ISSN 2618-1584 Vol. 4, Issue 6: 52-59, DOI: 10.25236/FMSR.2022.040610

Impairment of esophageal skeletal muscle in type XIX collagen mutant mouse

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Abstract: *Type XIX collagen is an extremely rare extracellular matrix collagen thought to be involved in the formation of basement membrane zones and is transiently expressed by differentiating muscle cells. Mice without collagen XIX exhibit an impaired muscle differentiation and function. Individual skeletal muscle fibers are ensheathed by a meshwork of collagen fibers comprising the endomysium. Such structures seem to play an important role in resisting imputes. Type XIX collagen-null mice have a poor survival, and surviving null mice exhibit a dilated esophagus after three months of age, especially in aged mice. We used scanning electron microscopy (SEM) and transmission electron microscopy (TEM) to examine the skeletal muscle morphology and reticular distribution of collagen fibers in the endomysium of the esophagus in mice. The aim of this research was to demonstrate the fine structures in the dilated portion of the esophagus in mutant mice. Our findings showed that the size and arrangement of skeletal muscle as well as the collagen fibrils of the endomysium in the dilated portion of the esophagus differed markedly from those in wild-type mice. In addition, skeletal abnormalities were seen in the non-dilated portion of the esophagus in the mutant mice. These findings suggest that type XIX collagen-containing fibrils influence morphogenesis during skeletal myogenesis in the murine esophagus.*

Keywords: *type XIX collagen, mutant mouse, skeletal muscle, esophagus, achalasia, basement membrane*

1. Introduction

In skeletal muscle, the entire muscle is wrapped in the connective tissues known as the epimysium. Extremely fine fibrous septa that extend inward from the epimysium consist of perimysium and the reticular fiber sheaths surrounding the individual skeletal muscle fiber are known as the endomysium. The reticular fibers associated with the basement membrane of the skeletal muscle fibers and the collagen fibers of the connective tissue are estimated to be 40 nm and 80-120 nm in diameter, respectively. Previous scanning electron microscopy (SEM) studies have shown that individual skeletal muscle fibers and cardiac muscle cells were covered by the dense meshwork of reticular fibrils running circularly[1-4]. An immunohistochemical study showed that the endomysium was intensely positive for anti-type III collagen[3]. Such structures seem to play an important role in resisting the stretching impetus.

A craniocaudal transition from smooth muscle to striated muscle occurs in the mouse fetal esophagus with the muscle eventually becoming striated in mature mouse except the lower esophageal sphincter (LES). Early studies suggested that the smooth-to-striated muscle development occurred through transdifferntiation[5]. However, recent studies have provided evidence of different pathways for both muscle types during esophagus development[6, 7].

Type XIX collagen is an extremely rare extracellular matrix component that is localized to the basement membrane zones[8]. Type XIX collagen presents in the muscular tissues of the esophagus and

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stomach at birth as well as in the myotome during embryogenesis and in the hippocampus in adulthood[9]. A previous study generated mice lacking type XIX collagen that were smaller than wild-type animals and showed a poor survival. The few surviving null mice showed a dilated esophagus (megaesophagus) after 3 months of age[9]. The progression of muscle differentiation from smooth to skeletal muscle in the esophagus failed in the null mice. Electron microscopy of these mutants revealed thick basement membrane around the smooth muscle cells and the excessive extracellular accumulation of collagen fibrils in the LES. However, no reports have described the electron microscopy findings of this area during esophageal dilatation.

In the present study, the morphological characteristics of the striated muscle fibers of the dilated portion of the esophagus in aged type XIX collagen mutant mice were investigated by the silver-impregnation method, immunohistochemistry, transmission electron microscopy (TEM) and SEM.

2. Methods

2.1 Animals, fixation and tissue preparation

All experimental procedures complied with the guideline of Animal Experiment of Oita University, Japan (approval No. L006001). Breeding was carried out in an animal laboratory facility at Oita University (room temperature: 22.5 ±2 ℃, lighting at 7: 00, extinction at 19: 00). Food and water were freely accessible, and the cages were exchanged weekly. Type XIX collagen mutant mice generated by Sumiyoshi et al[9] and wild-type mice (129/SvJ strain) of both sexes were used.

Of the 3 mutant mice used, the 24-month-old N/+ and 12-month-old △19/△19 male mice had esophageal dilation[9]; the other 18-month-old male △19/+ mouse has no apparent esophageal dilation. Normal 6-, 12-, 18-, 21-and 24-month-old mice also used. The mice were sacrificed under deep anesthesia with sodium pentobarbital (64.8 mg/ml; 0.01 ml/10 g body weight) and perfused through the left ventricle with 10% neutral formalin. The whole esophagus was removed. Tissue blocks about 3 mm in length were fixed in 10% neutral formalin for light microscopy and SEM, and small tissue blocks were immersed in cacodylate-buffered (pH 7.4) 2.5% glutaraldehyde and 2% paraformaldehyde (Karnovsky's fixative) for TEM.

2.2 Light microscopy

The paraffin blocks were cut into 5μm, and sections were stained with hematoxylin and eosin (HE) or stained with sliver according to the Bielschowsky-Gomori method[10]. They were also immunostained with anti-type III collagen antibody (Cosmo Bio, Tokyo, Japan), immersed in rabbit IgG gold (5nm) solution (Amersham Pharmacia Biotech, UK), and physically developed. All sections were observed under a light microscopy (Keyence BZ-9000).

2.3 Scanning electron microscopy (SEM)

Paraffin blocks of mouse esophagi were also used for SEM. They were deparaffinized with xylene, hydrated and again fixed in Karnovsky's fixative, and immersed in 2N NaOH at 37 ℃ for 3 hrs to digest extracellular matrix[3]. The samples were placed in 1% osmium tetroxide for 1 hr, 1% tannic acid/ 1% osmium tetroxide for 1 hr, dehydrated in a series of graded concentrations of ethanol and then dried by the t-butyl alcohol freeze drying method. The specimens were sputter-coated with gold, and examined at 15 KV under a scanning electronic microscope (Hitachi H-4800).

2.4 Transmission Electron microscopy (TEM)

After removal of the esophagus, small tissue blocks were immediately fixed in Karnovsky's fixative and for 2 hr at 4 ℃, and post-fixed in a mixture of 0.5 % potassium ferrocyanide and 2% osmium tetroxide for 2 hr at 4 ℃. They were dehydrated in a graded series of ethanol and embedded in epoxy resin. Thin section, 0.8 μm, was stained with uranyl acetate and lead citrate, and examined under a transmission electron microscope (Hitachi H-7650).

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3. Results

3.1 Structural differences in the muscle layer between the wild-type and type XIX collagen mutant mice

We first used silver staining of Bielschousky/Gomori, which show reticular fibers as black and collagen fibers red, in addition to hematoxylin/eosin staining for light microscopy. Collagen fibers in the submucosal tissue and the adventitia correspond to the epimysium, while those between muscles correspond to the perimysium. The endomysium wrapping each muscle fiber was clearly visible. The basic structure of the muscle layer in the mouse adult esophagus was composed of striated muscle fibers (Fig.1), and it extended near the cardia in both wild-type and type XIX collagen mutant mice.

The esophagus of the 24-month-old mutant mouse was enlarged, with a diameter＞4 mm, which was approximately 3-fold larger than the normal diameter (Fig.2a). The dilated region was composed of only a few layers of muscle fibers (Fig.2b). However, each muscle fiber was considerably larger than that in the non-dilated region (Fig.2c).

Although the esophagus was not macroscopically enlarged in the 12-month-old mutant mouse, light microscopy of cross sections of the esophagus showed that the expansion of the inner lumen was already present (Fig. 3a and 3b). Striated muscle fibers of the mutant mice were smaller in size and number than in the wild-type (Fig. 3c and 3d). The inner diameter of the esophagus was approximately 2.0-3.0 mm in wild-type mice, compared with approximately 3.0-4.0 mm in the mutant mice at the same level of the esophagus.

The 8N HCl treatment of the esophagus resulted in digestion of the connective tissue component and basement membrane, enabling visualization of the size and arrangement of the muscle fibers by SEM (Fig.4). The striated muscle fibers of the 18-momth-old wild-type mouse were neatly and densely arranged (Fig.4a), while those in the 18-month-old mutant mouse were extremely poor in density and ran in various directions (Fig. 4b).

TEM of the striated muscle fibers in the middle and lower esophagus in an 18-month-old wild-type mouse showed that striated muscle fibers had regularly- arranged myofibrils and many mitochondria (Fig.5a). In contrast, striated those same fibers in the highly expanded esophagus of a mutant-type were hypertrophic and occupied by myofibrils (Fig.5b). The disorder and collapse of the arrangement of myofibrils was observed in some muscle fibers (Fig.5b). Higher magnification views showed that the presence of electron-dense bodies was due to the degeneration of myofibrils (Fig.6a). In addition, inclusion bodies were also seen within the nucleus (Fig.6b).

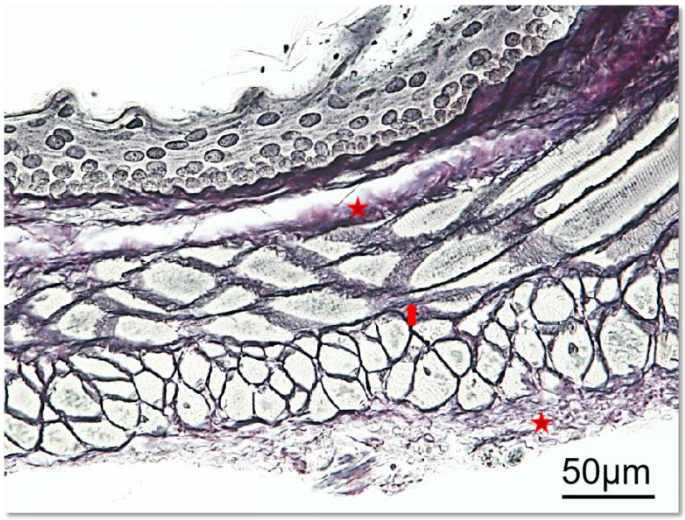

Figure 1: Light micrograph of the esophagus with silver staining in a 24- month-old wild-type mouse (+/+). The muscular layer is composed of striated muscle fibers, and each muscle fiber is wrapped in black reticular fibers. The epimysium and perimysium are indicated by an asterisk and an arrow, respectively.

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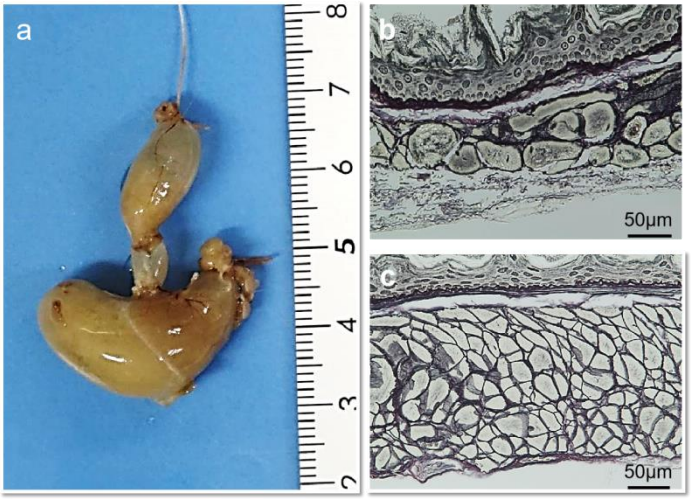

Figure 2: Macroscopic (a) and light microscopy images (b and c) of the esophagus of a 24-month-old mutant mouse (N/+). The esophagus is macroscopically enlarged (a). The diameter of the enlarged portion is approximately three times larger than that of the non-enlarged portion. There are fewer striated muscle layers in the enlarged portion (b) than in the non-enlarged portion (c). b and c: silver staining.

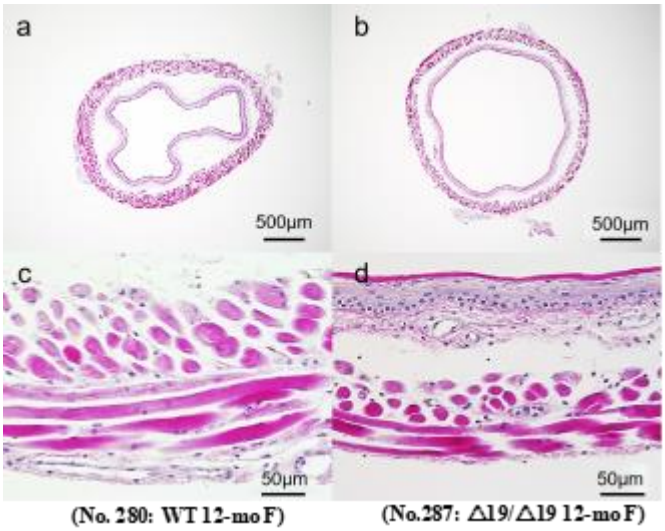

Figure 3: Light micrographs of the esophagus thoracic region in 12-month-old female wild-type (+/+) (a, c) and type XIX collagen mutant-mice (△19/△19) (b, d). The diameter of the esophagus is similar in both mice. However, the esophageal lumen in the mutant mouse is expanded and the striated muscle fibers in the mutant mouse (d) are smaller in size and number than those of the wild- type mouse (c). HE stainin.

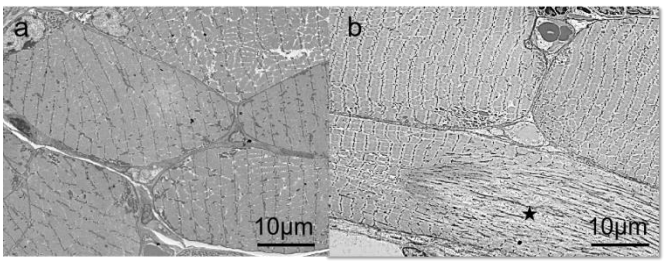

Figure 4: SEM images of the skeletal muscle layer of the esophagus in 18-month-old male mice treated with 8N HCl. In the wild-type mouse (+/+), the striated muscles fibers are arranged neatly and densely (a). In the mutant mice (△19/+), the muscular layer in the enlarged portion (b) consists of loosely arranged striated muscle fibers. Each fiber in the enlarged portion is larger than that in the wild-type mouse.

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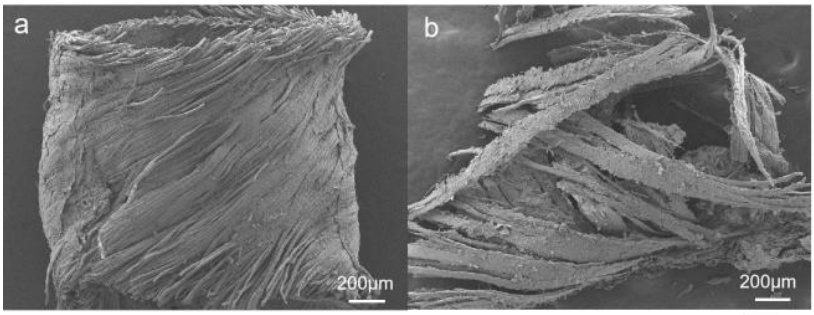

Figure 5: TEM images of the striated muscle fibers in 18-month-old male wild-type (a) and mutant mice (△19/+). In contrast to the wild-type mouse, the hypertrophic striated muscle fibers contain myofibrils in the mutant mouse. Muscle fibers with collapsed myofibrils can also be seen (asterisk).

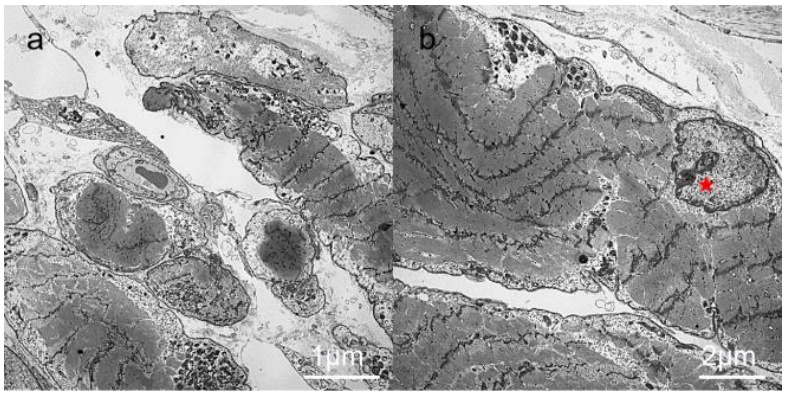

Figure 6: High magnification of TEM images in the outer muscular layer of the non-enlarged portion in the 18-month-old mutant mouse (△19/+). Markedly atrophic (a) and irregular arrangement (b) of myofibrils can be seen. A nuclear inclusion (asterisk) is also seen

3.2 Structural differences in the endomysium between wild-type and type XIX collagen mutant mice

Treatment with 2N NaOH enabled the three-dimensional visualization by SEM not only individual striated muscle fibers but also collagen fibers (Fig.7). The endomysium surrounding each striated muscle fiber of the esophagus in a wild-type mouse was stained black by silver staining (Fig.8a) and immunohistochemically showed a strong positive reaction to anti-type III collagen antibody (Fig.8b). In contrast, TEM showed that the striated muscle fibers were surround by the basement membrane and collagen fibrils with a diameter of 40 - 50 nm (Fig.8c). In the esophagus of the mutant mice, the three-dimensional architecture of collagen fibrils in the endomysium differed from that in wild-type mice or in the non-enlarged portion of mutant mice. The striated muscle fibers in the enlarged portion were larger than those in other sections, but the collagen fibrils of the endomysium were rather poor in density (Fig.9a, 9b and Fig.8d).

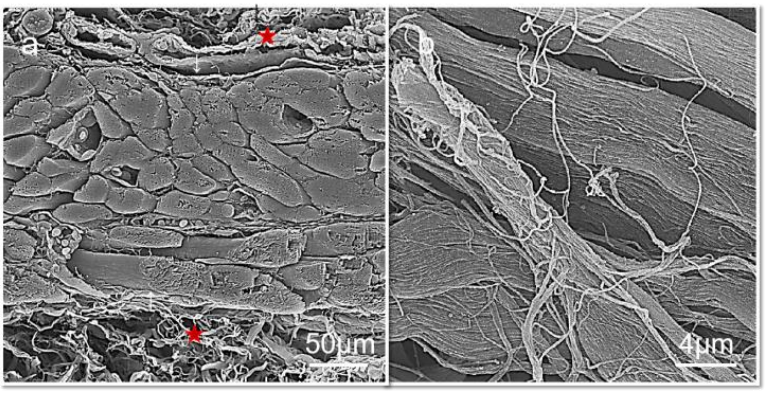

Figure 7: SEM images of the cross surface of the esophageal thoracic region in a 6-month-old wild-type mouse (+/+). The muscular layer is surrounded by connective tissue, which corresponds to the epimysium (arrows) (a) and consists of bundles of collagen fibrils (b).

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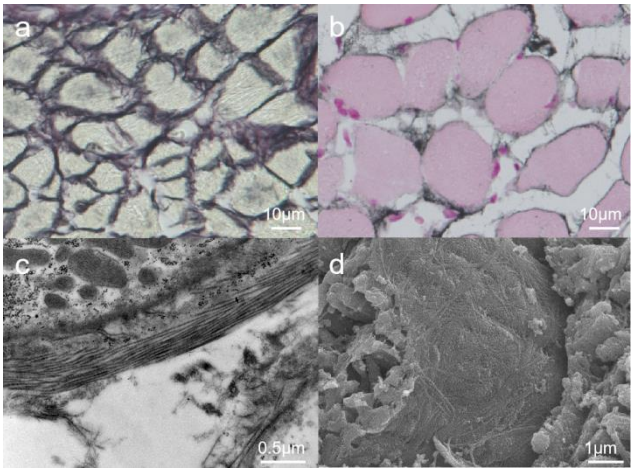

Figure 8: The esophagus endomysium of wild-type mice (+/+). (a) Silver staining of striated muscle fibers (24-month-old male). (b) Immunochemistry of anti-III collagen antibody (21-month-old female). (c) TEM observation of the basement membrane and collagen fibrils surrounded by striated muscle fibers (24-month-old male). (d) SEM observation of the three-dimensional endomysium of striated muscle fiber (24-month-old male).

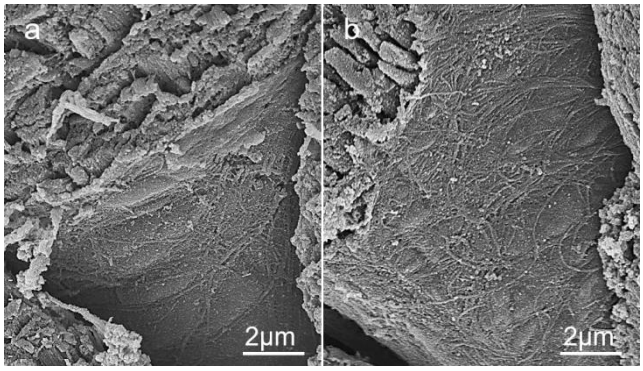

Figure 9: SEM images of the striated muscles in an 18-month-old male mutant mouse (△19/+). The reticular fibrils of the endomysium in the enlarged portion (a) are poorer in density than those in the non-enlarged portion (b) and wild-type in Fig. 8d.

4. Discussion

The esophagus functions to transport food by peristaltic contraction of the esophageal musculature and the transient relaxation of the LES. A previous TME study showed a thicker basement membrane around the smooth muscle cell (SMC) of the LES in null mice than in wild-type mice with greater intracellular spacing of mutant SMC than in wild-type controls[9]. The excessive extracellular accumulation of collagen fibrils was found to be particularly pronounced in aged mutant mice. These morphological changes were consistent with the abnormal findings of a physiological examination of the LES. However, the dilated portion of the esophagus itself and other portions of the esophagus were not examined. The findings of the present study using light and electron microscopy showed abnormalities of striated muscle in the dilated portion. In addition, abnormality of the tissues was also observed in the portions with a normal outer diameter of the esophagus.

Type XIX collagen belongs to the FACIT subfamily, which is associated with the surface of collagen fibrils[11]. Type IX collagen, which is a proto-type of FACIT, is covalently linked to the surface of cartilage collagen fibrils composed of type II and XI collagens[12]. Type XII and XIV collagen, other FACITs, are thought to be associated with type I collagen-containing fibrils in non-cartilaginous tissues[13]. However, no report has described which type of collagen associates with type XIX collagen to form collagen fibrils. Type XIX collagen is localized to the basement membrane zones, where fine reticular fibers exist, as shown by conventional silver staining. These fibers consist of at least type I and III collagens[3]. Therefore, it is postulate to form the fibrils that consist of I/III collagens and type XIX collagen in restricted region such as the esophageal muscle layer.

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Two types of mutant mice were used in this experiment. The half of volume of normal type XIX collagen chain is only thought to be synthesized in N/+ mouse[9]. In contrast, mutant chain is thought to be synthesized in the △19/△19 or △19/+ mouse. The 2N NaOH maceration method is useful for removing extracellular substances such as proteoglycan. Indeed, apparent abnormalities of the reticular fiber of the endomysium were observed in △19/+ mice using this method (Fig.9). Type XIX collagen molecule may be involved in the formation of supramolecular molecules, namely reticular fibers. The abnormality of fibers in the striated muscle was also shown using 8N HCl treatment. The craniocaudal transition from SMC to striated muscle was disturbed in null mice during developing stage[9]. Striated muscle reached the upper portion of the LES, which is composed of SMC, in both aged wild-type and mutant mice. However, we noted abnormalities in the striated muscle of mutant mice even in non-dilated the portions of the esophagus with a normal diameter. This suggests that the matrix containing type XIX collagen influences the morphogenesis of striated muscle in the esophagus.

Patients with achalasia show enlargement of the esophagus. Infectious, autoimmune, degenerative and genetic factors are believed to be involved in the onset of this disease[14]. A reduction in the network of interstitial cells of Cajal (ICCs) in the esophageal wall is associated with an impaired NOS expression in achalasia patients[15]. In these mutant mice, enteric neurons and ICCs showed normal numbers based on confocal micrographs[9]. The occurrence of familial achalasia, such as triple A syndrome, which is caused by mutations in the *ALADIN* gene leading to achalasia, alacrima and addisonism, suggests the involvement of genetic factors. At present, we are attempting to identify mutations in achalasia patients using whole-genome sequencing. Thus far, no mutations have been found to be associated with any candidate gene, including the type XIX collagen gene.

5. Conclusions

In conclusion, we demonstrated the abnormality of the skeletal muscle fibers and collagen fibers in the endomysium of the esophagus in type XIX collagen mutant mice using SEM and TEM. These findings suggest that type XIX-containing fibrils influence morphogenesis during skeletal myogenesis in the murine esophagus.

Acknowledgements

Funding information: This research was funded by The Natural Science Foundation of Hebei Province (grant number:H2021423019). Science and Technology Project of Hebei Education Department (grant number: BJ2021032). Hebei Province introduction of overseas students funding project (grant：number:C20210359).

Conflict of interest: The authors declare no conflict of interest. The authors alone are responsible for the content and writing of the article.

We thank Ms. S. Adachi, Ms. Ms. H. Sato, Ms. A. Uesono, Dr. T. Sasaki, Dr. N. Matsuo and the staff members of Department of Matrix Medicine, Faculty of Medicine, Oita University.

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