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Proteomics-based identification of novel proteins in temporal tendons of patients with masticatory muscle tendon–aponeurosis hyperplasia

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Abstract. Masticatory muscle tendon–aponeurosis hyperplasia (MMTAH) is a new disease associated with limited mouth opening that is often misdiagnosed as a temporomandibular disorder; subsequently, patients are mistakenly treated with irreversible operations. Due to the poor presentation and characterization of symptoms, the underlying pathological conditions remain unclear. We have previously conducted a proteomic analysis of tendons derived from one MMTAH subject and one facial deformity subject using two-dimensional fluorescence difference gel electrophoresis and liquid chromatography coupled with tandem mass spectrometry. However, the results were obtained for only one subject. The aim of the present study was to confirm the expression of specific molecules in tendon tissues from multiple subjects with MMTAH by applying two-dimensional polyacrylamide gel electrophoresis with matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Of the 19 proteins identified in tendons from both MMTAH and facial deformity patients, fibrinogen fragment D and beta-crystallin A4 were up-regulated, whereas myosin light chain 4 was down-regulated in MMTAH. We also found fibrinogen to be expressed robustly in tendon tissues of MMTAH patients. Our data provide the possibility that the distinctive expression of these novel proteins is associated with the pathology of MMTAH.

Key words: masticatory muscle tendon–aponeurosis hyperplasia; tendon, two-dimensional polyacrylamide gel electrophoresis; matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

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Masticatory muscle tendon–aponeurosis hyperplasia (MMTAH) is a newly described disease associated with limited mouth opening. The condition advances very slowly from adolescence; both mandibular angles exhibit hyperplasia, resulting in a characteristic square mandible.[1] MMTAH is diagnosed by a palpable dense band at the anterior border of the masseter muscle upon maximum mouth opening and a square mandible configuration. Hyperplasia of tendons and aponeuroses

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in MMTAH leads to a disturbance in muscle extensibility, resulting in limited mouth opening.[1] Common useful diagnostic markers of this disease are currently unavailable because of its poor objective symptoms.

Although MMTAH was formally approved as a disease at the conference of The Japanese Society of the Temporomandibular Joint in 2008, it is not well recognized in other countries. A lack of recognition of this disease among general practitioners results in misdiagnosis of the temporomandibular disorder, and patients have often been treated with irreversible operations. Therefore, there is a need to establish criteria for the correct diagnosis of MMTAH as soon as possible. From the viewpoint of histopathology, Chiba observed that the tendons and aponeuroses in MMTAH appear normal because of a lack of both inflammation and transformation; he therefore suggested that the excess tissue is a result of hyperplasia.[2] We have previously demonstrated that a good long-term recovery is reliably obtained in patients with MMTAH via resection of the hyperplastic masseter muscle aponeuroses and coronoidectomy for separation of the temporal muscle from the coronoid process.[3]

Tendons are structurally composed of tenocytes lying within a network of extracellular matrix. They are the tissues that connect the muscle to the bone and transmit forces generated by the muscle to the bone.[4] Although proteomic analysis has been performed on murine tendons, a lack of studies in man leaves it unclear as to which proteins are expressed in human tendons.[5] Facial deformity (FD), a disease that is defined as a congenital or acquired condition of the skeleton in the head and face region, requires surgical correction for the deformities.[6,7] In contrast to MMTAH patients, FD patients have normal tendons that do not exhibit hyperplasia.

Tendon tissues in MMTAH appear normal upon microscopic observation and gross pathology. However, the aberrant sound heard upon cutting with scissors indicates degenerative changes at the electron microscope or protein level.[3] Furthermore, MMTAH progresses bilaterally and is a juvenile-onset disease. We hypothesize that disease progression involves both environmental and genetic factors. We have previously conducted a preliminary study to identify candidate molecules as potential diagnostic markers in MMTAH using a two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) system and liquid chromatography coupled with tandem mass spectrometry. However, that study involved only one test subject and one control subject, and used crude tendons that contained muscle tissues.

The aim of the present study was to confirm the expression of specific molecules in tendon tissues from multiple subjects with MMTAH by applying another approach. We thus performed proteomic analysis of tendons from three test subjects and three control subjects using a 2D-polyacrylamide gel electrophoresis (2D-PAGE) system and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Materials and methods

Subjects

Tissue specimens from the temporal tendons of four subjects with MMTAH and four with FD whose tendon tissue showed no sign of hyperplasia and who had no limited mouth opening, were obtained from patients undergoing surgery (Table 1). None of the patients had underlying diseases. The dentist in charge provided all the patients or their guardians with an explanation of the study. The participants provided their written informed consent to participate in this study. Patients were free to withdraw from the study at any time. Informed consent was obtained from all subjects. Three samples from the MMATH group and three from the FD group (i.e., M1, M2, M3, F1, F2, and F3) were subjected to proteome analysis, and one sample from each group (i.e., M4 and F4) was subjected to histological analysis.

Sample preparation

Each tendon tissue (150–200 mg wet weight of tissue) with the muscle completely removed under a stereomicroscope, was homogenized using an SK mill (Tokken Inc., Japan) under liquid nitrogen cooling, and then lysed in a buffer containing 7 M urea, 2 M thiourea, 30 mM Tris (tris(hydroxymethyl)aminomethane), 3% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), and 1% Triton X-100. The resultant mixture was sonicated for 20 s. The homogenate was then centrifuged at 40,000 rpm for 60 min at 20 °C; the supernatant (0.5–3 ml) was subsequently dialyzed using a Slide-A-Lyzer Dialysis Cassettes kit at 3.5 K molecular weight cut-off (Thermo Scientific Inc., USA) overnight and then dried in a SpeedVac vacuum centrifuge (Thermo Scientific). The sample was the lysed again in the same buffer. Protein concentrations were determined using a Pierce 660 nm Protein Assay (Thermo Scientific).

Table 1. Basic patient characteristics.

Diagnosis	No.	Sex	Age, years
MMTAH	M1	Female	35
MMTAH	M2	Female	41
MMTAH	M3	Female	44
MMTAH	M4	Female	42
FD	F1	Female	25
FD	F2	Male	29
FD	F3	Male	19
FD	F4	Male	41

MMTAH, masticatory muscle tendon–aponeurosis hyperplasia; FD, facial deformity.

2D gel electrophoresis

2D gel electrophoresis was performed as described previously.[8] Briefly, the sample was mixed in a rehydration solution containing 8 M urea, 2% CHAPS, 2% immobilized pH gradient (IPG) buffer, 0.3% dithiothreitol (DTT), and a few grains of bromophenol blue dye, and the resulting mixture was vortexed. The first dimension was performed with an Ettan IPGphor II (GE Healthcare, USA) using IPG strips pH 3–10 L, 18 cm (GE Healthcare). The second dimension was performed with a Multiphor II 2-D (GE Healthcare) using ExcelGel SDS XL Gradient 12–14%, 245 cm × 180 cm (GE Healthcare). For the first dimension, an aliquot of sample supernatant containing 1.0 mg protein was loaded onto an IPG strip, and the strip was then allowed to rehydrate overnight. The first dimension was performed with the following voltage programme: 100 V for 12 h (step-and-hold), 500 V for 1 h (gradient), 1000 V for 1 h (gradient), 8000 V for 1 h (gradient), and 8000 V for 40 h (step-and-hold). Prior to the second-dimension separation, IPG strips were incubated for 20 min in an equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS), and bromophenol blue) containing 0.2% DTT, followed by equilibration in the same buffer containing 4% iodoacetamide and bromophenol blue dye instead of DTT. The IPG strip was then placed cathode side down on top of the SDS gel, and second-dimension electrophoresis was performed under the following conditions: 1000 V, 20 mA, and 40 W for 45 min; 1000 V, 40 mA, and 40 W for 5 min; and 1000 V, 40 mA, and 40 W for 160 min.

Protein spot image analysis

The separated protein spots were visualized using Coomassie brilliant blue (CBB) G-250, and the 2D gel electrophoresis