Masseter Muscle Properties Differ between the Left and Right Sides in Mandibular Class III Patients with Asymmetry

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Abstract: In this study on muscle characteristics and jawbone morphology, the relationship between masticatory muscle properties and jaw deformity was analysed in skeletal Class III patients with asymmetry. The subjects were 27 patients (21 women and 6 men) who had skeletal mandibular prognathism with mandibular asymmetry. The subjects’ ages ranged from 16 to 51 years, with an average of 23.5 years. Patients with lateral displacement of Me 3 mm or more and an ANB angle of 0° or less were included. Morphometric measurements were taken from CT images. Biopsy material was collected during surgery for immunohistochemical typing of muscle fibres, MyHC1, 2a, and 2x isoforms; measurement of MYH1, 2, 7, 8 mRNA expression. In CT morphometry, there was no difference in muscle thickness or cross-sectional area between the deviated and non-deviated sides, but both sides tended to have thinner muscles than the control group. In the typing of muscle fibres by immunohistochemical staining, type 2 fibres were found to be significantly more numerous on the deviated side than on the non-deviated side. Regarding protein quantities, MyHC1 was more abundant on the deviated side than on the non-deviated side. Regarding the expression of mRNA, MYH7 (type 1) and MYH1 (type 2x) showed no difference between the two sides, and MYH2 (type 2a) had a higher expression level on the deviated side than in the control group. The data suggested a tendency for Class III patients to have characteristics of a short face on the deviated side, and a long face on the non-deviated side. It seems that muscle type switching may be in progress.

Key words: Jaw deformity, Masseter, Muscle, Myosin heavy chain (MyHC)

Introduction

The cause of jaw deformities is unknown, but they develop through interactions between genetic and environmental factors1,2). In particular, it has been reported that genetic factors are deeply related to the Class III phenotype in patients3,4). Jaw deformities are often accompanied by asymmetry. Even in identical twins, the pattern of deformation is inconsistent. Therefore, it is difficult to explain the onset mechanism of jaw deformity with genotypic alone.

Environmental factors must be considered as well; for example, it is said that neuromotor effects during the growth period are related to bone morphology7), and the influence of muscle characteristics on the jawbone morphology seems to be large. Therefore, it is difficult to explain the onset mechanism of jaw deformity with genetic factors alone.

Muscle characteristics are susceptible to local effects such as stretching as well as genetic factors9). In this study, we investigated the relationship between masticatory muscle characteristics and jawbone deformation in patients with lower-jaw asymmetry.

Materials and Methods

Objective patients

The subjects were 21 females and 6 males who underwent sagittal split of ramus osteotomy for skeletal Class III malocclusion with mandibular asymmetry at Kyushu University Hospital from July 2015 to March 2018. The subjects ranged in age from 16 to 51 years, with an average of 23.4 years (Table 1). Cases in which Menton (Me) was laterally deviated by 3 mm or more were considered asymmetric10). Skeletal Class III was diagnosed when the ANB angle was 0° or less. Patients with benign diseases such as mandibular cysts with no morphological deformities on the maxillofacial area were selected, and 11 patients who agreed to collect masseter muscles were used as a control group.

The study protocol was reviewed and approved by the Ethics Committee of Kyushu University Hospital (Permission number; 29-583), and written informed consent was obtained from the study participants, including consent to participate and to publish the findings.

Morphological analysis of masseter muscle

From CT images taken before surgery, the thickness and cross-sectional area of the masseter muscles were measured at the height just below the lingula of the mandible, which is parallel to the orbit–meatus (OM) line.
Harvesting of masseter muscle

At the time of surgery, approximately 0.5 cm$^3$ of muscle tissue was collected from the anterior part of each masseter muscle. The tissue was wrapped in gauze moistened with saline and immediately transported to the laboratory for freezing. Tissues were treated with RNAlater Stabilization Solution (Invitrogen), and samples were stored in a -80 °C ultra-deep freezer until they were sectioned.
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Figure 3. Immunohistochemical staining for muscle type. a: Type 1 dominant masseter muscle. b: Type 2 dominant masseter muscle. Red staining shows MyHC 1 protein (type 1 myofibres), and green staining shows MyHC 2a+x (type 2 myofibres). Jaw deformity patients tend to have more type 2 fibres than normal controls.

Figure 4. Semi-quantitative analysis of protein content. a,c: Western blotting analysis detected by anti-MyH1 and anti-MYH7 antibody. Anti-actin antibody was used for normalization. b: MyHC1. d: MyHC2x. Both MyHC1 and MyHC2x tended to be more abundant on the deviated side than on the non-deviated side, and the difference was significant for type MyHC1.
Immunohistochemical staining

For frozen section preparation, the muscle tissue was immediately placed in a cryosection tray (#CRYO DISH No.2; ShoeiWorks Co., Ltd., Tokyo, Japan) containing OCT compound. The specimens were rapidly frozen in isopentane in liquid nitrogen. The tissue sections were prepared with a cryostat and immunohistochemically stained with an anti-MyHC antibodies provided by the Faculty of Agriculture of Kyushu University.\(^{10}\)

Staining was performed by the one-step method. Glass slides containing tissue sections were placed in a tall polypropylene Coplin staining jar filled with hot PBS solution and steamed in a food steamer for 5 minutes. After being heated, the specimens were cooled for 30 minutes at room temperature, and the slides were placed in another tall Coplin staining jar containing 1.0% Triton X-100 in PBS and incubated for 30 minutes at room temperature.

Slides were incubated in blocking buffer (1% BSA, 0.1% cold fish skin gelatin, 0.1% Triton X-100, 0.05% TWEEN 20, 0.05% sodium azide, and 100 mM glycine in 10 mM PBS) for 1 hour. Antibody solutions were prepared by mixing two conjugated antibody solutions containing antibodies specific for individual MyHC isoforms (MyHC 1, 2a and 2x).

After blocking treatment, the antibody solution was loaded onto each specimen and incubated overnight at 4 °C. The next day, the slide was washed with TBST, and the cover glass was mounted on the specimen with Fluorescent Mounting Medium (S3023; Dako). Measurements were performed with image measurement software (BZ-H4M, Keyence, Osaka, Japan).

RNA isolation and real-time quantitative RT-PCR

Total RNA was extracted from 100 mg of masseter muscle tissue using TRIzol Reagent (Life Technologies, Carlsbad, CA, USA). For the quantitative real-time polymerase chain reaction (qRT-PCR), the LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany) was used. The reaction conditions consisted of denaturation at 95 °C for 10 minutes, followed by 47 cycles of annealing at 60 °C for 10 sec and extension at 72 °C for 10 sec. Data were analysed using LightCycler Software Version 3.5 (Roche Diagnostics, Mannheim, Germany). The relative expression level was normalized to the mRNA level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers used are shown in Table 2.

Western blotting

Cell lysates from homogenate were analysed by Western blotting. Samples were rinsed with PBS and then lysed by sonication in sodium dodecyl sulfate (SDS) lysis buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, and 6% mercaptoethanol) containing a protease inhibitor cocktail (Sigma-Aldrich). The protein content of the lysates and fractionated samples was quantified using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein from each sample containing 30 μg of protein were electrophoresed on 7.5% SDS polyacrylamide gels and transferred electrophoretically onto nitrocellulose membranes (Bio-Rad Laboratories). After being washed with TBST (25 mM Tris-\(\text{HCl}\) [pH 8.2], 144 mM NaCl, and 0.1% TWEEN 20), the membranes were blocked with 5% nonfat skim milk in TBST at room temperature, and then incubated with specific antibodies.

Figure 5. Relative amount of mRNA expression. a: MYH1 (type 2x). b: MYH2 (type 2a). c: MYH7 (type 1). d: MYH8 (neonatal). There was no difference between the groups in the expression level of MYH7 (type 1) or MYH1 (type 2x). MYH2 (type 2a) was more highly expressed on the deviated side than in the control group. In MYH8 (neonatal), the bilateral expression levels were lower in patients than in the control group.
Immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary antibodies (DAKO, Carpinteria, CA, USA) and ECL detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The bands were quantitated using Quantity One Software (Bio-Rad Laboratories) after scanning by computer-assisted densitometry (ChemiDoc XRS-J; Bio-Rad). Quantitative analysis of western blotting was performed on using image J software (https://imagej.nih.gov/ij/).

**Statistical analysis**

The differences between the mean values were compared by means of t-tests. All statistical analyses were performed using JMP13 (SAS Institute Inc, NC, USA) p values of < 0.05 were considered statistically significant.

**Results**

**Masseter muscle morphology**

In Class III patients, there was no significant difference between the masseter muscle of the deviated side and that of the non-deviated side in terms of thickness or cross-sectional area. The bilateral thickness and cross-sectional area were smaller in Class III patients than in the control group (Fig. 1).

**Typing of muscle fibres by immunohistochemical staining**

Regarding the number of muscle fibers, type 1 fibres were found to be significantly less numerous on the deviated side than on the non-deviated side. Regarding the cross-sectional area of myofibres, there was no difference between the left and right. Type 1 fibres were thicker than type 2 fibres, and type 1 fibres accounted for most of the total area in both patients and controls (Figs. 2 and 3).

**Western blotting**

Both MyHC1 and MyHC2x tended to be more abundant on the deviated side than on the non-deviated side, and MyHC1 had a significantly greater abundance on the deviated side than on the non-deviated side (Fig. 4).

**qRT-PCR**

There was no difference between the groups in the expression level of MYH7 (type 1). There was no difference between the groups in MYH1 (type 2x). The expression level of MYH2 (type 2a) was higher on the deviated side than in the control group. In MYH8 (neonatal), the expression level was lower on both the deviated side and the non-deviated side than in the control group (Fig. 5).

**Discussion**

Asymmetry of the mandible may be a major complaint for patients, and it is difficult to achieve complete symmetry because the left and right sides differ in the interference of the bone fragments and the curvature of the bone in surgical treatment. Three-dimensional simulation and planning using computers are now possible, and the progress of surgical technique has improved treatment results. However, contour improvement is sometimes not sufficient to achieve good occlusion.

Controlling jaw growth and preventing mandibular asymmetry is extremely meaningful clinically, but since the onset mechanism is unknown, prevention is impossible at present.

The cause of mandibular asymmetry is unknown, but hyperplasia or hypoplasia of the mandibular head, mandibular branch, or body or deformity of the maxilla may affect the mandible. It has also been reported that there is a left-right difference in the phenotype of muscle, such as contraction characteristics. It is thought that genetic factors are involved in the onset of jaw deformities, however, it is difficult to explain left-right morphological differences, and it is assumed that some acquired, habitual, or local factors are also involved. Since hyperplasia of the mandibular head may be involved in neoplasia, hyper-/hypoplasia of the mandibular head was excluded from this study.

The largest local factor that affects the shape of the jawbone is thought to be muscle function. The muscles affecting the morphology of the jawbone include the masticatory muscles and the supratrochoid muscles in addition to the muscles of the lip, cheek and tongue. We focus on the masticatory muscles as the cause of the left-right asymmetry. The volume of the masseter muscle is said to have no left-right difference in mandibular asymmetry, but there are reports that the masseter of the non-deviated side is larger. Since muscle strength is proportional to the cross-sectional area of the muscle, the cross-sectional area and thickness were measured in this study. We found that there was no significant difference between the deviated side and the non-deviated side, but both were smaller (thinner) than the masseter of the control group. Since the decrease in masticatory muscle strength correlates with an open bite and dilatation of the gonial angle, and since gonial angle dilatation was often observed in Class III patients, we interpret the difference between patient and control masseters as an effect of mandibular prognathism more than left-right differences.

Regarding the number of muscle fibres, type 2 fibres were found to be significantly more numerous on the deviated side than on the non-deviated side. However, although there was no significant difference, the patient group tended to have more type 2 fibres than the healthy group. Type 2 fibres are “fast” muscle fibres that are poor in endurance and aerobic exercise, although they are superior in instantaneous power. Skeletal muscles are said to transition to fast muscle when mechanical stimulation is reduced by conditions such as space travel. Intermaxillary fixation and masticatory disorders are considered to cause a correspond decrease in mechanical stimulation for muscles in the oral region. Conversely, when exercise or training is performed, muscles become enlarged and transform into slow muscles. The results of this study also show that the masseter muscles of Class III asymmetric patients are macroscopically thin and atrophic muscles, which leads to faster muscles than those of healthy individuals who chew normally and have normal occlusion. Alternatively it is possible that slow muscle switching has not occurred. Sciote et al. reported a relative increase in type 2 fibres in short-faced patients and a relative decrease in type 2 fibres in long-faced patients. In short, the tendency of muscle characteristics resembled those of a short face on the deviated side and those of a long face on the non-deviated side.

Although muscle strength correlates with cross-sectional area, individual differences are large, and it is thought that various factors including MyHC type are involved. The masseter on the deviated side was inferred to be strong, suggesting a short face as opposed to a long face and mandibular protrusion on the non-deviated side.

Regarding protein content, type 1 and type 2x tended to be more abundant on the deviated side than on the non-deviated side, and the difference in type 1 was significant. This suggests that the switch to slow-stitch phenotype may be in progress on the deviated side. In the expression of mRNA, there was no difference between the groups in the expression level of MYH7 (type 1). There was no difference between the groups in MYH1 (type 2x), and the expression level of MYH2 (type 2a) was higher on the deviated side than in the control group. The shift from type 2x to type 2a may occur on the deviated side, and a switch to slow muscle was thought to be progressing. Muscles are known to shift...
toward a slow-twitch phenotype with exercise\textsuperscript{26}, the observed left-right difference may be because the occlusal force cannot be evenly shared between the left and right sides due to the bias of chewing on the deviated side.

Regarding MYH8 (neonatal type) expression, the mRNA level was lower on both the deviated side and the non-deviated side than in the control group. The neonatal type is observed particularly in masticatory muscles and is rarely found other skeletal muscles\textsuperscript{28}. It may reflect an element of regenerative ability. Patients with Class III jaw deformity may have poor muscle regeneration compared to healthy individuals.

Such changes in muscles are probably determined by the environment or habit rather than by genotype alone. In the future, it is expected that therapeutic intervention for masticatory muscles during the jawbone growth period will lead to prevention of mandibular asymmetry.

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Conflict of Interest

The authors have declared no COI exists.

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