Grouping patients for masseter muscle genotype-phenotype studies

Hadwah Abdelmatloub Moawad; Andrea C.M. Sinanan; Mark P. Lewis; Nigel P. Hunt

ABSTRACT

Objectives: To use various facial classifications, including either/both vertical and horizontal facial criteria, to assess their effects on the interpretation of masseter muscle (MM) gene expression.

Materials and Methods: Fresh MM biopsies were obtained from 29 patients (age, 16–36 years) with various facial phenotypes. Based on clinical and cephalometric analysis, patients were grouped using three different classifications: (1) basic vertical, (2) basic horizontal, and (3) combined vertical and horizontal. Gene expression levels of the myosin heavy chain genes MYH1, MYH2, MYH3, MYH6, MYH7, and MYH8 were recorded using quantitative reverse transcriptase polymerase chain reaction (RT-PCR) and were related to the various classifications. The significance level for statistical analysis was set at \( P \leq .05 \).

Results: Using classification 1, none of the MYH genes were found to be significantly different between long face (LF) patients and the average vertical group. Using classification 2, MYH3, MYH6, and MYH7 genes were found to be significantly upregulated in retrognathic patients compared with prognathic and average horizontal groups. Using classification 3, only the MYH7 gene was found to be significantly upregulated in retrognathic LF compared with prognathic LF, prognathic average vertical faces, and average vertical and horizontal groups.

Conclusion: The use of basic vertical or basic horizontal facial classifications may not be sufficient for genetics-based studies of facial phenotypes. Prognathic and retrognathic facial phenotypes have different MM gene expressions; therefore, it is not recommended to combine them into one single group, even though they may have a similar vertical facial phenotype. (Angle Orthod. 2012;82:261–266.)

KEY WORDS: Masseter muscle; Facial morphology; Myosin heavy chain gene expression

INTRODUCTION

One of the aims of masseter muscle (MM) genotype-phenotype studies has been to relate a specific craniofacial phenotype to a particular MM genetic profile, whether at transcriptomic\(^1,2\) or proteomic\(^3\) levels. This approach has been applied to assess the role of the MM in the primary cause and treatment outcome of patients with specific craniofacial discrepancies.\(^4\)

Variations in craniofacial form can arise as a consequence of variations in cranial,\(^5\) maxillary,\(^6\) mandibular,\(^7\) and dental\(^8\) components. This has urged clinicians to combine skeletal and dental features and to identify basic, combined, and comprehensive descriptive patterns, which are significantly different between average individuals and patients with craniofacial discrepancies.\(^7-9\) For example, a prognathic pattern describes basic single-dimensional criteria (horizontal), and prognathic long face (LF) explains two combined dimensional features (horizontal and vertical). Comprehensive description provides details of affected skeletal and dental components, such as
prognathic horizontal discrepancy with a retruded maxilla and LF seen vertically with a dental anterior open bite.

Despite the fact that patients commonly exhibit a combination of vertical and horizontal skeletal variations, rather than a single-dimensional discrepancy,9,10 previous human MM genotype-phenotype studies have mainly assessed vertical or horizontal facial phenotypes in relation to MM gene expression, with few6 or no7,8 average control patients for comparison.

Both groups of studies have focused mainly on a set of MM candidate genes called the myosin heavy chain genes (MYH). These include MYH1 (encoding fast-contracting IId/x MyHC), MYH2 (fast-contracting Ila MyHC), MYH3 (embryonic-MyHC), MYH6 (intermediate α-cardiac MyHC), MYH7 (slow-contracting I MyHC), and MYH8 (neonatal-MyHC). These genes have been selected because they encode previously identified contractile and unique myosin heavy chain proteins (MyHC) of MM11 and were found to be significantly different among patients with variable facial features.12

Nelson-Moon et al.,13 in a study of MM gene expression of MYH1, MYH2, MYH3, MYH6, MYH7, and MYH8 genes in relation to vertical facial development, recruited 15 patients, 9 of whom were LF. The remaining 6 patients exhibited horizontal facial discrepancies (retrognathia and prognathia) but with average vertical facial features and were considered as controls. Similarly, Suchak et al.,2 in a study of the same six MYH genes, recruited 9 patients with a wide range of vertical discrepancies and 1 average vertical control. Most vertical studies have included patients with horizontal discrepancies with average vertical facial features as controls. Therefore, it is likely that retrognathic LF and prognathic LF patients were combined into a single LF group. The results of both studies show no differences in MYH MM gene expression between LF patients and average vertical controls.

Gedrange et al.,14 on the other hand, tested MM gene expression of MYH1, MYH2, and MYH7 in relation to horizontal facial development, with 10 patients classified into 5 retrognathic and 5 prognathic cases regardless of their vertical facial phenotype. Results indicate that expression of both MYH2 and MYH7 was greater in retrognathic than in prognathic individuals. This indicates that prognathic and retrognathic patients have different MM gene expressions; however, it is not clear whether combining them into a single group, as was done in previous vertical studies, may camouflage the true effect of the vertical discrepancy or vice versa.

The aim of the present study was to use one set of data, including patients with variable facial forms and various classifications such as (1) only vertical, (2) only horizontal, or (3) both vertical and horizontal facial features, to assess whether the outcome of muscle genetic analysis is influenced by the method used to classify patients’ facial forms.

MATERIALS AND METHODS

Subjects

Following ethical approval (Ref. 05/Q0512/120 and 314/06/004), informed consent was obtained from 29 patients (8 males and 21 females; age range, 16–36 years) who were to undergo MM biopsy at the time of clinically necessary third molar removal. Only nonsyndromic, medically healthy, white patients with no previous history of orthodontic or orthognathic treatment and no obvious facial asymmetry were included. All patients had a pretreatment lateral cephalometric radiograph for hand tracing and analysis. Radiographs were taken at three hospital sites (UCL Hospital and Whipps Cross University Hospital [both in the UK], and Riyadh Military Hospital [Saudi Arabia]) using standardized cephalometric techniques.

Cephalometric Measurements

Seven cephalometric landmarks were identified (Figure 1) and were used to measure one horizontal (ANB angle) and one vertical variable (LAFH% [lower anterior face height]) (Figure 1). Both variables have been previously reported as the most significantly different vertical6 and horizontal7 cephalometric parameters between average individuals and patients with craniofacial discrepancies.

The Bland and Altman approach14 based on hand retracing of 25 radiographs, 2 weeks apart, was used to assess systematic and random errors associated with cephalometric analysis. Both measurements showed good correlation and agreement and no significant differences between paired readings.

Classification of Patient Groups

Based on clinical appearance and radiographic analysis, the 29 recruited patients were grouped using (1) basic vertical classification regardless of any horizontal discrepancy (LAFH% was used to confirm clinical vertical facial pattern [normal mean value ± 1 SD average vertical; above norm +1 SD LF; below norm −1 SD short face]); (2) basic horizontal classification regardless of any vertical discrepancy (ANB angle was used to confirm the clinical horizontal pattern [normal mean value ± 0 SD average horizontal; above norm +1 SD retrognathic; below norm −1 SD prognathic]); and (3) combined vertical and horizontal classification looking at both dimensional criteria.
recorded in the first two classifications. Normal cephalometric values were derived from published normal white values.\textsuperscript{16–17}

**Muscle Biopsy**

All muscle biopsies were obtained with the patient under general anesthesia before any orthodontic/orthognathic treatment was provided, following a standardized procedure\textsuperscript{18} at the time of removal of mandibular third molars. The incision was extended through the inner cheek to the anterior medial portion of the superficial belly of the MM. A biopsy measuring approximately $3 \times 3 \times 3$ mm was obtained from each patient, was maintained in a tube containing RNA-stabilizing reagent (RNALater Tissue Storage solution, Qiagen Ltd, West Sussex, UK), and was transferred to a $-80^\circ$C freezer until the time of RNA extraction.

**Total RNA Extraction and cDNA Synthesis**

Each muscle biopsy was cut, weighed (not greater than 30 mg each), and transferred into a clean tube containing ceramic beads (lysis matrix D, Q-BIOgene, Cambridge, UK) and 600 $\mu$L of RLT buffer (RNeasy mini kit, Qiagen). Physical disruption and homogenization were conducted using the FastPrep 120 reciprocating machine (Q-BIOgene) for 20 seconds, at speed 6, followed by cooling on ice for 5 minutes (repeated twice). The resulting lysate solution was processed using the manufacturer’s protocol of the RNeasy mini kit, including the on-column DNase digestion procedure (RNase-free DNase set, Qiagen), for 15 minutes. All samples had high total RNA integrity and quality (Bioanalyzer 6000 Nano kit, Agilent Technologies Ltd, West Lothian, UK). cDNA was synthesized in accordance with the manufacturer’s protocol for the high-capacity cDNA reverse transcription kit (Applied Biosystems, Warrington, UK).

**qRT-PCR**

To facilitate comparison with previous vertical\textsuperscript{2} and horizontal\textsuperscript{1} studies, MYH genes were selected for analysis using the technique of relative quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). This was conducted using TaqMan universal PCR master mix, TaqMan gene expression assays of MYH1, MYH2, MYH3, MYH6, MYH7, and MYH8 (GenBank mRNA accession numbers are described elsewhere),\textsuperscript{2} and the 7300 real-time PCR machine. Each gene of interest, including the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (all from Applied Biosystems), was tested in quadruplicate reactions. Normalization and calculation of gene expression values have been conducted using the $2^{-\Delta\Delta Ct}$ equation recommended by Livak and Schmittgen.\textsuperscript{19}

**Statistical Analyses**

Data were analyzed using the Statistical Package for the Social Sciences (SPSS), version 14.0 (SPSS Inc, Chicago, Ill), based on nonparametric data. The Mann-Whitney $U$-test and the Kruskal-Wallis test were used to assess the significance of differences in MM expression of selected genes between the classified groups. A $P$ value $\leq .05$ was considered statistically significant.

**RESULTS**

**Masseter Muscle Genotype-Phenotype Analysis in Relation to Different Classifications**

**Basic vertical classification.** When only vertical facial criteria were considered, there were 14 average vertical and 15 LF patients. None of the recruited patients exhibited a short face appearance (Table 1). None of the MYH genes was found to be statistically significantly different between LF patients and those with average vertical phenotype.

**Basic horizontal classification.** Based on their horizontal facial phenotype, regardless of vertical facial
Table 1. Cephalometric Values of Recruited Patients Based on Various Facial Phenotypic Classifications

<table>
<thead>
<tr>
<th>Phenotypic Classifications</th>
<th>ANB</th>
<th>LAFH%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Basic vertical</td>
<td>2.3****</td>
<td>±1.0</td>
</tr>
<tr>
<td>Retrognathic LF (n = 11)</td>
<td>2.3****</td>
<td>±1.0</td>
</tr>
<tr>
<td>Prognathic LF (n = 10)</td>
<td>6.4****</td>
<td>±5.7</td>
</tr>
<tr>
<td>Prognathic AVF (n = 3)</td>
<td>3.0****</td>
<td>±2.0</td>
</tr>
</tbody>
</table>

*a* ANB indicates A-point-nasion-B-point angle; LAFH%, lower anterior face height percentage; mm, millimeter; n, number of patients; SD, standard deviation; AVF, average vertical face; LF, long face; and V & H, vertical and horizontal.

**P < .0001.

**Combined vertical and horizontal classification.** Based on both vertical and horizontal facial features, patients were classified into 11 average vertical and horizontal faces, 5 retrognathic LF, 10 prognathic LF, and 3 prognathic average vertical faces (Table 1). Overall gene expression showed significant upregulation of only the MYH7 gene in retrognathic LF patients compared with both prognathic subgroups and average patients (Table 2) (Figure 2D). However, when gene expression analysis was conducted between paired groups, other genes were found to be significantly different (Table 2).

**DISCUSSION**

This study has demonstrated that outcomes of genotypic analyses can be affected by the phenotypic classification used.

When patients were classified based solely on vertical facial parameters (basic vertical classification), none of the MYH genes were found to be significantly different. These findings are similar to data from the previously discussed MM vertical studies of Suchak et al. and Nelson-Moon et al. One problem with this classification, as shown by our data, is that the LF group included patients with different horizontal discrepancies (5 retrognathic and 10 prognathic), which were not taken into account. Previously mentioned horizontal studies as well as our additional data confirm MYH MM gene expression variations between prognathic and retrognathic patients. Therefore, combining retrognathic LF and prognathic LF patterns into a single group may camouflage the true effect of vertical discrepancy. The need to segregate both horizontal patterns is mandatory during vertical classification, even though they may have a similar vertical facial appearance.

When the same patients were regrouped based only on horizontal patterns (basic horizontal classification), MYH3, MYH6, and MYH7 all were found to be significantly upregulated in retrognathic patients compared with prognathic and average individuals. These results concur with the findings of Gedrange et al., as discussed earlier. However, this latter study did not include MYH3 or MYH6 genes, nor were average horizontal controls included. The problem with this classification is that it does not assess vertical facial criteria of recruited subjects. This was demonstrated in the current study, in which the prognathic group included patients with both long and average vertical faces. Such combinations may lead to misinterpretation of the genotype-phenotype relationship.

Combined vertical and horizontal classification has not been used in previous MM genetic studies and has addressed two main issues in the current research. First, it has segregated retrognathic LF and prognathic
LF patterns, which was the main problem not addressed by the basic vertical classification. Second, it has differentiated between the prognathic LF and the prognathic average vertical face, which was the main problem not addressed by the basic horizontal classification. However, an obvious disadvantage of the combined classification is that the more subgroups were used, the smaller was the sample size. This was clearly noted in our data showing that the prognathic average face group had a reduced sample size (3 patients). Furthermore, MYH3, MYH6, and MYH7 genes showed upregulation in retrognathic patients when recruited subjects were classified based only on horizontal facial features; when patients were regrouped using this classification, only MYH7 was found to be upregulated in retrognathic LF patients compared with the other classified groups. It is interesting to note that when the analysis was conducted between pairs rather than three or four groups, MYH1, MYH2, and MYH3 gene expressions (Table 2) were found to be significantly different between pairs. This may have been attributed to large individual variations and small sample sizes.

When human MM genotype-phenotype studies are conducted, one has to take into account three main factors that may produce certain limitations to such studies, including the current one: (1) ethics, which is related mainly to the invasive nature of the MM biopsy procedure and subsequently limits the number of recruited patients; (2) clinical phenotypic variations, which often make it difficult to use simple classifications,

Figure 2. Masseter muscle gene expression variations between various facial phenotypes. Generally, large individual variations and skewed data were noted in most of the classified groups. Extreme cases were denoted as an asterisk; less extreme but still considered outlier samples were marked as circles. The small numbers on top of the asterisks and circles are indicative only of the sample code numbers. (A, B, and C) MYH3, MYH6, and MYH7 gene expressions, respectively, were upregulated in retrognathic patients compared with both average and prognathic individuals. (D) MYH7 gene expression was upregulated in the retrognathic long face group compared with average and both prognathic subgroups. V & H indicates vertical and horizontal.
compromise sample size, and may introduce large variations within the results; (3) genotypic variations, which can be seen on genomic (DNA), transcriptomic (RNA [gene expression]), and proteomic (protein) levels.

Genomic DNA variations are expressed as single nucleotide polymorphisms (SNPs) or mutations. Some of the MYH genes have been reported with mutations, SNPs, and various alleles. This may explain the wide range of individual variation noted in our data. However, with the absence of DNA analysis, it is not possible to confirm this explanation. Furthermore, MYH genes tend to respond to environmental factors and have the capability to shift their expression (on an RNA level) in response to different functional demands, such as orthognathic jaw surgery and an increased number of dental occlusal contacts (DOC).

This raises the question as to whether MYH MM gene expression may differ from that of LF patients with and without anterior open bite (AOB), in whom the number of DOCs is obviously less in AOB cases.

Masseter muscle genotype-phenotype studies are still in their infancy, and whether any of the MYH genes could be considered a predictor of a specific craniofacial phenotype or an indicator for possible relapse following jaw surgery would require further investigation on a larger sample size with less variability; analyses performed on genomic, transcriptomic, and proteomic levels; and long-term study design, including data obtained before and after surgery.

CONCLUSIONS

- Ideally, classification of patients for masseter muscle genotype-phenotype studies should be based on comprehensive craniofacial groupings, including clinical, dental, and radiographic criteria.
- If basic classifications are to be used, assessment of both vertical and horizontal features of recruited patients is recommended, and careful interpretation of the data is required.

ACKNOWLEDGMENTS

The authors would like to thank the Saudi Arabian Government, the British Orthodontic Society Foundation, and the Eastman Foundation for Oral Research and Training for sponsoring this project. Furthermore, thanks are due to the Consultant Maxillofacial Surgeons who performed the muscle biopsies: Mr Tim Lloyd, Mr Nayeem Ali, and Dr Khalid Abdel Wahab. We are also grateful to Professor David Moles and Mr Michael Roughton for their invaluable statistical advice.

REFERENCES