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ENPP1 and ESR1 Genotypes Associate with Subclassifications of Craniofacial Asymmetry and Severity of TMD

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Abstract

Introduction—We investigated if *ACTN3*, *ENPP1*, *ESR1*, *PITX1* and *PITX2* genes which contribute to sagittal and vertical malocclusion also contribute to facial asymmetries and TMD before and after orthodontic and orthognathic surgery treatment.

Methods—One hundred seventy four dentofacial deformity patients were diagnosed as symmetric or subdivided into four asymmetric groups according to PA cephalometric measurements. TMD exam diagnosis and Jaw Pain and Function-(JPF) questionnaires assessed presence and severity of TMD.

Results—Fifty two % of patients were symmetric and forty eight % asymmetric. The asymmetry classification demonstrated significant cephalometric differences between symmetric and asymmetric groups, and across the four asymmetric subtypes: Group 1 - mandibular body asymmetry, Group 2 - ramus asymmetry, Group 3 - atypical asymmetry and Group 4 - “C-shaped” asymmetry. *ENPP1* SNP-rs6569759 associated with asymmetry Group 1 ($p=0.004$), and rs858339 with asymmetry Group 3 ($p=0.002$). *ESR1* SNP-rs164321 associated with asymmetry Group 4 ($p=0.019$). These results are confirmed by Principal Component Analysis (PCA) that showed three principal components explaining almost 80% of the variation seen in the studied group. PC1 and PC2 were associated with *ESR1* SNP-rs3020318 ($p<0.05$). Diagnoses of disc displacement with reduction, masticatory muscle myalgia and arthralgia were highly prevalent in the asymmetry groups and all had strong statistical association to *ENPP1* rs858339. The average JPF scores for asymmetric subjects before surgery (JPF=7), were significantly higher than symmetric subjects (JPF=2). Patients with asymmetry Group 3 reported the highest preoperative JPF scores and Group 2 and 3 were most likely to be cured of TMD one year after treatment.

Conclusions—PA cephalometrics can classify asymmetry into distinct groups; identify probability of TMD and genotype associations. Orthodontic and orthognathic treatment of facial asymmetry is very effective at eliminating TMD in most patients.

INTRODUCTION

Genesis creating symmetry and breaking of symmetry are normal developmental biologic processes. In embryogenesis, symmetry is maintained until late gastrulation and early neurulation. The Nodal Pathway regulates signal transduction to maintain symmetric dorsal-ventral patterning of mesoderm and developing nervous system.¹ The Lefty proteins, a subclass of Transforming Growth Factor- β (TGF β) transcription proteins, act as extracellular antagonists to Nodal. During gastrulation, Lefty disrupts symmetry in germ layers to pattern normal situs positioning of the heart, lungs and gut coiling.² In the harmonious development of brachial arches immediately rostral, maintenance of symmetry is particularly important for natural midline fusion between right and left sides. It is well known that many genetic and environmental factors, acting separately or in unison, may produce facial clefting or developmental jaw defects in arch development.³ Due to the potential for congenital defects, different and more subtle influences on asymmetry might be active in normal facial development. *PITX2*, an upstream effector of Nodal and Lefty signaling pathways, has recently been identified as differentially expressed between left and right side masticatory muscles in adult humans with facial asymmetry.⁴ *PITX2* is necessary

for mesoderm-derived first branchial arch structures, including masticatory muscles, jaw bones and ectoderm induction for tooth formation.^{5–6} Therefore, it is likely that early developmental events persist into maturation, making asymmetry an etiologic and diagnostic challenge for clinical orthodontics.⁷

Craniofacial asymmetry may be considered as a type of dentofacial deformity, which arises during facial maturation at the same time sagittal and vertical jaw discrepancies become clinically evident. The association of skeletal asymmetry with Class II or Class III skeletal malocclusion varies with the type of human population being treated, ranging from 8-50%, indicating genetic influences from gastrulation to maturation play an important role in etiology.^{8–9} Skeletal malocclusions are complex trait conditions, developing from an interplay of genetic and functional (environmental) influences¹⁰; and new findings point toward a similar etiology for facial asymmetry.¹¹ In order to identify genetic contributions to skeletal malocclusion, a relatively precise description of phenotype and phenotypic variations is necessary. In orthodontics, there have been many approaches for classification of skeletal asymmetry utilizing either posterior anterior cephalograms or submentovortex radiographs; however, there is not a universally accepted method or classification system. Most recently, cone beam computed tomographic imaging has been utilized to more accurately describe and subclassify types of craniofacial asymmetry in dentofacial deformity populations.^{12–13} The classification system of Baek has identified four types of asymmetry with computed tomography, which arise from different growth imbalances of the jaws, teeth, nasal septum and cranial base.¹⁴ These new classification systems help remove much of the previous diagnostic uncertainty, since asymmetry can be masked by variations in head posture, canting of the occlusal plane or other dental and soft tissue compensations.^{14–15} This etiologic – based phenotypic classification may be useful in identifying gene single nucleotide polymorphisms (SNPs) associated with asymmetry.

We have been investigating how genetic variations associate with the development of skeletal malocclusions in the patient population undergoing orthognathic surgery at the University of XXXXX. This is a typical population of French patients, relatively young with a normal distribution of open, deep, Class II and Class III skeletal malocclusions. In our population and in other studies of dentofacial deformity patients, pain in the temporomandibular joint or masticatory muscles is a common and often debilitating comorbid condition¹⁶ estimated to occur in over 50% of patients with facial asymmetry.¹⁷ The Baek classification might also be useful in identifying an association between the subclasses of asymmetry and prevalence of TMD.

In previous studies, we identified significant associations between SNPs in *ACTN3* (α -actinin 3) and *ESR1* (estrogen receptor α) with Class II malocclusion and *ENPP1* (ectonucleotide pyrophosphatase/phosphodiesterase 1) with mandibular prognathism.^{10,18} We also found differences in gene expression for *PITX1* and *PITX2* between left and right masseter muscle in asymmetry.⁴ In this study, we explore associations between these genes and subclassifications of asymmetry. We also sought to determine if the subclassifications are related to the prevalence or severity of TMD before and after orthodontic treatment with jaw osteotomy.

METHODS

Patient Population

174 patients undergoing comprehensive orthodontic treatment with mandibular or mandibular and maxillary osteotomies for correction of jaw deformation malocclusion were recruited for study participation during their pre-surgical consultation at the University of XXXXX Department of Oral and Maxillofacial Surgery. At that time, the patients signed consent for participation according to human subject research protocols approved by the Committee for Personal Protection, University of xxxxx and the Institutional Review Board Committees at the University of XXXXXXXXXX and XXXXXX University. Patients provided a saliva sample collected in Oragene Kits for genotyping. Deidentified demographic information, panorex, lateral and posterior anterior cephalograms, and clinical examination information were compiled for analysis. The patient population had a mean age of 25.7 years, majority female (76%), and a normal mixture of sagittal (66% Class II, 33% Class III) and vertical jaw deformations (75% open bite, 25% deep bite). Sagittal and vertical malocclusion classifications were based on the Delaire Cephalometric Analysis of lateral cephalograms, which is particularly useful in planning the type of osteotomy repositioning necessary to correct malocclusion.¹⁹

Assessment of Asymmetry

Skeletal asymmetry was classified into four groups based on a new system adapted from Baek et. al 2012: Group 1 – lateralization of mandibular body only (“mandibular body asymmetry”); Group 2 – difference in ramus heights with menton deviation to the shorter ramus side (“ramus asymmetry”); Group 3 – difference in ramus heights with menton deviation to the longer ramus side, gonion contour more prominent on larger mandibular side and reverse maxillary canting (“atypical asymmetry”); Group 4 – difference in ramus heights with menton deviation to short ramus side and severe maxillary canting (“C-shaped asymmetry”) (Figure 1). Since this classification system was derived from CBCT images of orthognathic patients, we developed a PA (posterior anterior) cephalometric analysis, which allowed us to perform comparable measurements using digital two-dimensional images with Dolphin morphometric software.

Six cephalometric measurements were used: occlusal plane tilt, maxillary canting (JR or JL to ZR or ZL), menton deviation (A to Me to MSR), mandibular width to midsagittal plane (AG or GA to MSR), mandibular width to menton (AG or GA to Me), and ramal height (Table 1 and 2). In the maxilla, occlusal plane tilt was determined by the difference between Frankfort Horizontal and the horizontal line bisecting the buccal cusp tips of UR6 and LR6, as well as the buccal cusp tips of UL6 and LL6, measured in degrees (°). If the occlusal plane tilt was greater than 2°, the subject was considered to have maxillary canting. To further verify the maxillary canting was skeletal in etiology, left and right vertical distances from jugal process and frontozygomatic suture were compared. If the difference between the left and the right side was greater than 3 mm, the subject was considered to have maxillary canting. In the mandible, menton deviation was determined by the angle between midsagittal plane and the line connecting ANS and menton. If the angle was greater than 2°, the subject was considered to have mandibular facial asymmetry with menton deviation. To compare the

left and right mandibular width, the distance between antegonial notch and midsagittal plane was compared to the contralateral side. If the difference was greater than 2 mm, it was considered to have mandibular deviation. We also measured the distance between antegonial notch and menton, and compared it with its' contralateral side. Again, if the difference was greater than 2mm, it was considered to have mandibular body asymmetry. Lastly, ramal height of left and right side was compared. If the difference between left and right side was greater than 3 mm, ramus asymmetry was diagnosed.

Subjects were diagnosed as symmetric if there was no maxillary canting ($<2^\circ$), no menton deviation ($<2^\circ$), and no significant difference in ramal height (<3 mm). Asymmetric subjects were further divided into four groups. Group 1 subjects had menton deviation greater than 2° without any maxillary canting or any significant ramal height difference. Group 2 subjects had menton deviation greater than 2° with shorter ramal height on the deviated side. Group 3 subjects displayed "atypical symmetry", with shorter ramal height on the opposite of the deviated side, with slight maxillary canting towards or opposite of the deviated side. Group 4 subjects showed both shorter ramal height and maxillary canting towards the deviated side, displaying "C-shaped asymmetry" as described by Baek et al.¹⁴ Furthermore, principal components analysis (PCA) explaining more than 5% of the facial skeletal variation were selected for genotype-phenotype correlation analysis. Data were normalized and standardized using a linear model to assess the possible effects of age and sex and to consider the possibility of age-by-sex interactions.

Genotype Assessment

Saliva samples, one per patient, were collected in Oragene kits and prepared for DNA extraction and posterior genotyping using TaqMan chemistry and end-point analysis in an automatic sequence-detection instrument (ABI Prism 7900HT, Applied Biosystems, Foster City, CA), as described previously.¹⁰ Thirteen SNPs were selected for genotyping: in *ACTN3* rs1815739, and rs678397¹⁰; in *ENPP1* rs937300, rs6569759, rs858339 and rs1409181^{18,20–22}; in *ESR1* rs1643821, rs302318, rs3020377 and rs2077647^{18,23–26}; in *PITX1* rs1131611 and in *PITX2* rs2595110⁴ to determine if specific allelic variants are over-represented in subjects with malocclusion sub-classifications. Thirty-three additional anonym SNPs were genotyped.

Assessment of TMD

TMD was assessed using the routine clinical examination done by the Maxillofacial Surgeons before surgical treatment and entered into the Research Diagnostic Criteria for Temporomandibular Disorders (DC/TMD).²⁷ In addition, the Jaw Pain and Function (JPF) questionnaire was used to determine the presence and severity of TMD, as a subjective patient report. The JPF Questionnaire was developed as a screening tool to determine presence or absence of TMD conditions.²⁸ It consists of 8 questions relating to jaw pain and 5 questions related to jaw function. The questionnaire has been validated to reliably distinguish between normal and TMD subjects with up to 98% sensitivity and 100% specificity when a cut off score of 6 is used for responses.²⁹ The questionnaire has been translated for use in Germanic,³⁰ and we prepared a French version for use in Lille, as a standard assessment of presence and severity of TMD.¹⁸

One hundred twenty one patients have attended a one year post-treatment reevaluation appointment, where they completed a second JPF survey. Patients were divided into five groups for comparative purposes based upon difference in pre-surgical and one year post-treatment JPF scores. Patients were classified in the following five groups: *No Change* in TMD, *Improvement* of TMD if the JPF score decreased by 3 or more; *Worsening* of TMD if the JPF score increased by 3 or more; *Cured* of TMD if the JPF score was > 6 before surgery and < 6 after treatment; and *Iatrogenic* TMD if the JPF score was < 6 before surgery and > 6 after treatment. We used the terms “cured” or “iatrogenic” since a score > 6 is diagnostic for presence or absence of TMD with the JPF assessment.

Statistical Analysis—For cephalometric assessment of asymmetry, an unpaired t test was used to compare if differences between sides for individual cephalometric measurements were significantly different between symmetric and asymmetric patients. An ANOVA test was used between the four subclassifications of asymmetry to determine anatomical differences between groups. Tests for measurement error included intrarater reliability in cephalometric measurements (by repeating cephalometric tracing on 10% of the radiographs by 1 examiner [K.C.]; this resulted in an R^2 value of 0.98.

For genotype assessment, the characteristics of the population were presented with the usual rules of descriptive statistics: frequencies and percentages for categorical variables; mean and standard deviation for quantitative variables. The association between the change in JPF scores, TMD diagnosis and clinical or surgical characteristics of the population was assessed by Chi-square test for categorical variables or Fisher exact test in the case of small numbers. Quantitative variables were analyzed using Student’s t-test. When the distribution of the variable did not follow a normal distribution, a nonparametric Wilcoxon test was performed. After ensuring compliance with the Hardy-Weinberg equilibrium, the association of different SNPs was also sought by a Chi-square test or Fisher exact test when small numbers were present. For each SNP, the analysis was performed by considering the three genotypes separately, as well as by calculating the total number of alleles.

For PCA, SNPs were coded 0, 1, and 2 according to the number of minor allele copies. Multivariate linear regressions adjusting for age, sex, and ethnicity were performed to test for associations between each SNP (one at a time) and the selected principal components. The same Bonferroni threshold described above was used here. All analyses were performed with SPSS software for Windows (version 20.0; IBM, Armonk, NY).

For TMD assessment, an unpaired t-test was used to determine if there were significant differences in pre-surgical JPF scores between symmetric and asymmetric patients, and an ANOVA test was performed between the four asymmetric groups. An ANOVA was also used to compare if there were significant changes in the JPF score 1 year after treatment between the symmetric group and the four asymmetric groups. To further evaluate, a post-hoc t-test was done to compare individual JPF scores of asymmetric groups.

RESULTS

The patient population represented a normal demographic distribution of subjects seeking orthodontic and orthognathic surgery treatment of dentofacial deformity malocclusion from the geographic area of Northern XXXXXX and Southern XXXXXX. All patients treated for the condition were referred to the Oral and Maxillofacial Surgery Department at the University of xxxxx under the National Health Care Service of France. 52% of patients were diagnosed as symmetric and 48% were diagnosed as asymmetric. Segregating the asymmetric subjects into the four proposed subtypes was relatively easy to accomplish utilizing 11 cephalometric anatomic landmarks and 6 cephalometric measurements (Table 1 and Table 2, Figure 1). This classification system was validated by the very significant differences obtained by comparing the cephalometric measurements between symmetric and asymmetric groups, and asymmetric subtypes (Table 3). All six PA cephalometric measurements were compared between symmetric and asymmetric patients, and across the four asymmetry subclassification groups. For bilateral points, values are the difference between the left and the right side. The PA cephalometric analysis results demonstrated a significant difference in all six cephalometric measurements between symmetric and asymmetric patients. All mandibular measurements, such as menton deviation, mandibular width (GA or AG to MSR and GA or AG to Menton), and ramal height showed notably significant differences between symmetric and asymmetric patients, clearly indicating the presence of mandibular asymmetry.

Cephalometric measurements were also compared across different asymmetry groups (Figure 2). Generally, asymmetry group 4 showed a greater amount of canting and deviation when comparing cephalometric measurements. It had the most severe malformations with an occlusal plane tilt, maxillary canting and mandibular deviation that were proportionally much more imbalanced than in other asymmetry groups. Because group 4 had maxillary canting, the ANS to menton line used to measure the degree of chin deviation in the mandible was higher than in the other three groups $p = 0.003$ (Figure 2). The second characteristic evident with asymmetry was a worsening of ramal height differences from group 1 to group 4. Differences in PA Cephalometric measurements indicate the four subtypes may be considered as anatomically different forms of asymmetry that can be compared for differences in genotype variations within the study. We therefore compared differences in genotype for symmetric and asymmetric subjects and between asymmetric subtypes (Table 4).

Significant differences were detected for *ENPP1* and *ESR1* genotypes, but not for *ACTN3*, *PITX1* and *PITX2*. *ENPP1* SNP rs6569759 was significantly different for genotype and alleles for group 1 compared to other asymmetry subtypes. *ENPP1* SNP rs858339 was different for genotype between symmetric and asymmetric groups and for group 3 compared to other asymmetric groups. *ESR1* SNP rs1643821 was also significantly different for genotype and allele for asymmetry group 4 compared to the other asymmetric groups. Individuals with group 1 asymmetry were almost four times more likely to carry the G allele of *ENPP1* rs6569759 (OR=3.89; 95% confidence intervals 1.02–14.78). These results further support the appropriateness of the asymmetry subclassifications, as this phenotypic organization can be utilized to recognize meaningful genotypic differences.

Principal Component analysis (PCA) disclosed three principal components (PC1 to PC3) explaining more than 5% of the shape variation in the asymmetric subjects. The cumulative variation score of each component totaled 79.768% of the subject's variability (Table 5). (Supplemental table 1). PC1 comprised 39.721% of the sample variability and showed asymmetric individuals with low values to occlusal plane tilt angle and high values to maxillary canting. PC2 comprised 23.976% and disclosed low scores to occlusal plane tilt and mandibular width to midsagittal Plane (GA-MSR) and high scores to menton deviation (A-Me-MSR). PC3 showed 16.070% of shape variability and disclosed low scores to mandibular width to menton and high scores to ramal height. Regarding PCA and the subjects genotype, PC1 and PC2 were associated to SNP in *ESR1* (rs3020318) ($p=0.04$). (Supplemental table 2). To determine how variation in principle components related to PA cephalometric measurements we used the Varimax with Kaiser Normalization rotation method to plot components in rotated space. Variance in total cephalometric measurements for each symmetric or asymmetric patient was visualized by a component plot rotated in space (Figure 3). A second component plot visualized the variance in each individual cephalometric measurement for all patients (Figure 4).

Since TMD is often present in this patient population before initiation of treatment, we wanted to know if there was a higher prevalence within and between asymmetry subclassifications. TMD diagnosis was positive in only 3% of symmetric patients and very high in asymmetric (Table 5). Disc displacement with reduction was most common in asymmetric patients (78%), followed by 61% with myalgia of masticatory muscles, 33% with arthralgia and 12% with TMD-related headache. Disc displacement without reduction was the least common Axis I diagnosis at 6%. Overall the population is young, with an average age of 26 years and not presenting with fibromyalgia or pain related disability diagnosed in Axis II of the diagnostic criteria. Comparing genotypes to TMD diagnosis for disc displacement with reduction, myalgia and arthralgia, and not disc displacement without reduction or TMD-related headache since these groups have very few individuals; *ENPPI* rs858339 had significant associations for genotype and/or allele for all three TMD diagnosis (Table 6).

We utilized the JPF questionnaire as an efficient assessment tool for patient perception of presence and severity of TMD. Most symmetric subjects had little to no symptoms for TMD and an average JPF score of 1.97, which agreed with the clinical exam assessment for TMD (Table 7). Asymmetric subjects had an average JPF score of 6.87, which was significantly higher than in symmetric. Further, there were significant differences within asymmetric subtypes with Group 3 having the highest average score of 9.11 and Group 1 and 4 having the lowest average scores of about 4. Comparing JPF scores between groups, a t-test demonstrated very significant differences between symmetric and asymmetric patients $p<0.001$. Within the asymmetric patients an ANOVA comparison also revealed very significant differences between subclassifications. These results indicate there is more likely a chance for signs or symptoms of TMD when asymmetry is part of a patient's dentofacial deformity.

After treatment, we followed the subjects for one year to determine if TMD improved, worsened or if new conditions occurred. Overall, those without TMD remained so, with 52%

of the patients having no change in JPF score (Figure 5). Twenty percent of all patients were cured of TMD with post treatment JPF scores below 6 and 17% had an improvement with JPF scores decreased by 3 or more. Four percent of the patients had worsening of TMD after treatment with JPF scores increased by 3 or more. Finally, 7% could be diagnosed with occurrence of TMD with JPF scores < 6 before surgery and > 6 after treatment. All groups, including the symmetric group, had decrease in post-treatment JPF scores that were statistically significant by ANOVA comparison, $p < 0.001$. Further post hoc t-tests revealed significantly greater decreases in post-treatment JPF scores for asymmetry group 2 and 3 compared to the symmetric group, $p < 0.001$.

DISCUSSION

Craniofacial asymmetry, which arises from normal developmental processes, takes many shapes and forms, given the complexity of the nervous, skeletal, muscular and physiologic components that drive cognitive, sensory, stomatognathic and respiratory functions.³² A functional variation or genetic polymorphism influencing an individual or multiple components can lead to a localized skeletal asymmetry, or a generalized craniofacial asymmetry depending upon the cause and growth response over time.³³ Lateral skeletal distortions are known to occur in the skull,³⁴ cranial base,^{35–36} midfacial structures, including the vomer, pterygoid process, piriform apertures and maxilla,⁴ and in the mandible by hemimandibular elongation or hyperplasia.³⁷ These ubiquitous configurations of asymmetry have made simple classifications for patterns of craniofacial growth or etiology elusive. Yet a systematic classification system is needed to more accurately plan surgical corrections^{14,38} and serve as a phenotypic clustering for gene association studies.³⁹ Using either traditional tracings of PA cephalograms⁴⁰ or cluster analysis of 3-dimensional computed tomographic image analysis, a classification system with 4 groups or subtypes of craniofacial asymmetry have been formulated for Korean people.¹⁴ Our first objective was to determine if French people with asymmetry could also be subclassified using this diagnostic grouping. We were able to adapt the asymmetry classification system of Baek et al.¹⁴, which used 3-dimensional computed tomography, with a new cephalometric analysis of posterior anterior cephalograms for our patients. The diagnostic classification identified differences between groups due to the significant differences in cephalometric measurements. However, the French population differed from the Korean population in the percent distribution amongst the asymmetric groups. Koreans were most likely to have Group 1 or Group 2 asymmetry while the French were most likely to have Group 2 or 3. The French patients were almost equally matched with 52% of subjects undergoing orthognathic surgery being symmetric and 48% being asymmetric. To our knowledge, no similar estimates are available for Koreans, but in Singapore the prevalence of asymmetry ranges from 8 to 50% in orthognathic surgery patients.⁴⁰ These population differences most likely stem from three distinct influences. The first is the tendency for the percentage of asymmetry to differ in different combinations of Class II, III, open and deep bite malocclusions.⁴¹ The second is the almost certain likelihood that genetic differences in facial shape that mark race and ethnicity can also influence the distribution of asymmetry subclassifications.⁴ Finally, there were differences on the type of images being utilized in diagnosis, and some differences in the morphology of asymmetry sub-classification groups.

Posterior Anterior Cephalometric Measurement Errors

Although PA cephalometric analysis has been the traditional approach for diagnosis of asymmetry, variability of head positioning in the cephalostat may introduce radiographic projection errors, which diminish diagnostic reliability.⁴² Cone-beam computed tomography (CBCT) images are considered more reliable for diagnosis of asymmetry, but there is insufficient evidence to conclude that CBCT is superior to PA cephalograms for detecting transverse facial differences.⁴³ We routinely use PA cephalograms in surgical treatment planning of patients and attempt to minimize head rotation around the vertical Z-axis where the majority of projection error occurs⁴⁴. A second potential error is landmark identification due to unclear radiographic representation of anatomical locations. However, the four patterns of asymmetry are almost always discernible by visual observation, which helps decrease uncertainty as to where landmarks are located. There have been limited reports on landmark identification on PA cephalograms⁴⁵, but a recent report (Ulur et al., 2016⁴⁶) estimates rater reliability to be consistently high at 0.9 to 0.95, or above for most points. Our intrarater reliability had an R^2 value of 0.98, and is similar to Ulur. Therefore our methods may introduce some measurement error in diagnosis, but given the large differences in specific cephalometric measures between groups (Figure 2), was not a major influence on classification of subjects into asymmetry groups.

Genotype Associations

The second study objective was to determine if gene variations already identified as contributing to sagittal and vertical malocclusion (*ACTN3*, *ENPP1*, *ESR1*, *PITX1* and *PITX2*) might also associate with asymmetry (Table 4). We compared 12 SNPs to determine differences for genotype and allele between symmetric vs. asymmetric patients and between the asymmetric groups. Two SNPs were associated with differences in both genotype and alleles when compared between asymmetry groups. SNP rs6569759 in *ENPP1* was different in group 1 and SNP rs1643821 in *ESR1* was significantly different in group 4. The SNP rs858339 in *ENPP1* was very significantly different for genotype between symmetric vs. asymmetric subjects and between asymmetric groups for group 3.

ENPP1 has pleiotropic effects for mineralization and insulin signaling. The intronic SNP rs6569759 has previously been associated with an increased risk for type 2 diabetes, which results in insulin-mediated glucose metabolism, which affects fiber type composition of skeletal muscles.⁴⁷ The SNP has also been associated to changes in bigonial width dimension in Western Eurasians.⁴⁸ This corroborates our finding that rs6569759 is significantly different in group 1, which has asymmetry in mandibular body breadth, but not in ramus height. Rs858339 is an intronic SNP previously associated with variation in bone mineral density.⁴⁹ We have recently reported that rs858339 has a significant association with the occurrence of preoperative TMD in our patient population.¹⁸ *ENPP1* rs858339 TT genotype associated with pretreatment absence of TMD and the AT genotype as a pretreatment risk factor for presence of TMD as determined by JPF scores.¹⁸ In this study rs858339 has strong statistical association with the TMD diagnoses of disc displacement with reduction, masticatory muscle myalgia or arthralgia, further confirming its role in etiology (Table 5). The association of *ENPP1* to presence of TMD diagnoses or asymmetry group 3 is not yet informative as to the biological mechanisms responsible for occurrence.

The conditions of arthralgia and disc displacement may be related to *ENPP1* biomineralization functions and myalgia to insulin signaling in skeletal muscle, but more investigations will be necessary to confirm these possibilities.

ESR1 polymorphisms associate with skeletal Class II malocclusions and symptomatic osteoarthritis of the TMJ in Korean women.⁵⁰ The rs1643821 intron SNP contributes to the susceptibility for osteoporotic fracture in postmenopausal Chinese women.⁵¹ Since the incidence of TMD in asymmetry group 4 is relatively low, rs1643821 may be contributing to development of this type of asymmetry through variations in bone mineral density with growth, rather than by specific problems in the TMJ.

We used PCA as a secondary procedure for analysis of PA cephalometric measurements. Three components explained 80% of morphologic variation found from cephalometric analysis. Principal component (PC) one identified variability related to occlusal plane tilt and maxillary canting, component two to mandibular width and menton deviation and component three to ramal height (Supplemental table 1). A component plot rotated in space demonstrated a clustering of symmetric patients for overall variance in cephalometric measurements that was separated from asymmetric patients (Figure 3). Asymmetric patients also had greater variability in morphologic variation. A second component plot summarized how the variation in individual cephalometric measurements identified in Figure 2 related to each other morphologically. Left and right ramal height and left and right maxillary canting (JL-ZL with JR-ZR) clustered together, indicating that variation in these measures was similar between the left and right face. Two opposite measures also clustered together: AG-Menton with GA-MSR and GA-Menton with AG-MSR; indicating that menton deviation on one side of the face matched variance in mandibular body width on the opposite side. Variation in occlusal plane tilt was an independent morphologic entity which had limited relationship to other component variations. The relationship of ANS-Me-MSR was a second independent morphologic entity, which reflected the independent variation of nasal septal deviation relative to other facial structures.

Genotype analysis associated SNP rs3020318 in *ESR1* with principal components one and two. (Supplemental table 2). Although not directly comparable to the genotype associations for the four asymmetry groups, the PCA analysis provided a confirmatory finding. *ESR1* rs1643821 associates with asymmetry group 4 and rs3020318 with PC one and two. PC one and two relate to transverse canting and menton deviation respectively, and both of these morphologic features are found in group 4. rs3020318 is an intronic SNP listed as without any known functional effects. However, this marker belongs to a haplotype associated with greater cancer risk.⁵² There is growing evidence that the impact of genetic risk factors on breast cancer varies by hormone receptor status. Genetic variation of the estrogen metabolism pathway -particularly the genes involved in the production of estrogen through androgen conversion -influences the risk for the development of estrogen-sensitive breast cancer.⁵³ This could be also true for craniofacial deformities that include symptomatology in the temporomandibular joint, with variation in hormonal levels (*i.e.* estrogen) influences the risk for development of TMD.

While there is not a specific polymorphism associated with *PITX2* and facial asymmetry, differences in gene expression were previously identified in right and left masseter muscle samples from asymmetric patients⁴. The variation in *PITX2* gene expression likely has an association with facial asymmetry, and warrants further research. One possibility to be investigated is that *PITX2* may interact with *ENPP1* to produce differences in mineral density and bone growth between facial sides.

Asymmetry and pre- or post-operative TMD

There is broad consensus among orthodontists that little to no relationship exists between specific types of malocclusions and development of TMD.⁵⁴ Yet for many people with jaw deformations, pain in the TMJ or masticatory muscles is a common and debilitating comorbid condition estimated to occur in over 50% of patients with facial asymmetry.^{16–17} Our results affirm this finding. The JPF scores for asymmetric subjects were significantly higher than symmetric subjects ($p > 0.0001$). Further, asymmetry groups 2 and 3 were most likely to have pre-operative TMD present before orthodontic treatment was initiated. In order to determine if the musculoskeletal imbalances were the root cause of TMD, we followed these patients at least one year after orthodontic treatment to see if the condition resolved or persisted. Group 2 and 3 were most likely to be cured of TMD at one year follow-up or at least had significant symptom improvement (Figure 5). There is growing evidence, confirmed on different patient populations that dentofacial deformities have higher prevalence of TMD,⁵⁵ and when treated with combined orthodontic and surgical procedures, usually have better masticatory function and improved TMD symptoms, especially for the relief of pain^{56–57}.

These findings lead to the conclusion that orthodontic and orthognathic treatment of craniofacial asymmetry helps cure or alleviate TMD for the majority of patients. However, as with any clinical treatments there were a small percentage of patients who had significant worsening or presentation of TMD symptoms in the retention phase (7%). Most of these (80%) were subjects without asymmetry, who we would not have expected this to occur. Other post-treatment conditions, such as condylar remodeling or condylar resorption could be influencing this rare TMD occurrence and will be studied in the future. Do to the small number of subjects with this result, 5 total, many more patients will need to be followed for further characterization of post-treatment development of TMD. Overall, these treatments are highly effective at correcting skeletal malocclusions, producing a physiologic balance that alleviates TMD symptoms.

CONCLUSIONS

1. A new posterior anterior cephalometric analysis utilizing six measurements to detect differences in facial sides has been developed to distinguish four main classifications of asymmetry that are common in dentofacial deformity patients.
2. TMD prevalence is much higher in patients with asymmetry compared to dentofacial deformity patients without asymmetry. The most common TMD presentations were disc displacement with reduction, masticatory muscle

myalgia and arthralgia. Two of the four asymmetry groups had both high positive diagnosis for TMD and subjective patient reporting of symptom.

3. SNP genotype rs6569759 in *ENPP1* associated with asymmetry group one and rs858339 with asymmetry group three.
4. SNP genotype rs1643821 in *ESR1* associated with asymmetry group four. rs3020318 in *ESR1* associated with PC one and two, which relate to maxillary canting and menton deviation.
5. SNP genotype rs858339 in *ENPP1* associated with presence of disc displacement with reduction, masticatory muscle myalgia and arthralgia.
6. Orthodontic and orthognathic treatment of asymmetry elevates TMD symptoms for at least one year into retention in most of the patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights for review

1. A new asymmetry classification system utilizing PA cephalograms, producing 4 diagnostic groups.
2. Pre-treatment TMD is elevated in some of the asymmetric diagnostic groups.
3. *ENPP1* and *ESR1* genotypes and alleles associate with 3 of the 4 asymmetry groups.
4. Orthodontic and orthognathic treatment of asymmetry elevates TMD symptoms for at least one year into retention in most patients.
5. New insights into what types of asymmetry predispose to TMD; a basis for future studies.

Figure 1. Prototypes for four asymmetry subtypes and illustration of PA cephalometric tracing
 Group 1 - mandibular body asymmetry, Group 2 - ramus asymmetry, Group 3 - atypical asymmetry, and Group 4 - C-shaped asymmetry. Landmarks used for cephalometric analysis labeled on Group 4.

Figure 2. Histogram of cephalometric measurement comparisons by different asymmetry groups
Average values for each cephalometric measurement were compared across different asymmetry groups. *For bilateral points, the difference between left and right side was used and denoted as a positive value. Average values are expressed in mm for maxillary canting, GA or AG-MSR, GA or AG-Menton and ramal height, and degrees for occlusal plane tilt and menton deviation.

Figure 3.

A Component Plot in Rotated Space representing variation in all cephalometric measurements of individual patients, classified as either symmetric or asymmetric, and their interrelationships for Principal Components one to three.

Figure 4.

A Component Plot in Rotated Space representing variation in individual cephalometric measurements and their interrelationships for Principal Components one to three.

Figure 5. One year post-treatment changes in JPF score by asymmetry classification

A. Distribution of patients based on difference in pre-surgical and one year post-surgical JPF scores: 1) No Change in TMD, 2) Improvement of TMD if the JPF score decreased by 3 or more; 3) Worsening of TMD if the JPF score increased by 3 or more; 4) Cured of TMD if the JPF score was > 6 before surgery and < 6 after treatment; and 5) Iatrogenic TMD if the JPF score was < 6 before surgery and > 6 after treatment. **B. Distribution of symmetric and asymmetric groups in “No Change” category. C. Distribution of symmetric and asymmetric groups in “Cured” category. D. Distribution**

of symmetric and asymmetric groups in “Worsening” category. E. Distribution of symmetric and asymmetric groups in “Iatrogenic” category. F. Distribution of symmetric and asymmetric groups in “Improvement” category.

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Table 1

Landmarks used for PA cephalometric analysis

Landmarks	Definitions
ANS	Anterior nasal spine
AG/GA	The highest point in the antegonial notch (left and right)
Co	Condylion; Most superior point on condylar head
Crista Galli	Most superior point at its intersection with the sphenoid
Gonion/Most Lateral Ramus	Most inferior, posterior, and lateral point at the gonial angle of the mandible
JL/JR	Bilateral points on the jugal process at the intersection of the outline of the tuberosity of the maxilla and zygomatic buttress
Me	Menton; most inferior point at symphysis
Midsagittal Plane	A plane bisecting the head and face through the crista galli, ANS, and genial tubercles in a symmetric face
Occlusal Plane	Horizontal line bisecting UR6 and LR6 as well as UL6 and LL6
UR6/UL6 LR6/LL6	Buccal cusp tip of right/left maxillary molar Buccal cusp tip of right/left mandibular molar
ZL/ZR	Medial aspect of frontozygomatic suture (Bilateral)

Table 2

Measurements used for evaluation of facial asymmetry in maxilla and mandible

Measurements	Definitions
Maxilla:	
Occlusal Plane Tilt (°)	Difference between Frankfort Horizontal and the horizontal line bisecting UR6 and LR6 as well as UL6 and LL6
Maxillary Canting JL or JR – ZL or ZR (mm)	Difference between vertical distance from left or right jugal process between left or right frontozygomatic suture
Mandible:	
Menton Deviation A-Me-MSR (°)	Angle formed between midsagittal plane and line going through ANS and menton
Mandibular Width to Midsagittal Plane GA or AG-MSR (mm)	Distance between left or right antegonial notch and midsagittal plane
Mandibular Width to Menton GA or AG-Menton (mm)	Distance between left or right antegonial notch and menton
Ramal Height (R or L) (mm)	Linear distance between condylion to most lateral ramus (gonion)

Table 3

Cephalometric measurement comparison between symmetric and asymmetric subjects and statistical

Measurements	Symmetric	Asymmetric	<i>P</i> value [*]
Occlusal Plane Tilt (°)	0.87 ± 0.59	1.36 ± 1.47	0.0040
Maxillary Canting JL or JR – ZL or ZR (mm)	0.68 ± 0.44	2.70 ± 5.17	0.0003
Menton Deviation A-Me-MSR (°)	0.31 ± 0.29	4.47 ± 2.10	<0.0001
Mandibular Width to Midsagittal Plane GA or AG-MSR (mm)	1.51 ± 1.31	14.2 ± 12.8	<0.0001
Mandibular Width to Menton GA or AG-Menton (mm)	1.51 ± 2.69	21.4 ± 14.9	<0.0001
Ramal Height (mm) significance	1.41 ± 0.79	14.5 ± 12.1	<0.0001

* Unpaired t-test derived from mean and SD.

Table 4

Comparison of SNP genotypes by symmetry subclassifications

Gene	SNP	Genotype	Asymm-1	Asymm-2	Asymm-3	Asymm-4	Symmetric	Symmetry vs. Asymmetry	
								p value - genotype	p value - allele
<i>ENPP1</i>	rs9373000	AA	4 (57)	17 (65)	9 (41)	6 (67)	35 (54)		
		AG	2 (29)	7 (27)	10 (46)	3 (33)	28 (43)		
		GG	1 (14)	2 (8)	3 (13)	0 (0)	2 (3)	0.24	0.83
	<i>p</i> - value genotype		0.33	0.21	0.15	0.62			
	<i>p</i> - value allele		0.23	0.46	0.13	0.19			
	rs6569759	GG	5 (72)	6 (21)	5 (22.5)	2 (22)	11 (17)		
		AG	1 (14)	12 (46)	1	5 (56)	38 (58)		
		AA	1 (14)	8 (33)	5 (22.5)	2 (22)	16 (25)	0.27	0.38
	<i>p</i> - value genotype		0.004	0.56	0.83	0.92			
	<i>p</i> - value allele		0.016	1	0.66	0.76			
	rs858339	TT	1 (16)	3 (9)	0 (0)	0 (0)	20 (30)		
		AT	3 (42)	10 (36)	13 (59)	4 (44)	12 (18)		
		AA	3 (42)	15 (55)	9 (41)	5 (56)	33 (52)	0.002	0.55
	<i>p</i> - value genotype		0.33	0.13	0.002	0.33			
	<i>p</i> - value allele		0.27	0.77	0.14	0.24			
	rs1409181	CC	1 (14)	5 (12)	5 (22.5)	1 (12)	9 (15)		
		CG	4 (57)	15 (55)	1	7 (76)	36 (55)		
		GG	2 (29)	8 (33)	4 (18.5)	1 (12)	20 (30)	0.53	0.31
	<i>p</i> - value genotype		0.99	0.88	0.41	0.41			
	<i>p</i> - value allele		0.92	0.69	0.21	0.49			
<i>ESR1</i>	rs1643821	GG	1 (16)	3 (9)	3 (13)	3 (32)	4 (7)		
		AG	3 (42)	14 (50)	10 (46)	5 (56)	36 (55)		
		AA	3 (42)	11 (41)	9 (41)	1 (12)	25 (38)	0.23	0.3
	<i>p</i> - value genotype		0.63	0.72	0.48	0.019			
	<i>p</i> - value allele		0.58	0.8	0.76	0.02			

Gene	SNP	Genotype	Asymm-1	Asymm-2	Asymm-3	Asymm-4	Symmetric	Symmetry vs. Asymmetry	
								p value - genotype	p value - allele
	rs3020318	TT	3 (43)	12 (46)	12 (55)	1 (12)	24 (37)		
		CT	4 (57)	13 (45)	7 (32)	7 (76)	28 (43)		
		CC	0 (0)	3 (9)	3 (13)	1 (12)	13 (20)	0.39	0.27
	<i>p</i> - value genotype		0.47	0.46	0.4		0.16		
	<i>p</i> - value allele		0.17	0.29	0.23		0.4		
	rs3020377	GG	3 (43)	13 (45)	12 (55)	3 (33)	27 (41)		
		AG	4 (57)	12 (46)	6 (26.5)	6 (67)	31 (48)		
		AA	0 (0)	3 (9)	4 (18.5)	0 (0)	7 (11)	0.78	0.51
	<i>p</i> - value genotype		0.65	0.8	0.23		0.28		
	<i>p</i> - value allele		0.22	0.51	0.73		0.91		
	rs2077647	TT	2 (29)	2 (8)	3 (13)	1 (12)	7 (11)		
		CT	2 (29)	20 (71)	11 (51)	6 (67)	34 (52)		
		CC	3 (42)	6 (21)	8 (36)	2 (21)	24 (37)	0.63	0.53
	<i>p</i> - value genotype		0.37	0.2	0.98		0.67		
	<i>p</i> - value allele		0.71	0.52	0.93		0.59		

Table 5

Percentage of TMD diagnosis among classification groups

	Symmetric (n=90)	All Asymmetric (n = 84)	Group 1	Group 2	Group 3	Group 4
DDR *	3 %	78 %	11 %	29 %	52 %	23 %
Myalgia	4	61	11	33	44	23
Arthralgia	1	33	0	17	19	8
Headache **	0	12	0	9	7	8
DD w/o R ***	0	6	0	3	11	0

* DDR = Disc displacement with reduction

** Headache attributed to TMD

*** DD w/o R = Disc displacement without reduction with limited opening

Table 6

ENPP1 rs858339 genotypes by DC-TMD Classification

Gene	SNP	Genotype	No TMD	DDR	Myalgia	Arthralgia
<i>ENPP1</i>	rs858339	TT	50 (67)	9 (35)	0 (0)	0 (0)
		AT	21 (28)	16 (62)	14 (61)	7 (78)
		AA	4 (5)	1 (3)	9 (39)	2 (22)
	<i>p</i> - value genotype		0.33	0.009	0.01	0.01
	<i>p</i> - value allele		0.27	0.02	0.11	0.056

Table 7

Pre-surgical JPF scores by symmetry classification

	n	Mean JPF Score	SD	p value [*]
Symmetric	90	1.97	2.53	<0.0001
Asymmetric	84	6.87	5.43	
Group 1	9	3.75	4.09	0.009
Group 2	35	6.94	5.46	
Group 3	27	9.11	5.62	
Group 4	13	4.00	3.61	

* Comparison between symmetric and asymmetric groups with unpaired *t*-test. Comparison across the four groups with ANOVA.