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IRF6 and *SATB2* Polymorphisms in Non-Syndromic Orofacial Clefts among Bangladeshi Patients

(Polimorfisme *IRF6* dan *SATB2* pada Klef Orofasial Bukan Sindrom dalam kalangan Pesakit Bangladesh)

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ABSTRACT

Non-syndromic cleft lip and/or palate (NSCL/P) is a common congenital craniofacial anomaly resulting from the complex interaction of genetic and environmental factors. Although several genetic variants have been associated with NSCL/P, population-specific evidence from Bangladesh remains limited. Therefore, this study aimed to describe the demographic characteristics of Bangladeshi patients with NSCL/P and to investigate the distribution of selected single nucleotide polymorphisms (SNPs) in interferon regulatory factor 6 (*IRF6)* and special AT-rich sequence-binding protein 2 (*SATB2)* among patients with NSCL/P and a control group. Sixty-one parents or guardians of patients with NSCL/P were interviewed regarding demographic characteristics, socioeconomic background and family history of cleft. Dried blood spot (DBS) samples were collected, and genomic DNA was extracted using the QIAamp DNA Micro Kit (Qiagen). Five SNPs in *IRF6* (rs2235377, rs2235373, rs2235371, rs861019, and rs2013162) and two SNPs in *SATB2* (rs142825652 and rs137853127) were genotyped using TaqMan®-based assays. Allele and genotype frequencies were determined using TaqMan Genotyper software. Fisher's exact test and odds ratios (ORs) were used to evaluate the distribution of the selected variants between the NSCL/P and control groups. From the total of 61 patients, 84% of the cleft patients were males and 16% were females with a ratio 5:1. All patients were reported to be from low socioeconomic background and claimed no family history of cleft incidence. For *IRF6* rs2235377, the C allele frequency was 0.64 in the NSCL/P group compared with 0.53 in the control group (OR = 1.57, P = 0.33). For rs2013162, the A and C alleles occurred at equal frequencies in the NSCL/P group (0.50 each), whereas the C allele was more frequent in the control group (0.58). The remaining *IRF6* variants showed no statistically significant differences in allele frequencies between groups (P > 0.05). Both *SATB2* SNPs showed identical allele frequencies between the NSCL/P and control groups, with the G allele observed at a frequency of 1.00 in both groups. Male patients and UCL were predominant among the Bangladeshi patients studied. Selected *IRF6* SNPs showed differences in genotype distribution, but no significant allele-level association was observed, while the *SATB2* variants were not associated with NSCL/P. Despite the limited sample size, this study provides preliminary population-specific data on cleft characteristics and genetic variation in Bangladesh, which may support future genetic studies and improve understanding of NSCL/P in this population. Larger sample size is needed to validate these findings.

Keywords: Bangladeshi; cleft lip and/or palate; gene; *IRF6*; non-syndromic

ABSTRAK

Klef bibir dan/atau lelangit tanpa sindrom (NSCL/P) merupakan anomali kraniofasial kongenital yang lazim dan berpunca daripada interaksi kompleks antara faktor genetik dan persekitaran. Walaupun beberapa varian genetik telah dikaitkan dengan NSCL/P, bukti populasi khusus dari Bangladesh masih terhad. Oleh itu, kajian ini bertujuan untuk menerangkan ciri demografi pesakit NSCL/P Bangladesh serta mengkaji taburan polimorfisme nukleotida tunggal (SNP) terpilih dalam faktor kawalaturan interferon 6 (*IRF6*) dan jujukan-pengikat protein 2 khusus kaya AT (*SATB2*) dalam kalangan pesakit NSCL/P dan kumpulan kawalan. Enam puluh satu orang ibu bapa atau penjaga pesakit NSCL/P telah ditemu bual berkaitan ciri demografi, latar belakang sosioekonomi dan sejarah keluarga klef. Sampel darah kering (DBS) dikumpulkan dan DNA genom diekstrak menggunakan QIAamp DNA Micro Kit (Qiagen). Lima

SNP dalam *IRF6* (rs2235377, rs2235373, rs2235371, rs861019 dan rs2013162) dan dua SNP dalam *SATB2* (rs142825652 dan rs137853127) telah digenotip menggunakan kaedah berasaskan TaqMan®. Frekuensi alel dan genotip ditentukan menggunakan perisian TaqMan Genotyper. Ujian tepat Fisher dan nisbah *odds* (OR) digunakan untuk menilai taburan varian terpilih antara kumpulan NSCL/P dan kumpulan kawalan. Daripada jumlah 61 orang pesakit, 84% pesakit sumbing adalah lelaki manakala 16% adalah perempuan, dengan nisbah 5:1. Kesemua pesakit dilaporkan daripada latar belakang sosioekonomi rendah dan tidak mempunyai sejarah keluarga klef. Bagi SNP *IRF6* rs2235377, frekuensi alel C ialah 0.64 dalam kumpulan NSCL/P berbanding 0.53 dalam kumpulan kawalan (OR = 1.57, P = 0.33). Bagi rs2013162, alel A dan C menunjukkan frekuensi yang sama dalam kumpulan NSCL/P (0.50 setiap satu), manakala alel C lebih kerap dalam kumpulan kawalan (0.58). Varian *IRF6* yang lain tidak menunjukkan perbezaan yang signifikan secara statistik dalam frekuensi alel antara kumpulan (P > 0.05). Kedua-dua SNP *SATB2* menunjukkan frekuensi alel yang sama antara kumpulan NSCL/P dan kawalan, dengan alel G diperhatikan pada frekuensi 1.00 dalam kedua-dua kumpulan. Pesakit lelaki dan UCL merupakan kumpulan yang paling dominan dalam kalangan pesakit Bangladesh yang dikaji. Walaupun terdapat perbezaan dalam taburan genotip bagi SNP *IRF6* terpilih, tiada perkaitan yang signifikan pada aras alel diperhatikan, manakala varian *SATB2* yang dikaji tidak menunjukkan perkaitan dengan NSCL/P. Walaupun saiz sampel adalah terhad, kajian ini menyediakan data awal populasi khusus berkaitan ciri klef dan variasi genetik di Bangladesh yang dapat menyokong kajian genetik pada masa hadapan serta meningkatkan pemahaman tentang NSCL/P dalam populasi ini. Kajian dengan saiz sampel yang lebih besar diperlukan untuk mengesahkan penemuan ini.

Kata kunci: Bangladesh; klef bibir dan/atau lelangit; gen; *IRF6*; tanpa sindrom

INTRODUCTION

Orofacial clefts are the most common human congenital disorders (Blanton et al. 2010; Mangold et al. 2009) that affect mainly low social economic status groups. The management of cleft starts from birth until adulthood which results in constant social and psychological insult to the patients and families (Bueno et al. 2011). Therefore, it is vital to understand the pathogenesis of deformity for an optimum care and counseling to the patients and families. Orofacial clefts involve both genetic and environmental factors in which the end results would be different across geographical, racial and population differences (Mangold et al. 2009; Stanier & Moore 2004).

Two genes that are well-known, play an important role in cleft development, interferon regulatory factor 6 (*IRF6)* and special AT-rich sequence-binding protein 2 (*SATB2)* were used in this study. Multiple studies have shown strong evidence for the contribution of *IRF6* variants to the risk of non-syndromic oral clefts across various ethnic groups, with increased risk among those with cleft lip only (Rahimov et al. 2008; Salahshourifar et al. 2012; Zucchero et al. 2004). The identified allele may contribute to genetic susceptibility to NSCL/P, however, its presence alone is unlikely to determine cleft development, given the complex and multifactorial nature of the condition. *SATB2* has also been widely investigated in worldwide population related to cleft study. Previous studies on multiplex NSCL/P families in different populations have showed evidence for linkage in 2q and 2p region (Marazita et al. 2002; Zeiger et al. 2003). In addition, deletion at 2q31-q33 region of *SATB2* has delineated the medial region of maxillary process to form the palatal shelf within the primitive oral cavity (FitzPatrick et al. 2003).

This study provided scientific knowledge to the cleft study and delivered output for the betterment of bottom billion society. Building on four years of multi-institutional collaboration with organisations such as MERCY Malaysia and Dhaka Community Hospital in Bangladesh, this study strengthens established partnerships and expands existing activities through international collaboration, particularly within the Asia-Pacific region.

MATERIALS & METHODS

DEMOGRAPHIC DATA COLLECION

Consented parent or guardian was interviewed during the screening by specialists. This study was approved by The Human Research Ethics Committee, Universiti Sains Malaysia (FWA Reg. No: 0000718, IRB Reg. No: 00004494). The questions being asked were the socio-economis status of the family and the history of cleft in the family. Gender and type of cleft of 61 patients aged between 3 months and 2 years were observed and documented by the specialists.

DNA EXTRACTION

DNA extraction with QIAamp DNA micro kit (Qiagen)

Three punched-out circles (2 mm each) from a dried blood spot were placed to the bottom of 1.5 µL tube, and 180 µL of Buffer ATL and 20 µL proteinase K was added. The tube was sealed thoroughly using parafilm and it was incubated at 56 °C for 60 min. At each 10 min, the tubes were mixed using vortex. Then, the tube was spun briefly at 8000 rpm for 1 min to collect any remaining solution from the caps. After that, 200 µL buffer AL was added and mixed using pulse-vortexing for 10 s. It was then

incubated in water bath at 70 °C for 10 min and vortexed for 10 s every 3 min. DNA quality was determined using both NanoDrop with absorbance of 260/280.

SNP GENOTYPING

SNP selection

Five SNPs of *IRF6* were selected: rs2235377, rs2235373, rs2235371, rs861019, and rs2013162 and 2 SNPs of *SATB2*: rs142825652 and rs137853127. Primer sequences targeting the selected *IRF6* and *SATB2* SNPs were designed to encompass the respective variant positions. The primer sequences, including the polymorphic nucleotide represented in square brackets, were presented in Table 1.

Preparation for reaction mixture

Three pieces of DBS (2 mm diameter each) that equals to ≈ 3 ng were placed at the bottom of 96-well plate. All these components were mixed to each well: 2X TaqMan® GTXpress™ Master Mix, 20X TaqMan genotyping assay mix and DNase-free water to the total volume of 8 µL.

Quantitative PCR (qPCR)

qPCR was performed using TaqMan® GTXpress Master Mix for relative quantitation of the selected SNPs. qPCR was run using StepOne™ system with following parameters: Polymerase activation at 95 °C for 20 s, denaturation at 95 °C for 3 s (40 cycles), annealing and extension at 60 °C at 20 s (40 cycles).

Bioinformatics analysis

The data was calculated and determined using TaqMan Genotyper software. From the allelic discrimination plot, allele frequency was determined. Statistical analysis, Fisher's exact test was done using Epi Info version 3.5.4 to determine if there are associations between two categorical variables.

RESULTS AND DISCUSSION

DEMOGRAPHY

The cleft patients were screened prior to surgery. From the total of 61 patients been treated, 51 (84%) were males and 10 (16%) were females (Figure 1). This sex distribution brought the ratio of cleft incidence of male to female by 1:5 ratio. There were various age groups identified among the cleft patients. The distribution of patients based on age was shown in Figure 2. The highest one was the patient age 2 years old with 17 out of 61, followed by 13 of them at 1 year old, 11 babies age from 8-11 months old and 5 of them each at age of 3-6 months and 4 years old. The others were 3 children at the age of 3 years old, two of them each at 7 and 15 years old and one for each at 6, 11, and 18 years old.

The type of cleft in each patient was determined. Type of cleft was divided into 5 types; Unilateral cleft lip (UCL), Unilateral cleft lip and palate (UCLP), Bilateral cleft lip (BCL), Bilateral cleft lip and palate (BCLP), and Cleft palate only (CP). From Figure 3, it was identified that 39.34% of patients had UCL followed by CP with 24.59%. Following this, 16.39% of the patients had BCL and 14.75% of them had UCLP. The most least identified was BCLP with 4.92%. In addition, all patients were from low socioeconomic backgrounds, and none reported a family history of CL/P.

Non-syndromic cleft lip and/or palate (NSCL/P) is a major birth defect worldwide. It arises from the interplay of genetic and environmental factors. In Bangladesh, there are quarter of million people suffer from cleft lip and/or palate deformity. Birth prevalence and incidence of clefts vary among populations based on ethnic background (Cooper, Ratay & Marazita 2006). It was remarkable that Asian and Native North American descent population has the highest birth prevalence with NSCL/P by 2 per 1000 births compared to the other populations. Following this, Caucasian populations had NSCL/P prevalence of 1 per 1000 births and African

TABLE 1. Primer sequences used for genotyping of *IRF6* and *SATB2* SNPs

Gene	SNP	Primer sequence
IRF6	rs2235377	TTCCCTTGACCGCTCAAAGATTCTG[C/T]ATGGAGAAAAGGAGGGGTCTCAGCT
IRF6	rs2235373	AGTGGAAGGAATGTACTTCCAGAGA[A/G]TGATTCCCACGATCAACTTTCTGAG
IRF6	rs861019	TTGTCTTTCCCTTGACCGCTCAAAG[A/G]TTCTGTATGGAGAAAAGGAGGGGTC
IRF6	rs2013162	TGTCCTGGATGGGAACATGGTGCTG[A/C]GACTGATCCAGCTCATCTTCCTCAT
IRF6	rs2235371	ATATGCTCAGGACCTGGGAATTTGA[C/T]CTGCTCCAGGCTGACGGGACCAAAG
SATB2	rs137853127	ACACAATAGTCTGAAAGGTTTTCTC[G/A]TTCCACTCTTTCCACTGTTAAGAGA
SATB2	rs142825652	CCAGGGCTCATGATGGGCTGTAATG[C/T]GGGCACTTGGTTTCGGATTGGAGTA

populations had the lowest NSCL/P prevalence with 1 per 2500 births (Cooper, Ratay & Marazita 2006; Vieira et al. 2008). In Bangladesh, with a population of approximately 150 million, an estimated 300,000 individuals are living with unrepaired clefts (Paul et al. 2007). The large number of untreated cases has been attributed to limited access to specialized cleft care, a shortage of trained cleft surgeons, inadequate multidisciplinary services and financial constraints, particularly among underserved and rural populations.

During the visit, we managed to obtain 61 completed profile details from the cleft patients. The sex distribution of cleft was approximately 1:5 which means it was 5 times higher in male than in female. This ratio was higher than expected. Previously, it had been reported that the sex ratio was 1:2 in which it was two times higher in male compared to female. Similar study on Jordanian children had found significant higher CL/P prevalence in male than in female patients (Al Omari & Al-Omari 2004). Therefore, suggestive evidence concludes that the cleft prevalence was more frequent in male than in female. However, the ratio might vary, can be higher or lower in different populations throughout different geographical, ethnic or lifestyle backgrounds.

About one-third of patients who underwent cleft lip surgery were at the age of 2 years old. A higher proportion of patients presented at 2 years old compared with those aged 3-6 months, the age range generally recommended for cleft lip surgery, thus, suggesting delayed access to surgical treatment. The main factor associated with the late surgery was an economical issue. However, the number of treated patients aged between 8-11 months was average about one-fifth of the patient, in which 8-9 months of age is an ideal age to repair cleft palate. As opposed to our finding, the cleft mission conducted by other team in Bangladesh within year 2000-2004 had found that the most patients underwent cleft operation were in age group of 10-15 years old. The teenagers especially girls had the priority as they need the cleft repair before getting married (Paul et al. 2007). It was apparent that all patients came from low socio-economic family supported our hypothesis that the cleft deformity in Bangladesh was associated with socio economic background.

The most common type of cleft among the Bangladeshi is unilateral cleft lip (UCL). However, previous report had documented the most common cleft is unilateral cleft lip and palate (Boo & Arshad 1990). On the other hand, study conducted in South East of Ghana had found similar finding as 36% of the patients had UCL, which is higher than the other type of clefts and 16% had UCLP (Agbenorku et al. 2011).

GENOTYPING SCREENING

SNP genotyping assay was carried out to determine allelic discrimination for Interferon regulatory factor-6 *(IRF6)* and *SATB2* genes. Five selected SNPs for *IRF6* (rs2235377, rs2235373, rs861019, rs2013162, rs2235371) and 2 SNPs for *SATB2* (rs142825625, rs137853127) were used in the study. The interface of TaqMan Genotyper software showed the frequency of alleles detected. The figure illustrates the three *IRF6* genotypes: homozygous C/C, homozygous T/T, and heterozygous C/T (Figure 4). Although rs2235373 and rs861019 are reported as A/G polymorphisms, only the A allele was detected in both the CL/P and control groups in the present study, resulting in a 100% A allele frequency and no observed allelic variation. Meanwhile, for *SATB2*, homozygous G/G alleles were 100% detected and none heterozygous A/G and homozygous A/A alleles were detected in both CL/P and control group (Figure 5).

It was detected that the frequency for allele C was higher (0.64) in CL/P group than allele frequency of C (0.53) in control group for rs2235377 *IRF6* (Table 2). However, in rs2235373 and rs861019, frequency for allele A (1.0) was only detected in control and CL/P group. None of allele G was detected in both groups. Meanwhile rs2013162 had frequency of allele A and C by half in CL/P group (A = 0.5 and C = 0.5) and allele frequency of C was slightly higher (0.58) compared to allele A (0.42) in control group. In rs2235371, allele frequency of C (CL/P = 0.79 and Control = 0.75) was higher in both groups.

The estimated odds ratios (ORs) for all SNPs were greater than 1, suggesting a potential positive association between the investigated alleles and CL/P in this study population. However, none of the associations reached statistical significance, as indicated by the corresponding confidence intervals and $p>0.05$. Thus, the present findings do not provide sufficient evidence to conclude that these variants are associated with an increased risk of CL/P, and the observed elevations in ORs may reflect sampling variation, particularly given the relatively small sample size.

For SNPs of *SATB2* (rs142825652 and rs137853127), it was found that the allele frequency of G (1.0) was similar in CL/P and control group (Table 3). Low odds (OR < 1) were also detected. This confirmed that the group was not significantly associated with allele type, P value > 0.05. Therefore, these two SNPs of *SATB2* were not associated with CL/P formation in Bangladeshi.

Genotype frequencies for each SNP were also calculated. The genotype distribution of *IRF6* rs2235377 differed significantly between NSCL/P patients and controls, $p = 0.003$). The C/C genotype was observed in 40.9% of NSCL/P patients compared with 5.6% of controls, while the C/T genotype was observed in 45.5% and 94.4%, respectively. The T/T genotype was detected in 13.6% of NSCL/P patients but was absent among controls (Table 4). Similarly, the heterozygous AC genotype of rs2013162 in *IRF6* was observed in 62% of individuals with CL/P and 74% of controls, indicating a higher frequency in the control group than in the CL/P group.

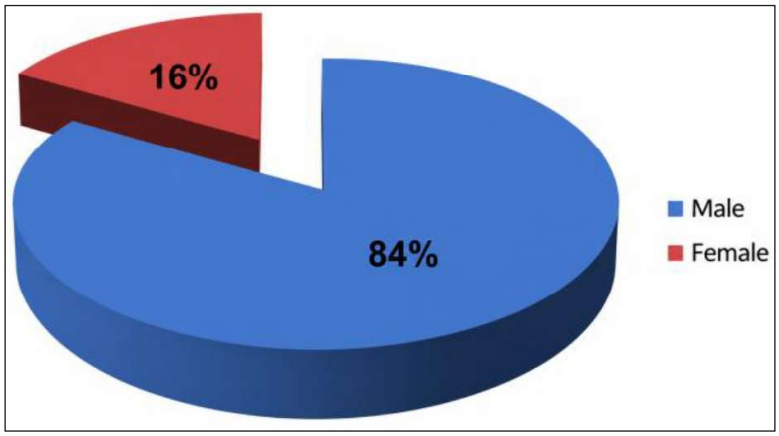


FIGURE 1. The percentage of cleft lip and/or palate incidence in male and female

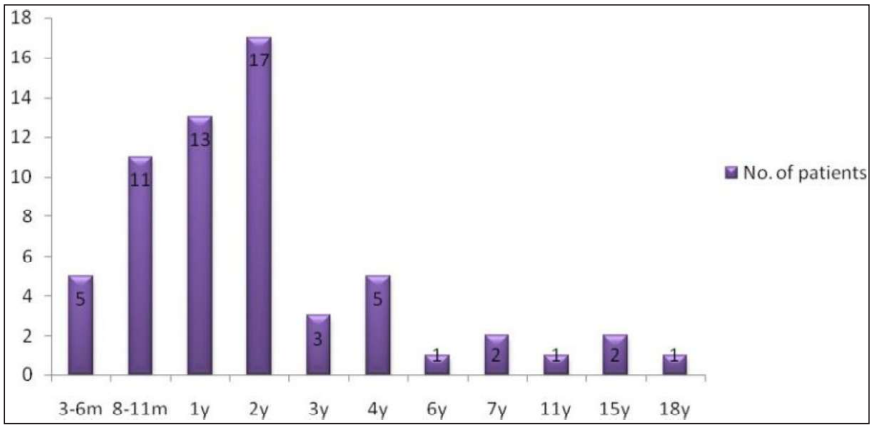


FIGURE 2. The bar graph showed the number of patients that have been treated starts from 3 months to 18 years old

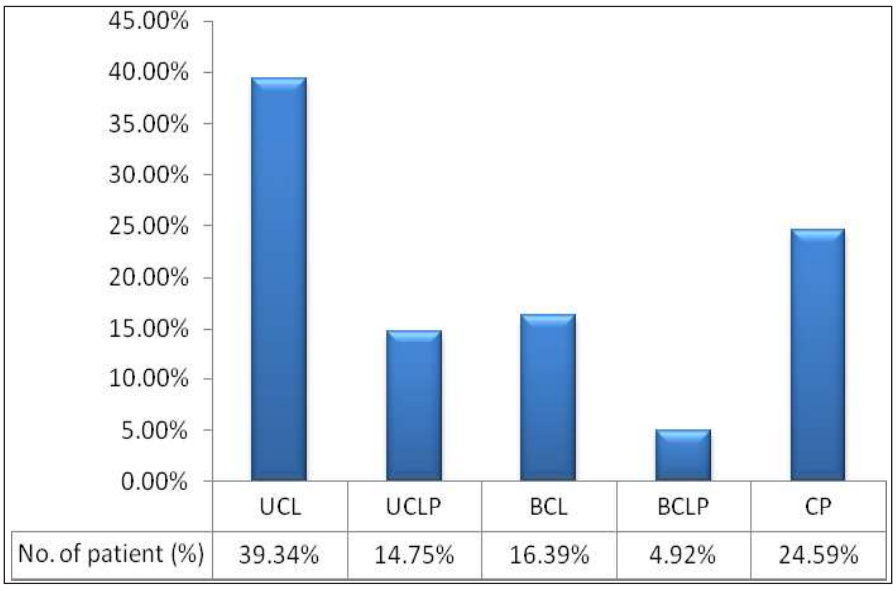


FIGURE 3. The type of cleft was categorized into 5 types; Unilateral cleft lip (UCL), Unilateral cleft lip and palate (UCLP), Bilateral cleft lip (BCL), Bilateral cleft lip and palate (BCLP) and cleft palate only (CP)

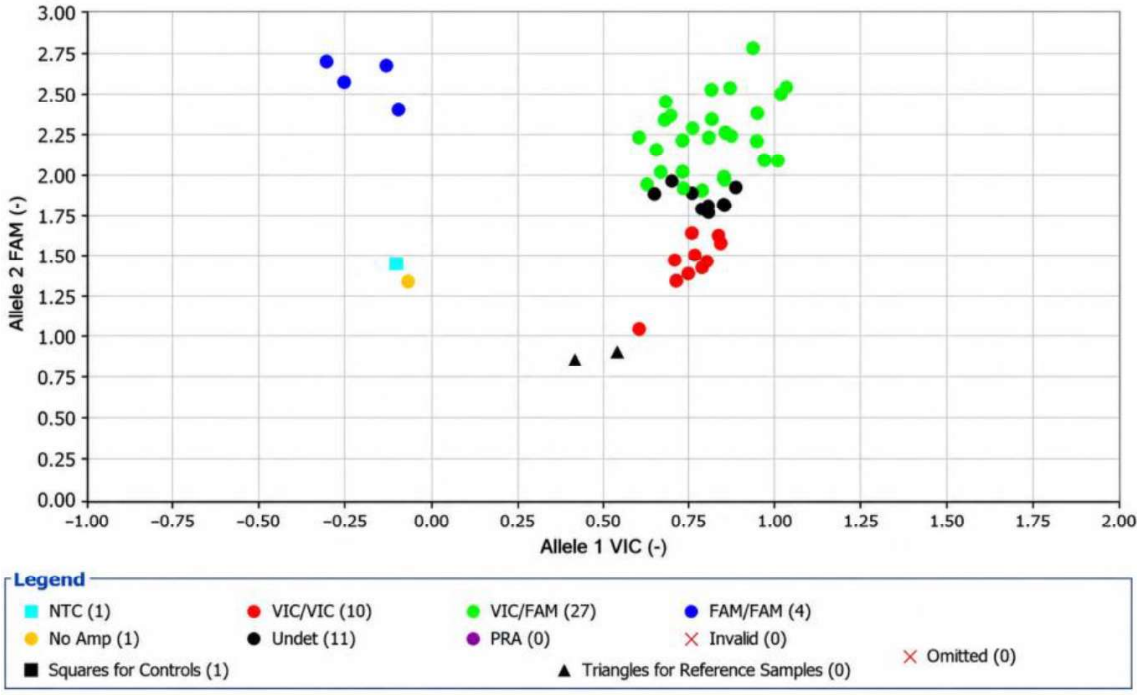


FIGURE 4. Alleles of C/C (red dots), T/T (blue dots), C/T (green dots) and undetermined (black dots) were shown in the interface of TaqMan Genotyper software for *IRF6*

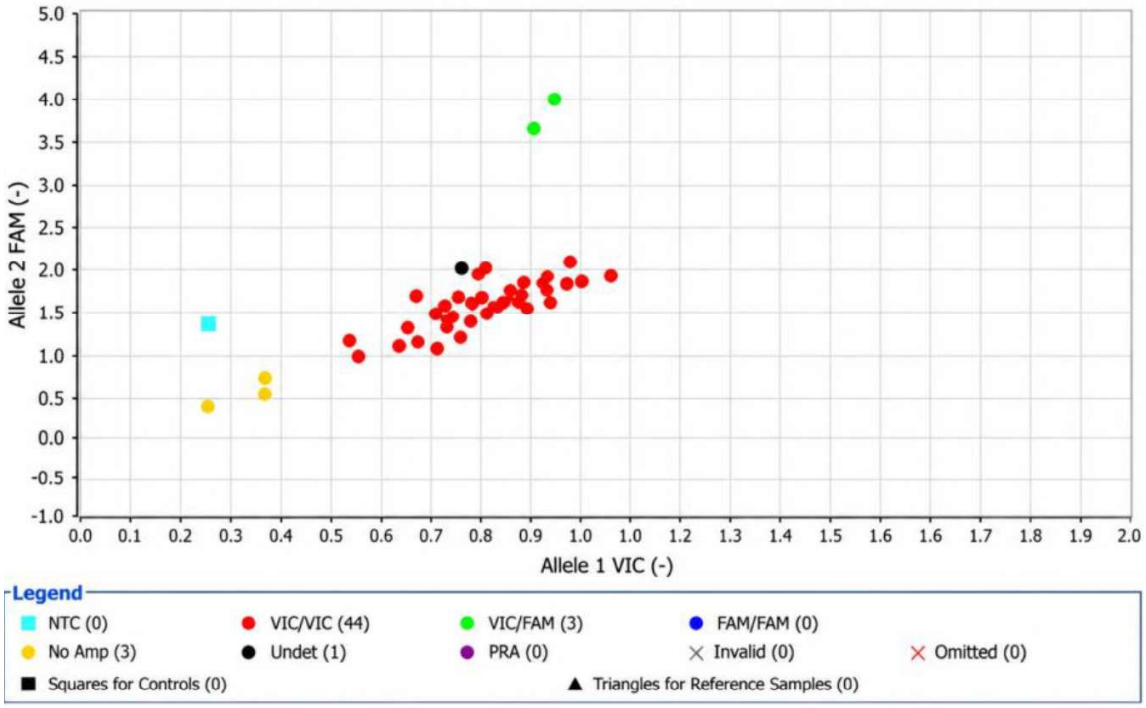


FIGURE 5. Multiple red dots indicate homozygous A/A were mostly present for *SATB2*. Very little heterozygous A/G in green dots and none homozygous G/G were present

On contrary, genotype frequencies for *SATB2* were found similar in both SNPs; rs142825652 and rs137853127 for both groups (CL/P and control) with no heterogeneity exhibiting the dominant allele of G (Table 4).

Based on our previous study, *IRF6* and *SATB2* were identified as candidate genes potentially involved in the pathogenesis of CL/P in the Malay population (Salahshourifar et al. 2012). In the present study, the same genetic variants within *IRF6* and *SATB2* were investigated to assess their association with CL/P among patients from the Bangladeshi population.

However, we found that there was no significant difference of allele frequencies in CL/P patients compared to control in all SNPs of *IRF6* and *SATB2*. The differences observed between the present and previous studies may reflect population-specific genetic variation, as allele and genotype frequencies of specific polymorphisms can differ across ethnic and geographical populations.

However, genotype frequencies was found significant in SNP of *IRF6* rs2235377. CL/P group had higher frequencies of homozygote dominant while control had higher frequencies of heterozygote. A slight different of heterozygote frequecies between CL/P and control group in rs2013162 *IRF6* was also detected. These findings depicted there was a genetic variation in *IRF6* gene among the Bangladeshi population that may at risk to

TABLE 2. Allele frequencies from five SNPs of *IRF6*

SNP	Alleles	Allele frequency				
		CL/P	Control	p value	Odds Ratio (OR)	95% Confidence Interval (CI)
rs2235377	C/T	C = 0.64	C = 0.53	0.33	1.57	0.63
rs2235373	A/G	A = 1.0	A = 1.0	0.48	2.63	1.00
rs861019	A/G	A = 1.0	A = 1.0	0.89	1.21	1.00
rs2013162	A/C	A/C = 0.50	C = 0.58	0.48	1.38	0.50
rs2235371	C/T	C = 0.79	C = 0.75	0.74	1.27	0.79

*Fisher's exact test, P<0.05

TABLE 3. Allele frequencies from two SNPs of *SATB2*

SNP	Alleles	Allele frequency				
		CL/P	Control	p value	Odds Ratio (OR)	95% Confidence Interval (CI)
rs142825652	A/G	1.0	1.0	0.73	0.60	1.00
rs137853127	A/G	1.0	1.0	0.88	0.80	1.00

*Fisher's exact test, P<0.05

TABLE 4. Genotype frequencies for each SNP of *IRF6* and *SATB2*

Gene	SNP	Alleles	Genotype Frequency			
			CL/P	Control	HWE	p value
IRF6	rs2235377	CT	0.45	0.94	1.00	0.003
		CC	0.41	0.06		
	rs2235373	AA	1.0	1.0	N/A	N/A
	rs861019	AA	1.0	1.0	N/A	N/A
	rs2013162	AC	0.62	0.74	0.40	0.42
	rs2235371	CC	0.58	0.50	1.00	1.00
SATB2	rs142825652	GG	1.0	1.0	N/A	N/A
	rs137853127	GG	1.0	1.0	N/A	N/A

N/A, not applicable because the SNP was monomorphic

develop CL/P formation. In addition, the monomorphic nature of several SNPs in both study groups limited their statistical evaluation, as the absence of allelic and genotypic variation precluded meaningful assessment of genotype distribution and Hardy–Weinberg equilibrium.

IRF6 belongs to a family of transcription factors. *IRF6* mutation has been found in Van Der Woude Syndrome (VWS), popliteal pterygium syndrome (PTS) with the presence of orofacial cleft phenotype (Kondo et al. 2002; Zhang et al. 2020). Several previous studies including linkage analyses, pedigree analysis, candidate gene analyses, and genome-wide association studies have found polymorphisms near *IRF6* and a rare variant in patients with NSCL/P and thus confirmed the relationship between *IRF6* and NSCL/P (Beaty et al. 2013; Park et al. 2007; Rahimov et al. 2008; Sun et al. 2015; Zhao et al. 2018).

The important role of *SATB2* during craniofacial development was proven in multiple studies whereas they found disruption of *SATB2* regulation during human craniofacial development which led to the combined cleft palate, micrognathia, dental anomalies and learning difficulties (FitzPatrick et al. 2003; Rosenfeld et al. 2009; Urquhart, Black & Clayton-Smith 2009). Similar loss of function of *SATB2* in mice was detected in human that caused craniofacial and skeletal defects (Britanova et al. 2006; Dobreva et al. 2006). In addition, one study conducted in Thailand has discovered that the pathogenic *SATB2* point mutation was associated with craniofacial dysmorphism (Leoyklang et al. 2007). Deletions involving the 2q32–q33 region, which encompasses *SATB2*, have been reported in patients with cleft palate and other craniofacial abnormalities (Balasubramanian et al. 2011; Rosenfeld et al. 2009; Van Buggenhout et al. 2005). These observations provide supporting evidence for the involvement of SATB2 in craniofacial development and the rationale for investigating SATB2 polymorphisms in CL/P.

In contrast, our findings were contradicted as we found similarities in *SATB2* genotype in NSCL/P and normal groups. Therefore, further studies shall be done to rule out the *SATB2* mutation. The investigated *SATB2* polymorphisms were not associated with CL/P among the Bangladeshi population.

CONCLUSION

In Bangladesh, the NSCL/P incidence the most common among males than females (1:5) and the most common type of cleft diagnosed was UCL. Although most patients received treatment at approximately 2 years of age, considerably later than the recommended age for cleft repair, effective treatment was successfully provided. Based on genetic study of NSCL/P, two genotype variants of *IRF6* were associated with Bangladeshi population meanwhile the variants in *SATB2* was not associated. As for limitation, the number of samples were limited due to the time constraint and lack of facility in Bangladesh. Further study shall be carried out in future using larger group of patients to further confirm these findings.

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APPENDICES

Sample Size Calculation

Determination of sample size is based on the single proportion formula.

Sample size, $n = \left[\frac{z}{\triangle}\right]^2 p\ (1\text{-}p)$

where z = 1.96; Level of Significance, α = 0.05; Absolute precision (△) = 3%; p = expected proportion of individual in the sample; Anticipated population proportion (p) = 3% (Alam 2005; Melnick et al. 1986; Normastura et al. 2008)

Sample size, $n = \left[\frac{1.96}{}\right]^2 0.03\ (1\text{-}0.03)\ 0.05$

$= \left[\frac{1.96}{}\right]^2 0.03\ (0.97)\ 0.05$

$= 15$

Total samples = 45