Original Article – Comparative Study

Single Nucleotide Polymorphisms in Runt-related Transcription Factor 2 and Bone Morphogenetic Protein 2 Impact on Their Maxillary and Mandibular Gene Expression in Different Craniofacial Patterns - A Comparative Study

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Abstract

Introduction: This study aimed to evaluate if single nucleotide polymorphisms (SNPs) in runt-related transcription factor 2 (*RUNX2)* and bone morphogenetic protein 2 (*BMP2)* are associated with different craniofacial patterns. Furthermore, we also investigated if *RUNX2* and *BMP2* expression in the maxilla and mandible are differently expressed according to facial phenotypes and influenced by the SNPs in their encoding genes. Orthognathic patients were included. **Materials and Methods:** Lateral cephalometric radiographs were used to classify facial phenotypes based on Steiner's ANB and Ricketts' NBa-PtGn angles. Bone samples from 21 patients collected during orthognathic surgery were used for the gene expression assays. DNA from 129 patients was used for genotyping the SNPs rs59983488 and rs1200425 in *RUNX2* and rs235768 and rs1005464 in *BMP2.* The established alpha was 5%. **Results:** A statistically significant difference was observed in the relative BMP2 expression in the mandible between Class I and III participants ($P = 0.042$). Homozygous GG (rs59983488) had higher RUNX2 expression ($P = 0.036$) in the mandible. In maxilla, GG (rs1200425) had a higher BMP2 expression ($P = 0.038$). **Discussion:** In conclusion, BMP2 is expressed differently in the mandible of Class I and Class III participants. Genetic polymorphisms in *RUNX2* and *BMP2* are associated with their relative gene expression.

Keywords: Craniofacial morphology, facial type, gene expression, polymorphism, skeletal class

Introduction

Craniofacial growth and development is a complex process involving many molecular aspects.[1,2] The growth process of the maxilla and other midfacial bones is associated with the growth of the mandible and the cranial base.[3,4] Variations in craniofacial growth lead to different sagittal and vertical morphological patterns.[5-7] Sagittal patterns are most frequently classified into skeletal malocclusion Class I, II, and III,[8] whereas vertical patterns are differentiated into mesofacial, dolichofacial, and brachyfacial facial growth patterns.[9]

Many molecular and genetic factors are responsible for triggering the growth process.[1,2,4] Among them, runt-related transcription factor 2 (*RUNX2*) and bone morphogenetic protein 2 (*BMP2*)[2] should be highlighted due to their important role in the craniofacial development.

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RUNX2 is a gene that encodes a transcription factor essential for bone formation and skeletal morphogenesis. This protein plays a role in the regulation of factors involved in skeletal gene expression, such as the expression of bone sialoprotein, collagen type 1 α (*COL1A1*), and osteocalcin (*BGLAP2*).[9]

BMP2 is a gene that encodes ligands of the transforming growth factor-beta superfamily of proteins.[2,10] These ligands are responsible for bone and cartilage formation due to its role in osteoblast and chondroblast differentiation.[9] BMP signaling pathway is associated with the fusion of facial patterns.[2,11]

Single nucleotide polymorphisms (SNPs) have the potential to alter all steps of gene expression depending on their genomic location. When they are within transcriptional regulatory elements, SNPs can affect mRNA (messenger RNA) expression,[12] impacting the phenotype. SNPs in many genes have been associated with different sagittal and vertical craniofacial patterns in humans,[5-7,13] including SNPs in *RUNX2* and *BMP2*, which were associated with different maxillary and mandibular phenotypes.[13]

Although many studies have been evaluating the association between SNPs in genes with different craniofacial patterns, the interplay among SNPs, gene expression and craniofacial pattern has not been explored yet. Therefore, in this study, we evaluated if SNPs in *RUNX2* (rs59983488 and rs1200425) and *BMP2* (rs235768 and rs1005464) are associated with different craniofacial patterns in patients presenting for orthognathic surgery. Furthermore, we also investigated if *RUNX2* and *BMP2* expression (mRNA) in the maxilla and mandible are differently expressed according to facial patterns and are influenced by the SNPs in their encoding genes. Thus, SNPs as well as the relative gene expression of *RUNX2* and *BMP2* in bone samples from the maxilla and mandible were explored in patients submitted to orthognathic surgery.

Materials and Methods

Ethical considerations

This study was approved by the ethical committee of the Federal University of Parana (26502919.6.0000.0093). All procedures performed in the study were conducted in accordance with the ethics standards given in 1964 Declaration of Helsinki, as revised in 2013. All the participants provided written informed consent for the participation in the study.

Sample selection and study design

Patients with finalized orthodontic preparation were invited to participate consecutively during a 2-year period. This cross-sectional study followed three steps: (1) Gene expression analysis using maxillary and mandibular bone samples from 21 patients to explore if *RUNX2* and *BMP2* are differentially expressed according to craniofacial patterns; (2) Functional analysis to evaluate, if SNPs in *RUNX2* and *BMP2* are potentially involved in the expression variations of RUNX2 and BMP2 within the maxilla and mandible; (3) Genotyping analysis of DNA samples from 129 patients to evaluate, if SNPs in *RUNX2* and *BMP2* are associated with craniofacial patterns.

Adult patients referred to orthognathic surgery were included. Patients were invited to participate at universities and a private office (Curitiba, PR, Brazil). The oral and maxillofacial surgeon invited the patients to participate. Participants' systemic condition was classified according to the American Society of Anaesthesiologists (ASA) Physical Status Classification System. Syndromic patients and patients with ASA III or higher were excluded.

Cephalometric analysis

All patients presented a pretreatment digital lateral cephalometric radiograph, which was analyzed by a single expert surgeon. Radiographs were taken in the centric jaw relationship. Dolphin 2D® software (Dolphin Imaging software, Microsoft) was traced for the following angular measurements:

- Facial axis (Ricketts') (NBa-PtGn°): angle between Nasion-Basion (NaBa) and Pterygomaxillary fissure-Gnathion (PtGn) lines
- ANB° (Steiner) (ANB°): angle formed between A point-Nasion (AN) and Nasion-B point (NB) lines.

Patients were classified according to the ANB° as Class I (0°–4°), Class II (>4°) and Class III (<0°), and according to NBa-PtGn° as mesofacial (87°–93°), dolichofacial (<87°), or brachyfacial (>93°).

Sample collection for relative gene expression analysis of runt-related transcription factor 2 and bone morphogenetic protein 2

Bone samples (surgical waste) were collected from maxilla and/or mandible, depending on the surgical plan (i.e., mono-or-bimaxillary surgery). Maxillary samples were collected from any region in the area of the Le Fort I osteotomy (as long as they were interfering with maxillary adaptation) after the downfracture. In the mandible, samples were collected from the bilateral sagittal split osteotomy region. The samples were stored in tubes containing RNAlater solution and frozen immediately after surgery.

Relative gene expression analysis are described in the Supplementary Material and was previously reported by Olsson *et al*.[14]

Briefly, the target genes *RUNX2* (Hs00231692_m1) and *BMP2* (Hs00154192_m1) and the reference genes *ACTB* (Hs01060665_g1) and *GAPDH* (Hs02758991_g1) were used. The 2-Δ Cycle Threshold method was used to determine the relative levels of mRNA expression.

Sample collection for genotyping analysis

Saliva samples were collected for DNA extraction following a previously described method.[15] The SNPs described in Table 1 were blindly genotyped (described in the Supplementary Material) as previously reported.[16] An internal consistency of 100% was obtained by randomly repeating 10% of all samples.

Statistical analysis

Statistical analysis was performed using the Prism GraphPad8.2 package (GraphPad Software, Inc., San Diego, CA-USA). Data normality was assessed with the Kolmogorov − Smirnov and Shapiro − Wilk tests.

One-way analysis of variance or *t*-test was used to compare the mean and standard deviation (SD) relative gene expression. The Chi-square test was used to compare the SNPs according to the phenotypes. A correlation of gene expressions was tested using the Spearman's correlation test. An established alpha of 0.05 was adopted.

Results

A total of 129 individuals were included: 86 (66.7%) females and 43 (33.3%) males. Thirty-two (24.8%) were Class I, 24 (18.6%) were Class II, and 73 (56.6%) were Class III. Regarding NBa-PtGn°, 70 (54.3%) were brachyfacial, 37 (28.7%) mesofacial, and 22 (17.1%) dolichofacial.

Bone samples from 14 mandibles and 17 maxillas from 21 individuals (8 males and 13 females) were used in this analysis. Regarding skeletal malocclusion, 7 participants were Class I, 4 were Class II, and 10 were Class III. Eight participants were mesofacial, 7 dolichofacial, and 6 brachyfacial.

Figure 1 shows the RUNX2 and BMP2 expression in the mandible and maxilla ($P = 0.783$).

Figure 2 demonstrated the relative RUNX2 and BMP2 expression according to the skeletal malocclusions. A statistically significant difference was observed for BMP2 expression in mandibular bone from Class I and III participants ($P = 0.042$).

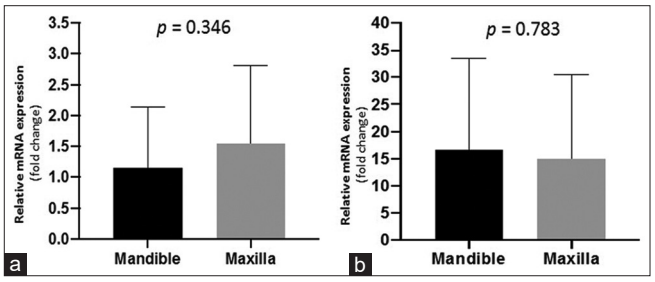

Figure 1: Relative mRNA expression according to each dental arch (mandible and maxilla). (a) Mean and standard deviation of runt-related transcription factor 2. (b) Mean and standard deviation of bone morphogenetic protein 2

Table 1: Characteristics of the single nucleotide polymorphisms studied

Gene	Chromosome	Reference sequence	Position/function	Base change (context sequence)	Global MAF
RUNX2	6	rs59983488	Intron variant	GGG[G/T] AGT	0.1787
		rs1200425	Intron variant	TTT[G/A] GAA	0.4297
BMP2	20	rs235768	Missense R (Arg)>S (Ser)	CAG[A/T] CTT	0.2332
		rs1005464	Intron variant	GCC[A/G] GCC	0.2232

Table 2: Mean mRNA levels in the mandible and maxilla according to single nucleotide polymorphisms genotypes

Arch	SNPs	Genotypes and mean (SD) relative mRNA expression			*P*§
Mandible	*RUNX2*				
	rs59983488	GG	GT	TT	
		1.58 (1.02)	0.31 (0.27)	-	0.036*
	rs1200425	AA	AG	GG	
		1.30 (1.52)	0.95 (0.63)	1.28 (0.71)	0.874
	BMP2				
	rs235768	AA	AT	TT	
		10.7 (4.50)	12.71 (15.98)	28.21 (19.75)	0.230
	rs1005464	AA	AG	GG	
		3.73 (-)	21.36 (23.81)	17.60 (15.03)	0.686
Maxilla	*RUNX2*				
	rs59983488	GG	GT	TT	
		1.74 (1.50)	1.23 (0.61)	-	-
	rs1200425	AA	AG	GG	
		0.96 (0.56)	1.38 (0.81)	3.17 (2.15)	0.038*
	BMP2				
	rs235768	AA	AT	TT	
		13.98 (17.94)	19.68 (16.84)	13.34 (17.07)	0.789
	rs1005464	AA	AG	GG	
		40.96 (-)	15.90 (15.83)	13.37 (14.86)	0.258

§ANOVA or *t*-test were used, *Means statistically significant difference. SNPs: Single nucleotide polymorphisms

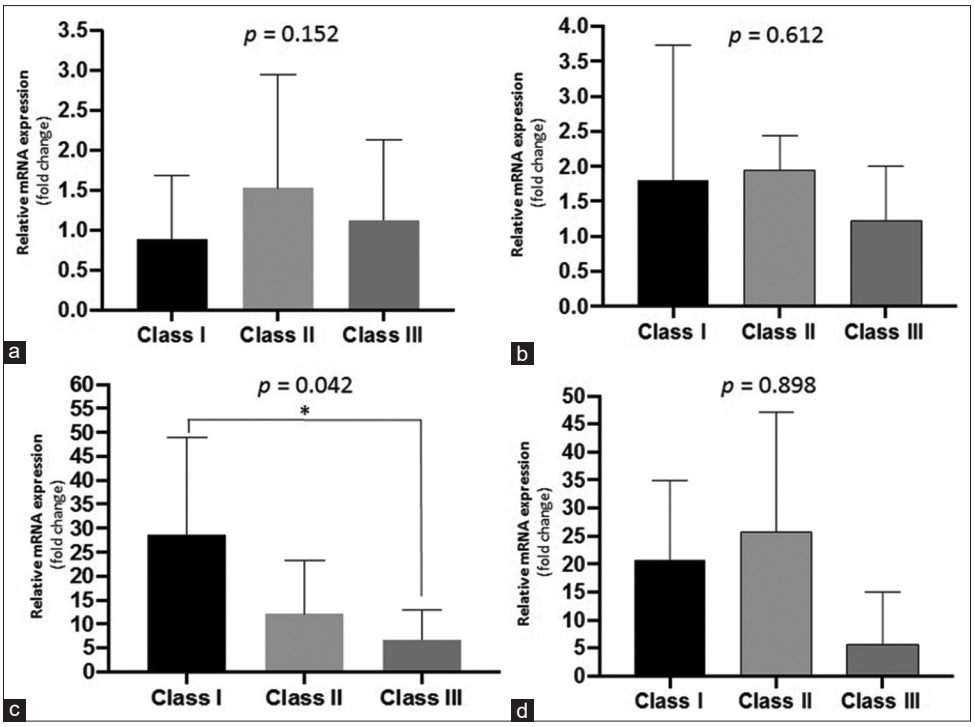

Figure 2: Relative mRNA expression according to the skeletal malocclusions. (a) Runt-related transcription factor 2 expression in the mandible. (b) Runt-related transcription factor 2 expression in the maxilla. (c) Bone morphogenetic protein 2 expression in the mandible. (d) Bone morphogenetic protein 2 expression in the maxilla

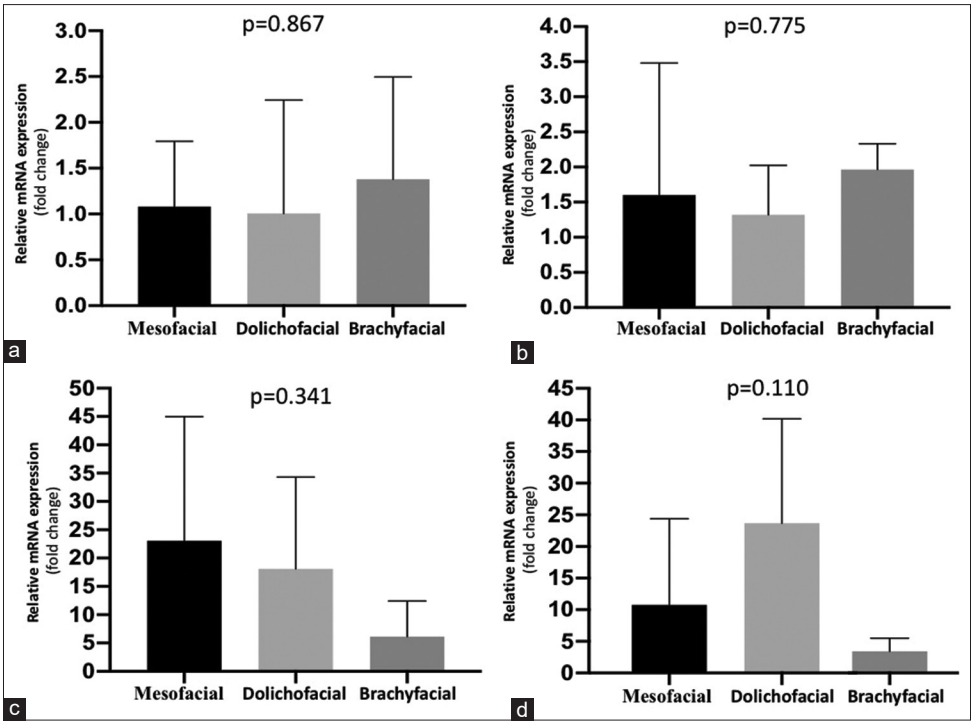

Figure 3: Relative mRNA expression according to the facial growth patterns. (a) Runt-related transcription factor 2 expression in the mandible. (b) Runt-related transcription factor 2 expression in the maxilla. (c) Bone morphogenetic protein 2 expression in the mandible. (d) Bone morphogenetic protein 2 expression in the maxilla

Figure 3 demonstrated the relative RUNX2 and BMP2 expression according to the facial growth patterns ($P > 0.05$). Mean and SD for each comparison are presented in Supplementary Table 1.

There was no correlation between *RUNX2* and *BMP2* expression neither in the mandible ($r = 0.227$; $P = 0.379$) nor in the maxilla ($r = 0.389$; $P = 0.574$).

In *RUNX2*, the genotype distribution in rs59983488 was: GG = 14, GT = 6 and TT = 0; while in rs1200425 was: AA = 6, AG = 10, and GG = 4. In *BMP2*, in the rs235768 was: AA = 5, AT = 8 and TT = 6, while in rs1005464 was: AA = 1, AG = 6 and GG = 13.

Table 2 demonstrates the *RUNX2* and *BMP2* expression according to the genotypes. A statistical significance was observed in rs59983488 ($P = 0.036$) and in rs1200425 (GG vs. GA, $P = 0.038$).

Due to associations and borderline associations observed in the first steps of this study, a genotyping analysis was performed in the total sample of 129 individuals. Table 3 shows genotype frequencies according to the phenotypes. No association was observed ($P > 0.05$).

Discussion

In the past years, there was an increase in SNP-phenotype[5-7] and genome-wide association studies (GWAS),[16,17] in which many genes/SNPs were linked to some craniofacial phenotypes, including *RUNX2*[16-18] and *BMP2*.[19-21] However, the understanding of the functional impact of SNPs on gene expression within craniofacial tissue is still largely unexplored. Here, we provide novel findings of the impact of *RUNX2* and *BMP2* SNPs on their maxillary and mandibular gene expression in different craniofacial patterns.

In our study, BMP2 was differentially expressed in the mandible according to the skeletal malocclusion. Mandibular bone samples from skeletal Class III patients showed a lower BMP2 expression, when compared to skeletal Class I. Interestingly, SNPs in *BMP2* are associated with mandibular retrognathism.[13] BMP2 is an essential regulator of osteogenesis, which directly regulates target gene expression[22] crucial for proper development. In osteoblasts, the target genes of BMP2 encode many transcription factors, including RUNX2,[23] known as highly important for the determination of craniofacial pattern.

RUNX2 function in osteoblast differentiation is affected by many regulatory genes.[24] RUNX2 also interacts with several coregulatory transcription factors, forming complexes that regulate the transcription of many bone-related factors in osteoblasts.[24] Mutations in *RUNX2* and expression levels of RUNX2 may be involved in the development of several craniofacial defects.[25] SNPs in *RUNXs* are involved in different craniofacial patterns.[13] Although our results of RUNX2 expression in the bone were not statistically different across skeletal patterns, the gene expression distribution conforms to previous evidence in the literature suggesting that RUNX2 is worth further investigation. Therefore, we performed a functional analysis in our study and found that the GG genotype of rs59983488 was associated with a higher expression of RUNX2 in the mandible.

Table 3: Genotype frequencies according to the respective craniofacial pattern

Phenotype	Genotypes, *n* (%)			*P*
	***RUNX2* rs59983488**			
	GG	**GT**	**TT**	
Skeletal malocclusion				
Class I	21 (72.4)	8 (27.6)	0	Reference
Class II	12 (57.1)	7 (33.4)	2 (9.5)	0.189
Class III	41 (63.1)	24 (36.9)	0	-
Growth pattern				
Mesofacial	16 (55.2)	13 (44.8)	0	Reference
Dolichofacial	14 (66.7)	5 (23.8)	2 (9.5)	0.104
Brachyfacial	44 (67.8)	21 (33.2)	0	0.243
Phenotype	**Genotypes, *n* (%)**			***P***
	***RUNX2* rs1200425**			
	AA	**AG**	**GG**	
Skeletal malocclusion				
Class I	8 (25.8)	14 (45.2)	9 (29.0)	Reference
Class II	6 (28.6)	8 (38.1)	7 (33.3)	0.878
Class III	13 (18.5)	34 (48.6)	23 (32.9)	0.707
Growth pattern				
Mesofacial	7 (20.6)	14 (41.2)	13 (38.2)	Reference
Dolichofacial	8 (38.1)	8 (38.1)	5 (23.8)	0.314
Brachyfacial	12 (17.9)	34 (50.7)	21 (31.1)	0.657
Phenotype	**Genotypes, *n* (%)**			***P***
	***BMP2* rs235768**			
	AA	**AT**	**TT**	
Skeletal malocclusion				
Class I	4 (12.9)	14 (45.2)	13 (41.9)	Reference
Class II	2 (9.5)	8 (38.1)	11 (52.4)	0.752
Class III	8 (11.9)	30 (44.8)	29 (43.3)	0.987
Growth pattern				
Mesofacial	4 (12.1)	13 (39.4)	16 (48.5)	Reference
Dolichofacial	1 (4.8)	8 (38.1)	12 (57.1)	0.476
Brachyfacial	9 (13.8)	31 (47.7)	25 (38.5)	0.542
Phenotype	**Genotypes, *n* (%)**			***P***
	***BMP2* rs1005464**			
	AA	**AG**	**GG**	
Skeletal malocclusion				
Class I	2 (6.4)	11 (35.5)	18 (58.1)	Reference
Class II	3 (14.3)	5 (23.8)	13 (61.9)	0.500
Class III	3 (4.2)	23 (32.9)	44 (62.9)	0.848
Growth pattern				
Mesofacial	2 (6.1)	11 (33.3)	20 (60.6)	Reference
Dolichofacial	4 (19.0)	4 (19.0)	13 (61.9)	0.235
Brachyfacial	2 (2.9)	24 (35.3)	42 (61.8)	0.874

χ^2 was used

It is important to highlight that although our study did not demonstrate an association between the SNPs in *BMP2* and *RUNX2* and the craniofacial pattern, it does not mean that they are not involved in craniofacial phenotypes. Genetic variants in *RUNX2* produce a deficient *RUNX2* protein

leading to cleidocranial dysostosis,[26] which affects dental arches.[27] Furthermore, disruptions in BMP signaling cause Treacher-Collins syndrome, which clinically presents remarkable craniofacial alterations.[2] Adhikari *et al*., (2016) performed a GWAS for facial features (using photographs) in Latin Americans and observed that SNPs in *RUNX2* affected nose morphology. Recently, Küchler *et al*. (2021) performed a SNP-phenotype association study in Brazilians, also using cephalometrics to determine the phenotypes, and observed that SNPs in *RUNX2* and *BMP2* were involved in a variety of craniofacial phenotypes, including skeletal Class II, mandibular retrognathism, mandibular protrusion, and dolichofacial phenotype.[13] It is possible that our sample led to a type II error in the genotype analysis, as some phenotypic groups presented a small sample size.

In the general population, skeletal Class I is more prevalent than Class II, followed by Class III.[28,29] Our sample was mainly composed by Class III individuals due to the fact that only patients seeking orthognathic surgery were included.[30] This might also explain the difference between the results observed here and in a previous study[13] evaluating orthodontic patients, in which SNPs in *RUNX2* and *BMP2* were associated with different craniofacial patterns.

Conclusion

BMP2 is expressed differently in the mandible of Class I and Class III participants. SNPs in *RUNX2* and *BMP2* are involved with their relative gene expression in the mandible and maxilla, respectively.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflicts of interest

There are no conflicts of interest.

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Supplementary Material

Supplementary Table 1: Mean and standard deviation for each analysis

Gene	Arch	Mean (SD)	*P*
	Gene expression in maxilla and mandible [data from Figure 1]		
Runx2	Mandible	1.14 (0.99)	0.346
	Maxilla	1.55 (1.26)	
BMP2	Mandible	16.57 (16.93)	0.783
	Maxilla	14.95 (15.58)	
Gene	**Arch**	**Mean (SD)**	***P***
	Gene expression in the maxilla and mandible according to skeletal malocclusion [data from Figure 2]		
RUNX2	Mandible		
	Class I	0.89 (0.79)	>0.05
	Class II	1.52 (1.42)	
	Class III	1.12 (1.00)	
	Maxilla		
	Class I	1.79 (1.94)	>0.05
	Class II	1.95 (0.49)	
	Class III	1.21 (0.78)	
BMP2	Mandible		
	Class I	28.59 (20.37)	>0.05
	Class II	12.09 (11.22)	
	Class III	6.79 (6.12)	
	Maxilla		
	Class I	20.57 (14.31)**	0.042
	Class II	25.63 (21.55)	
	Class III	5.56 (9.48)*	
Gene	**Arch**	**Mean (SD)**	***P***
	Gene expression in the maxilla and mandible according to the facial pattern [data from Figure 3]		
RUNX2	Mandible		
	Mesofacial	1.08 (0.71)	>0.05
	Dolichofacial	1.01 (1.24)	
	Brachyfacial	1.38 (1.12)	
	Maxilla		
	Mesofacial	1.60 (1.88)	>0.05
	Dolichofacial	1.32 (0.71)	
	Brachyfacial	1.97 (0.36)	
BMP2	Mandible		
	Mesofacial	23.06 (21.93)	>0.05
	Dolichofacial	18.10 (16.23)	
	Brachyfacial	6.16 (6.26)	
	Maxilla		
	Mesofacial	10.78 (13.61)	>0.05
	Dolichofacial	23.68 (16.49)	
	Brachyfacial	3.42 (2.07)	

SD: Standard deviation; * Means statistically significant difference

PCR Method

Relative gene expression analysis of runt-related transcription factor 2 and bone morphogenetic protein 2

For relative gene expression analysis, mirVana miRNA isolation kit (Ambion/Life Technologies™) was used to perform RNA isolation. The spectrophotometer NanoDrop™One (Thermo Fisher Scientific, Massachusetts, USA) was used to assess purity and RNA concentration. The High-Capacity® cDNA Reverse Transcription Kit (Applied Bio-systems, Foster City, CA, USA) was used. For the reaction, 400 ng of RNA, 0.8 μL of dNTP Mix ×25, 2.0μL of RT Buffer ×10, 2 μL of ×10 RT Random Primers and 1.0μL of MultiScribe® enzyme (50 U/μL) were used in a final volume of 20μL.

Both the gene expression and the reverse transcription reaction were run in a StepOnePlus Real-Time Polymerase Chain Reaction System (Applied Biosystems). The target genes *runt-related transcription factor 2* (Hs00231692_m1) and *bone morphogenetic protein 2* (Hs00154192_m1) TaqMan™ Gene Expression Assay primers and probes were used. As reference genes we used *ACTB* (Hs01060665_g1) and *GAPDH* (Hs02758991_g1). Amplification was performed under the following conditions: 95°C for 2 min, followed by 40 cycles of 95°C for 1s and 60°C for 20s. The 2-Δ Cycle Threshold method was used to determine the relative levels of mRNA expression.

Genotyping analysis

The spectrophotometer NanoDrop™One (Thermo Fisher Scientific, Massachusetts, USA) was used to assess purity and DNA concentration. The SNPs were blindly investigated using TaqMan™ technology (Applied Bio-systems, Foster City, CA, USA) in the same real-time PCR system described above, following the reaction: 4 ng DNA/reaction, 1.5μL Taqman PCR master mix and 0.075μL SNP assay (Applied Biosystems, Foster City, CA, USA) in a total volume of 3 μL. The thermal cycle was 10 min hold-cycle at 95°C and 40 amplification cycles of 15 s at 92°C and 1 min at 60°C. An internal consistency of 100% was obtained by randomly repeating 10% of all samples.