homozygotes was higher in cases.

Proportions of C and T alleles of the *TGFβ3* rs2268625

A comparison of the allele frequencies is shown in **Table 6** and in **Figure 7**. The frequency of the wild type C allele was 0.2468 in the case group, compared to 0.3061 in the controls. The frequency of the T mutated allele was 0.7532 in cases, compared to 0.6939 in the control group.

A higher frequency of the wild type C allele was observed in controls compared to cases. Although, a higher frequency of mutated allele T was observed in cases compared to controls (which may be indicating a possible role of the *TGFβ3* rs2268625 C/T polymorphism in etiology of *NCL/P*) this difference was not statistically significant ($\chi^2=2.2115$, $p=0.1369$).

Table 6 C and T allele frequencies of the *TGFβ3* rs2300607 C/T in cases and controls.

Alleles—No (%)

Sample type	C	T	Total
Cases	117 (24.68)	357 (75.32)	474 (100.00)
Controls	60 (30.61)	136 (69.39)	196 (100.00)

$\chi^2=2.2115$; $p=0.1369$

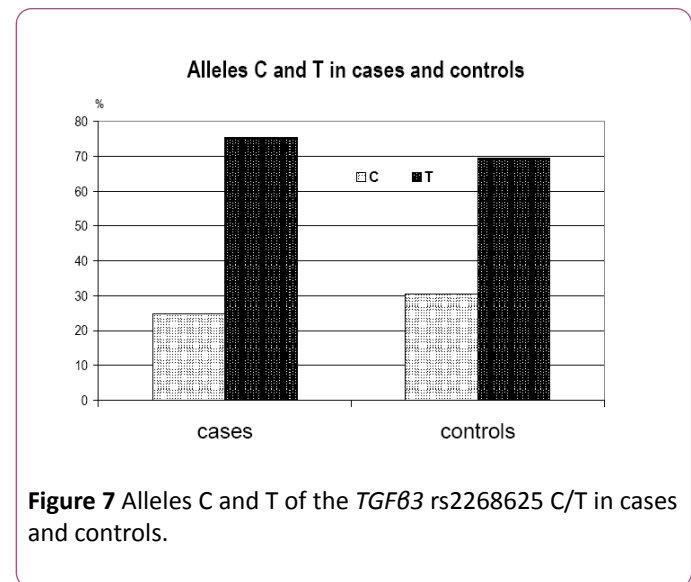


Figure 7 Alleles C and T of the *TGFβ3* rs2268625 C/T in cases and controls.

Combination of the *TGFβ3* rs2300607 A/T and rs2268625 C/T genotypes

The distribution of combined genotypes of the *TGFβ3* rs2300607 A/T and rs2268625 C/T was ascertained for 212 cases and 87 controls. The most frequent genotype combination in both cases and controls was AA/TT (46.22% in cases and 41.37% in controls) (**Table 7**).

Table 7 Distribution of combined genotypes of the *TGFβ3* rs2300607 A/T and rs2268625 C/T in cases and controls.

Genotypes—No (%)									
Sample type	AACC	AACT	AATT	ATCT	ATTT	TTCC	TTCT	TTTT	Total
Cases	0	2(0.94)	98(46.22)	7 (35.38)	1 (5.19)	1 (6.13)	(1.89)	9(4.25)	21 (100.00)
Controls	1(1.15)	(1.15)	36(41.37)	36(41.38)	7 (8.05)	5 (5.75)	1(1.15)	0	87 (100.00)

The second most frequent was AT/CT (35.38% in cases and 41.38% in controls).

We observed no combination of wild type genotypes AA/CC in cases and only one in controls. More, we did observe combination of double mutated homozygotes TT/TT only in

cases (4.25%). No such genotype combination was observed in controls. There was no genotype combination AT/CC observed neither in cases nor in controls.

There were slight differences in proportions of haplotypes rs2300607 A/T and rs2268625 C/T, however none of them was

found significantly different when cases and controls were compared (**Table 8**).

Table 8 Distribution of haplotypes of the *TGF β 3* rs2300607 A/T and rs2268625 C/T in cases and controls.

Haplotypes–No (%)					
Sample type	AC	AT	TC	TT	Total
Cases	79 (9.32)	493 (58.13)	135 (15.92)	141 (16.63)	848 (100.00)
Controls	42 (12.07)	196 (56.32)	58 (16.67)	52 (14.94)	348 (100.00)

Discussion

The identification of factors that contribute to the etiology of *NCL/P* is important for prevention, treatment planning, and education. With an increasing number of couples seeking genetic counseling as part of their family planning, the knowledge of how specific genes and environmental factors contribute to formation of *NCL/P* has gained increased importance [23]. *TGF β 3* is one of several candidate genes that have been implicated in the etiology of clefts.

In this study, the distribution of *TGF β 3* rs2300607 A/T genotypes and also rs2268625 C/T genotypes significantly differ between cases and controls ($p=0.0206$ for rs2300607 A/T and $p=0.041$ for rs2268625 C/T). More specifically, for rs2268625 C/T, the proportion of homozygotes for the wild type allele (CC) genotype was significantly lower in cases ($p=0.024$) compared to controls. In addition, a higher proportion of the mutated T allele homozygote (TT) was observed in cases compared to controls.

When allele frequencies were compared for both mutations, the higher proportion of mutated allele was found in cases compared to controls. There was a marginally statistically significant difference for *TGF β 3* rs2300607 A/T and for rs2268625 C/T, even when more than three-quarters of the cases carried the mutated T allele. These results suggest that both mutations of the *TGF β 3* gene are involved in the etiology of *NCL/P* in the population under investigation.

Ichikawa [17] investigated the possible relationship between *NCL/P* in a Japanese population and multiple polymorphisms of the *TGF β 3* gene. The results showed significant association between *NCL/P* and the *TGF β 3* rs2300607 A/T polymorphism. Although IVS-1572 (rs2268625) alone did not show a significant difference between cases and controls, the haplotype "A/A" for rs2300607-rs2268625 showed significant association.

The results of our study represent the first finding of an association between the *TGF β 3* rs2268625 C/T polymorphism and *NCL/P* and confirmed Ichikawa's finding of the association between *TGF β 3* rs2300607 A/T polymorphism and *NCL/P*.

Both studies utilized a case control design. The sample size of cases was larger in our study ($n=237$) compared to

Ichikawa's study ($n=112$), while our control sample was smaller for rs2268625 C/T ($n=98$) compared to 192 controls in Ichikawa's investigation. Analysis of DNA samples from additional unaffected individuals is on-going with the aim to establish a larger control sample and to confirm our results.

Interactions between the *TGF β 3* gene and other candidate genes, like *RFC1*, *MTHFR*, *MSX1* and others, also need to be investigated to increase the understanding of the genetic etiological component of *NCL/P*. The Guatemalan population investigated in this study was studied by Costanzo et al. [24] The *RFC1* A80G polymorphism was found to be associated with *NCL/P* in this population. Also, in the same population, *MSX1* CA-repeats polymorphism was found associated with *NCL/P* [25]. A joint analysis of *MSX1* and *TGF β 3* in South American children by Vieira [14] suggested that there may be an interaction between these two genes which increases cleft susceptibility. A similar interaction between *MSX1* and *TGF β 3* in the Guatemalan population may need to be investigated.

The rs2300607 A/T SNP and rs2268625 C/T SNP of *TGF β 3* are, interestingly, located in intron 1 of *TGF β 3* gene. The mechanism of how the intronic rs2300607 A/T SNP and rs2268625 C/T SNP of *TGF β 3* can contribute to clefting is still to be clarified.

It has been shown that genetic and environmental factors are ethnicity specific, and in many places in the world, location specific [23]. Thus a specific protocol for cleft prevention has to be worked out based on genetic and nutritional studies of each specific population group in order to be effective [26]. The Guatemalan population is mainly of Mayan descent as eluded earlier. Other populations with different ethnic background may have different genetic predisposition to *NCL/P*. Thus, future research on other populations is needed to determine the nature of the *TGF β 3* gene polymorphism's involvement in the etiology of *NCL/P*.

Summary and Conclusion

The etiology of *NCL/P* is currently considered to be the result of interactions between environmental and genetic factors. The genetic risk appears to be derived from at least several different genes. In this study, we investigated a possible role of the *TGF β 3* gene in the etiology of *NCL/P*.

Specifically, we examined the AT allele variation of *TGFβ3* rs2300607A/T SNP and CT allele variation of *TGFβ3* rs2268625 C/T SNP. We studied a sample of individuals from Guatemala City, Guatemala, who were affected with *NCL/P* and compared them to unaffected individuals from the same location.

The χ^2 test revealed a statistically significant difference between distributions of *TGFβ3* rs2300607 A/T SNP genotypes in cases and controls ($p=0.0206$). A higher proportion of mutated allele T was found in cases and this difference was on the margin of statistical significance stated for this study ($p=0.0554$).

A statistically significant difference in the distribution of *TGFβ3* rs2268625 C/T genotypes with a higher proportion of TT homozygotes was found in cases compared to controls ($p=0.041$) and a lower proportion of CC homozygotes in cases compared to controls ($p=0.024$). A higher frequency of mutated allele T was observed in affected individuals compared to unaffected controls (this difference was not statistically significant).

The results of our study showed, for the first time, the association between the *TGFβ3* rs2268625 C/T polymorphism and *NCL/P* and confirmed for Guatemalan population the association between *TGFβ3* rs2300607 A/T polymorphism and *NCL/P* found by Ichikawa in Japanese [17].

Thus, our findings suggest that the rs2300607 A/T and rs2268625 C/T polymorphisms of the *TGFβ3* gene are involved in the etiology of *NCL/P* in the Guatemalan population.

The results of this research may provide a guide for the direction of future research and genetic counseling. The potential for gene-gene and gene-environment interactions should be investigated. Further studies, preferably on larger samples, including other candidate genes and assessing haplotypes within the Guatemalan population and in other populations, are needed. Further, the identification of genes involved in causing *NCL/P* is the first step in formulating preventative measures that will aim to reduce the occurrence of this condition in Guatemala.

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