

Three-Dimensional Imaging of Palatal Muscles in the Human Embryo and Fetus: Development of Levator Veli Palatini and Clinical Importance of the Lesser Palatine Nerve

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Background: After palatoplasty, incomplete velopharyngeal closure in speech articulation sometimes persists, despite restoration of deglutition function. The levator veli palatini (LVP) is believed to be significantly involved with velopharyngeal function in articulation; however, the development and innervation of LVP remain obscure. The development of LVP in human embryos and fetuses has not been systematically analyzed using the Carnegie stage (CS) to standardize documentation of development. **Results:** The anlage of LVP starts to develop at CS 21 beneath the aperture of the auditory tube (AT) to the pharynx. At CS 23, LVP runs along AT over its full length, as evidenced by three-dimensional image reconstruction. In the fetal stage, the lesser palatine nerve (LPN) is in contact with LVP. **Conclusions:** The positional relationship between LVP and AT three-dimensionally, suggesting that LVP might be derived from the second branchial arch. Based on histological evidence, we hypothesize that motor components from the facial nerve may run along LPN, believed to be purely sensory. The multiple innervation of LVP by LPN and pharyngeal plexus may explain the postpalatoplasty discrepancy between the partial impairment in articulation vs. the functional restoration of deglutition. That is, the contribution of LPN is greater in articulation than in deglutition. *Developmental Dynamics* 245:123–131, 2016. © 2015 Wiley Periodicals, Inc.

Key words: levator veli palatini muscle; human embryo; human fetus; phase-contrast imaging; Kyoto collection; palatoplasty; lesser palatine nerve

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Introduction

Cleft lip and palate (CLPs) are among the most common congenital facial anomalies. In Japan, the prevalence rate of CLPs is 1.8 per 1,000 births, and it is higher globally (Vanderas, 1987; Kochi, 2007). While the surgical intervention in cleft lip is often focused on the cosmetic aspect, the surgical operation in cleft palate also involves repair of the muscles involved in the velopharyngeal function. Nevertheless, after palatoplasty, some patients exhibit velopharyngeal dysfunction that particularly affects speech. To resolve the problem, it is critical to

understand the anatomy of the palatal muscles as well as the innervation of the palate.

The palatal muscles consist of five muscles including the tensor veli palatini muscle (TVP) and the levator veli palatini muscle (LVP). The LVP is the muscle responsible for the elevation of the soft palate and is thus critically involved in the speech and deglutition functions (Fara and Dvorak, 1970; Dickson, 1972), and it is also believed that the reconstruction of the LVP is important in palatoplasty (Kriens, 1969; Huang et al., 1998; Masuoka et al., 2011). Understanding of the anatomy and development of the LVP is important to ensure an effective repair of these structural anomalies. To date, most studies on the development of the palatal muscles have focused on the relationship between the auditory tube and the TVP (Ross, 1971; Seif and Dellon, 1978; De la Cuadra Blanco et al., 2012). Moreover, the number of reports on

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the LVP is fewer than those on the TVP (Broomhead, 1951; Domenech-Ratto, 1977). Information on the innervation of the LVP is indispensable for the operation of palatoplasty; that is, it is essential to determine which nerves should be preserved during the operation. However, innervation of the LVP remains controversial (Futamura, 1906; Cords, 1910; Edgeworth, 1916, 1923; Gasser, 1967; Nishio et al., 1976; Sonobe, 1982).

Human embryology started in the 1880s, and today there are several collections of human embryos around the world (Yamada and Takakuwa, 2012). The human embryological development changes have been characterized morphologically and classified into 23 standardized stages, named Carnegie stages (CS). For example, CS 1 represents the fertilized egg, and CS 23 marks the end of the organogenesis (O'Rahilly and Müller, 1987). Before the introduction of the CS, human development was discussed based on crown-rump length (CRL). Before the CS system, previous studies on the development of the LVP used the CRL, and there were discrepancies in the reported timing of the developmental LVP between reports.

In this study, we visually examined serial histological sections in detail, and obtained images of the LVP in the embryos and early fetuses, using nondestructive three-dimensional (3D) imaging. We conducted morphological analyses in three orthogonal planes, using staged human embryos and fetuses. To the best of our knowledge, this is the first image-scan focused on the early developmental LVP. As a result, the present study clarifies the development of the LVP at each CS, using histological serial sections. In addition, we also investigated the innervation of the LVP and emphasized the importance of the lesser palatine nerve during palatoplasty.

Results

Imaging and Development of the LVP

To elucidate the normal development of the LVP, histological investigation of the perioral regions was performed using 32 cases of serially sectioned human embryos from the Kyoto collection. At CS 20, the oral cavity and the auditory tube (AT) were easily visually identified (Fig. 1). In the perioral region, the tensor veli palatini muscle adjacent to the masticatory muscles were already observable at CS 20 (data not shown), but no muscular structures could be detected histologically in the region between the oral cavity and the AT (Fig. 1). In contrast, a group of immature myoblasts that will ultimately contribute to the formation of the anlage of the LVP was identified near the aperture of the AT to the pharynx at CS 21 (Fig. 1, arrow). We also examined the embryos at CS 19 and failed to detect any sign of the LVP at this stage; suggesting that the LVP starts to develop at CS 21. A summary of our observations of the LVP anlage in the present study is shown in Table 1. In the embryo specimen at CS 21, the anlage of the LVP was imaged just under and medial to the AT, using phase-contrast X-ray computed tomography (PCT) (Fig. 1). The LVP was examined in a few slices only; therefore, 3D reconstruction could not be performed. Using magnetic resonance imaging (MRI), the LVP could not be identified, because the samples were too small to detect the LVP due to lower resolution of MRI than PCT (data not shown).

At CS 23, the LVP was also identified histologically. The LVP was examined in all the embryos after CS 21. The LVP was clearly visible in CS 23 compared with CS 21, owing to its devel-

opmental growth. The coronal view of the LVP in histological sections was almost identical to that of CS 21 (data not shown). As with CS 21, the LVP at CS 23 was not identified in the MRI because the samples were too small. However, the structure was identified when using PCT (Fig. 2). PCT has higher resolution than MRI, so PCT resolved the visibility issue. The PCT provided clearer images of the LVP at CS 23 compared with CS 21. We were thus able to generate 3D images from the PCT section images (Fig. 2). At this developmental stage, the soft palate has not fused yet, as examined in the histological sections and PCT images. The LVP travels behind and under the AT from one half of the auditory tube to near the aperture of the AT. From the 3D images, it is obvious that the LVP runs behind and under the AT; that is, it runs in parallel with AT over its full length, and the course of the LVP resembles that in adults.

Next, we examined the LVP in the fetus. The sample at the fetal stage was not suitable for PCT scanning because of the upper limit of size. A sample of fetus is too large to scan by PCT; therefore, MRI scans were performed. The LVP was clearly identifiable in the MRI images (Fig. 3). We constructed 3D images from the image stacks (Fig. 3), creating a 3D image using the same software as for CS 23. The positional relationship between the LVP and AT seemed very similar to that of the embryo at CS 23, although the relative length of the LVP was greater in the fetus in comparison with the embryo at CS 23.

Innervation of the LVP

Next, we visually examined the innervation of the LVP. Neither the MRI nor the PCT scans allowed the detection of nerve fibers, because of the insufficient spatial resolution of these two imaging modalities. Therefore, histological observation was performed using serial sections. In the embryonic stages, nerve fibers going into the LVP were not observable. In the early fetal stage, in the CRL 40.7-mm fetus, nerve fibers were detected in close association with the insertion of the LVP (Fig. 4, large arrow). At this stage, the TVP winds around the pterygoid hamulus. To identify the origin of the fibers, several adjacent sections were carefully examined. The palatine nerve, surrounded by the primordium of the palatine canal, was easily identified, and it went into the palatine foramen. (In the adult human, the lesser palatine nerve [LPN] exits the palatine canal through the lesser palatine foramen, continues medially and posteriorly and innervates the soft palate [Fig. 5].) At this early fetal stage, the greater and lesser palatine foramina have not yet divided and only one foramen was thus detected (Fig. 4). The nerve fiber continues medially and posteriorly, and finally appeared to enter the LVP anlage. This course of the nerve fiber was consistent with the LPN. No other nerve fibers were observed around the LVP in this fetus. There were no serial sections available from older or larger fetuses in our collection.

Discussion

3D Imaging of Human Embryos

Morphological changes during development occur in three dimensions, and several 3D-imaging techniques have been developed and have facilitated our understanding of such dynamic changes (Yamada et al., 2012). In the development of the palate, all past morphological studies have been performed using

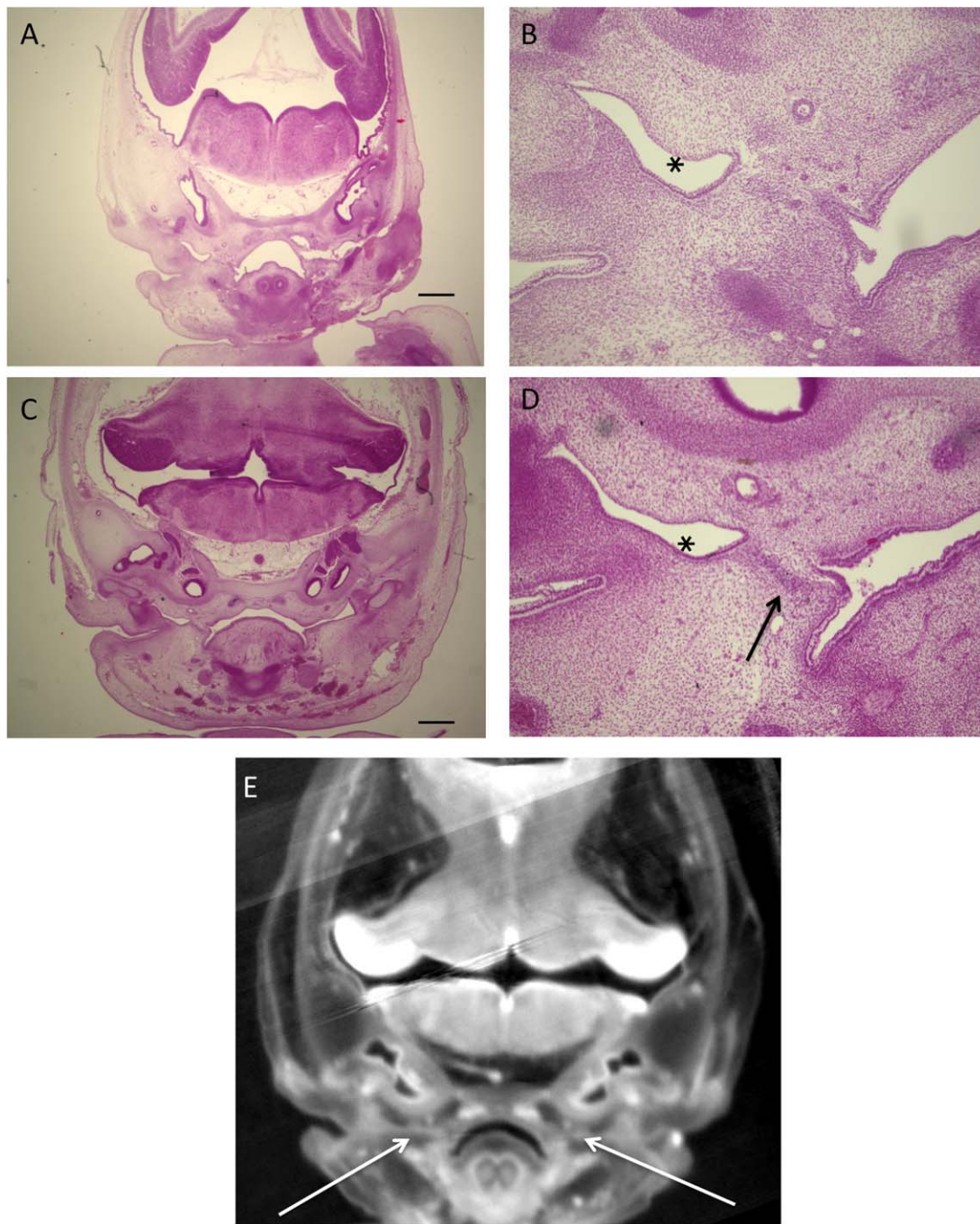


Fig. 1. Early development of the LVP. **A,B:** Histological specimen of CS 20, CRL18-mm embryo (#16264). **C,D:** Histological specimen of CS 21, CRL 20.3-mm embryo (#17429). **E:** The PCT image of CS 21, CRL 18.1-mm embryo (#28066), coronal plane. Arrowheads point to LVP, asterisk points to the auditory tube. Scale bar = 500 μm.

histological serial sections (Broomhead, 1951; Ross, 1971; Domenech-Ratto, 1977; Seif and Dellon, 1978; Klueber and Langdon, 1979; Iwase and Kitamura, 1985; De la Cuadra Blanco et al., 2012). In this study, MRI and PCT were used as nondestructive 3D-imaging modalities. The PCT imaging system used in this study was limited by the fact that the imaging sample size must be a maximum of approximately 15 mm × 15 mm × 20 mm; therefore, MRI was used with fetuses that allowed adequately sized samples. The sectional images and 3D reconstructions (Figs. 2 and 3), allowed easy understanding of the mutual location

within the skull of the LVP and the AT. This is, therefore, the first published example that analyzes morphological changes of the palatal muscles in human embryos using 3D-imaging techniques.

Development of the LVP Based on the CS

The developmental stages of human embryos are classically determined according to the CS (O'Rahilly and Müller, 1987, 2010), although the CRL-based classification had been used before the CS was developed, and at a time when the age of the

TABLE 1. Summary of Observations of the LVP in the Kyoto Collection

LVP not observed				LVP observed			
CS	Number	CRL (mm)	Direction of section	CS	Number	CRL (mm)	Direction of section
20	1311	17.6	Frontal	21	13375	17.2	Frontal
	16264 ^a	18	Frontal		12155	17.9	Frontal
	16564	18	Frontal		5767	18.3	Transverse
	4198	18.2	Frontal		11302	18.8	Frontal
	17186	18.7	Frontal		41	19	Frontal
	1580	18.8	Frontal		11315	19.9	Transverse
	3855	18.8	Frontal		17429 ^a	20.3	Frontal
	1770	18.9	Transverse		2021	21.4	Sagittal
	6444	20.7	Sagittal		11235	22	Transverse
	1061	22	Frontal		2314	22.6	Frontal

^aFigure 1.

samples was estimated by Streeter's computation method (Streeter, 1920, 1948, 1951). Today Streeter's computation method is deemed not to be appropriate, and O'Rahilly and Müller (2010) advocate that the CRL-based classification be abandoned in embryology. All past studies of the palatal muscles using human embryos were performed before the CS era and were based on the CRL system (Domenech-Ratto, 1977; Iwase and Kitamura, 1985). The previous studies on the development of the LVP are summarized in Table 2. There is a discrepancy with the 20-mm CRL embryo, however. On the other hand, if we try to map the CRL developmental stages onto the CS, each CS exhibits individual differences relative to the CRL, as shown in Table 3 (O'Rahilly and Müller, 1987). Therefore, the size of the 20-mm CRL embryo corresponds to CS19~21 (see Table 3). In the present study, human embryos at CS 19~23 were examined, and none of the embryos at CS 20 exhibited the LVP, while the anlage of the LVP was observed in all embryos at CS 21 (Table 1). If the 20-mm CRL embryo in Domenech-Ratto's paper is at CS 21 and the 20 mm CRL embryo in Iwase's paper is at CS 19 or 20, these observations are consistent with our observation that the LVP starts to develop at CS 21.

Developmental Origin and Innervation of the LVP

Innervation of the LVP is another topic of high interest. It is common knowledge that the TVP is derived from the first branchial arch, and is innervated by the trigeminal nerve (Broomhead, 1951; Domenech-Ratto, 1977). It is widely accepted that the LVP is derived from the fourth–sixth branchial arch (Cords, 1910; Edgeworth, 1916, 1923), and this idea has been adopted by textbooks (Patten, 1953; Arey, 1966). Therefore, of the derivatives of the fourth–sixth branchial arch, the LVP is innervated by the pharyngeal plexus. However, morphological analyses suggest that the LVP is innervated by the facial nerve (Futamura, 1906; Gasser, 1967), and electrophysiological experiments revealed that the LVP is innervated by multiple nerves such as the facial, glossopharyngeal, and vagus nerves (Nishio et al., 1976; Sonobe, 1982). It was also revealed that the greater petrosal nerve is included in the course of the facial nerve for the LVP (Ibuki et al., 1978). The greater petrosal nerve joins onto the pterygopalatine ganglion, and the LPN emerges

from the ganglion, as parasympathetic components; however, the greater petrosal nerve could have motor fibers (Foley, 1947). In our human fetus specimen at 40.7 mm CRL, the LPN fiber seems to supply the LVP (Fig. 4), and in a past report, similar findings emerged (Iwase and Kitamura, 1985). In addition, Shimokawa et al. (2005) reported in a cadaver study that the lesser palatine nerve innervates the LVP, and they identified the terminal branch of the LPN and its neuromuscular junction in the LVP by immunostaining. Therefore, it is possible that the facial nerve innervates the LVP by means of LPN as a motor nerve (Fig. 5).

In our 3D imaging study, the LVP in the embryonic and fetal stages was shown to run along and adjacent to the AT. The AT is derived from the first branchial groove between the first and the second branchial arch. The facial nerve develops around the second branchial arch; therefore, the proximal positional relationship of the LVP, the AT, and the facial nerve is compatible. Futamura (1906) reported the LVP derived from the second branchial arch from the view of the relationship with the platysma muscle innervated by the facial nerve, and Dellon (1989) explained that the LVP derived from the second branchial arch based on the positional relationship between the LVP and the AT. Our results (Figs. 1–3) also correspond with the explanation above, and there is another report about the functional relationship between the LVP and the AT (Ishijima et al., 2002). The AT is derived from the first branchial groove, and the TVP is derived from the first branchial arch. Here we suggest that the LVP may be derived from the second branchial arch. Thus, the tripartite relationship between the AT, LVP, and TVP during development is an interesting new topic.

Although there are many studies that do not support the LVP as being innervated by the facial nerve in experimental animals such as *Macacus sinicus* (Beavor and Horsley, 1888), dog (Rich, 1920), or cat (van Loveren et al., 1983; Keller et al., 1984), Sonobe (1982) demonstrated with both electrophysiological experiments and morphological investigations using rats that the LVP is innervated by the facial, glossopharyngeal, and vagus nerves. There is an acceptable explanation for the failure of other studies to find and identify the component of the facial nerve in the LVP (Shimokawa et al., 2005). Specifically, the pharyngeal plexus

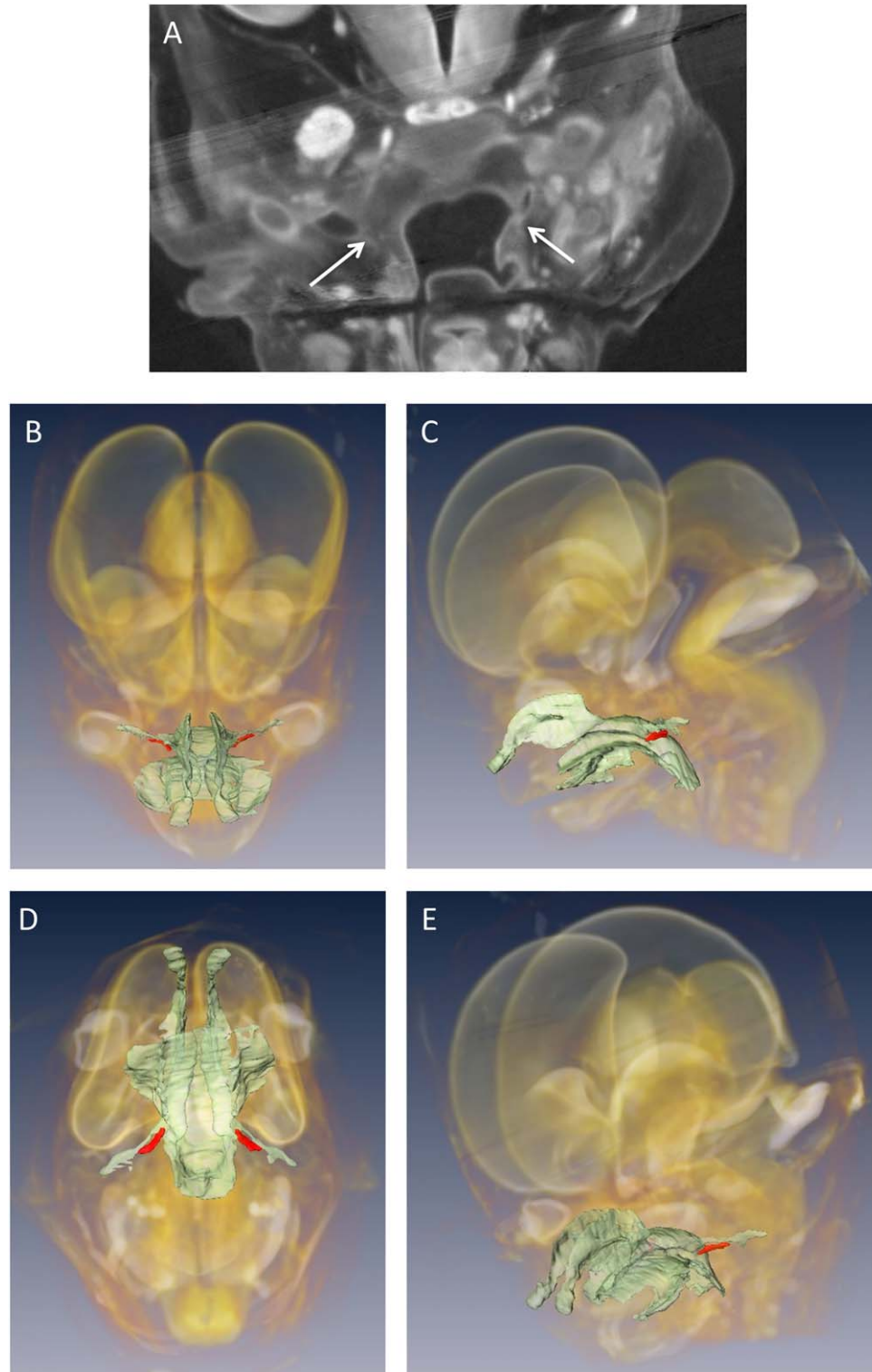


Fig. 2. CS 23, the CRL 29.4-mm embryo (#22761). **A:** The PCT image, coronal slice. Arrowhead points to the LVP. **B–E:** The 3D image from the images of PCT. LVP (red) The nasal cavity, the pharynx, and the auditory tube (yellow green) B: frontal view; C: lateral view; D: caudal view; E: left anterior oblique view.

innervates most of the superior part (near the origin) of the LVP, and the LPN innervates a very small inferior part of the LVP (near the insertion); that is, the component of the facial nerve is often too small to find and identify.

Function of the LVP Innervated by the LPN

It is widely accepted that the LVP is innervated by the glossopharyngeal and vagus nerves by means of the pharyngeal plexus. In this study, we suggest that the LVP is also innervated by the

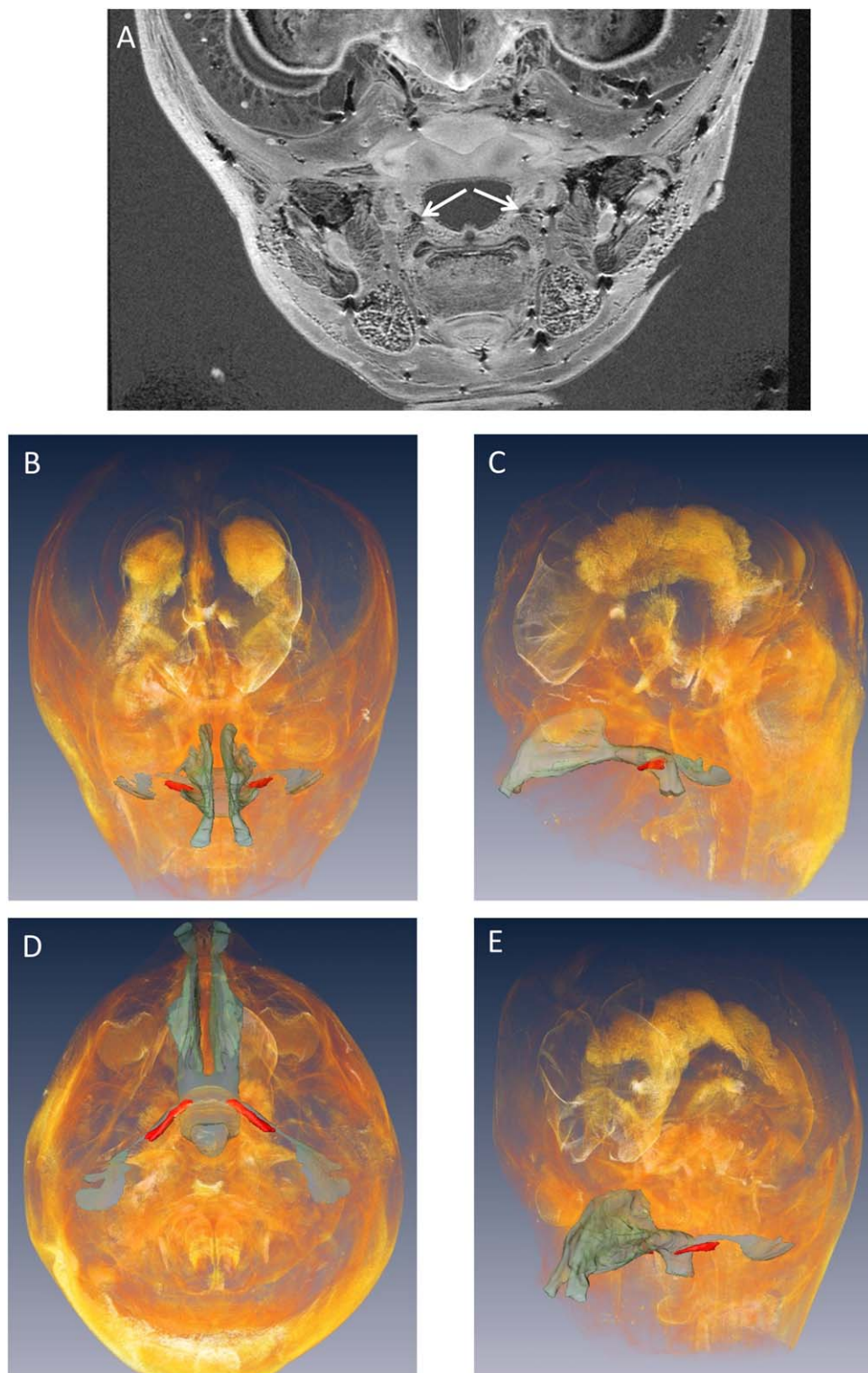


Fig. 3. At 13 weeks 5 days of gestation, the CRL 65-mm fetus (#F1289). **A:** The MRI image, coronal slice. Arrowhead points to the LVP. **B–E:** The 3D image from the images of the MRI. Coloring is the same as that in Figure 2. **B:** frontal view; **C:** lateral view; **D:** caudal view; **E:** left anterior oblique view.

facial nerve by means of the LPN (Fig. 5). The LVP is involved with the velopharyngeal function; the velopharyngeal function operates in swallowing and speech. Podvinec (1952) has proposed a hypothesis that in speech, the facial nerve dominates in coordi-

nating the movements of the palate and the muscles of the face, and that in swallowing, the glossopharyngeal and vagus nerves ensure the harmony of movement with the muscles of the pharynx and the esophagus. Some published studies of patients after

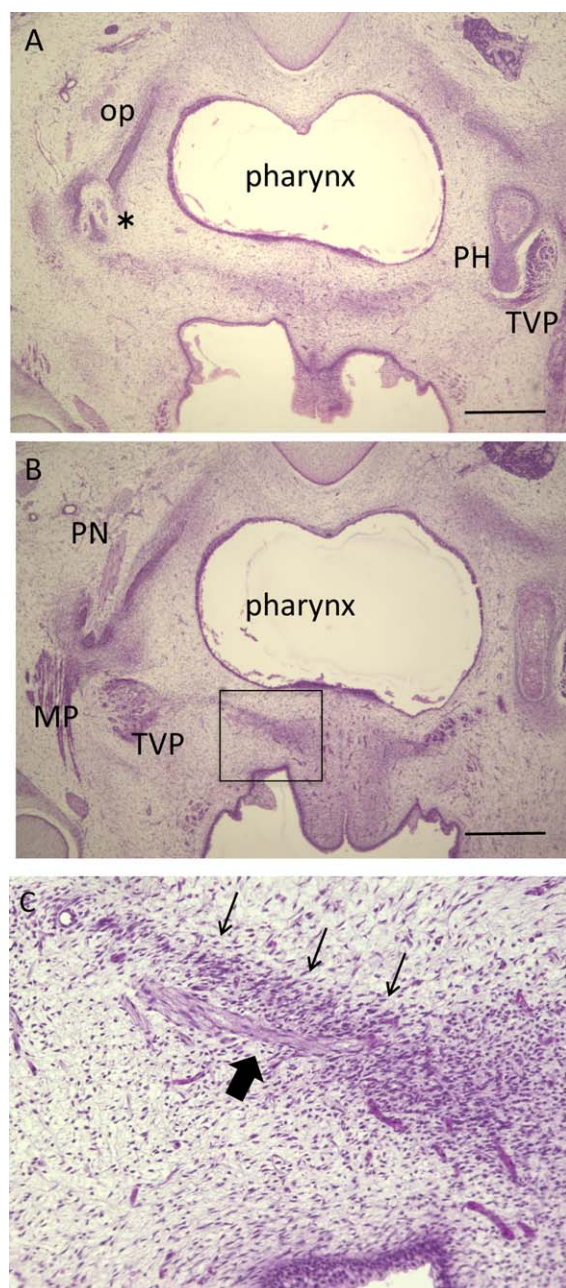


Fig. 4. A–C: Histological specimen of the CRL 40.7-mm fetus (#14202). C: Close-up image of the boxed area in B. Small arrows point to the LVP. The large arrow points to the lesser palatine nerve. The asterisk points to the palatine foramen. Op, os palatinum; PH, pterygoid hamulus; TVP, tensor veli palatini muscle; MP, medial pterygoid muscle; PN, palatine nerve. Scale bar = 500 μ m.

palatoplasty support the notion that the velopharyngeal closing pattern in articulation is different from that in deglutition. That is, the velopharyngeal function in deglutition depends on the constriction of the pharyngeal muscles innervated by the pharyngeal plexus rather than the movement of the soft palate, including the LVP (Moll, 1964; Yamaoka, 1973). The clinical phenomenon suggests a relationship between the LVP and the facial nerve such that insufficient movement of the soft palate on one side is often accompanied by facial palsy on the same side (Toyoda and Tachibana, 1986).

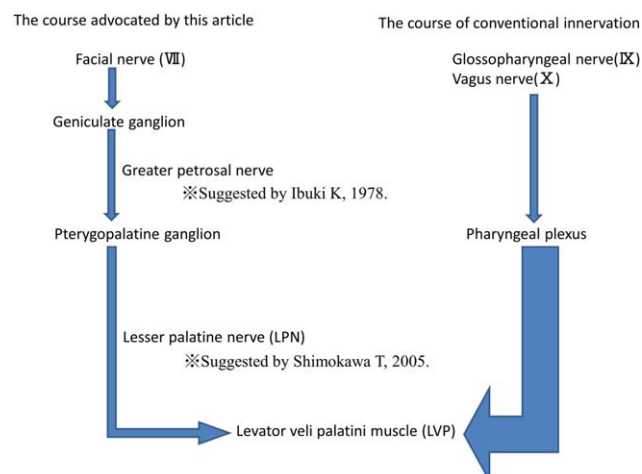


Fig. 5. Schematic showing the course of the innervation of the LVP.

In humans, the velopharyngeal function in deglutition is indispensable for living, and the muscular movements in deglutition are detectable during fetal development in the uterus. In contrast, the velopharyngeal speech function is acquired through learning within a few years after birth. It is interesting that the patients with both cleft palate and mental retardation often have speech disorders after palatoplasty, because of the assumed relationship between speech learning and the soft palate's movement (Kitamura, 1993). If articulation and deglutition are directed by different neural pathways, such as the LPN and pharyngeal plexus, this discrepancy in speech and swallowing functioning is understandable. On the other hand, the LVP is present only in mammals (Edgeworth, 1935), and "speech" is unique to humans; thus, it is reasonable that the innervation of the LVP should be studied from the perspective of evolution and comparative anatomy.

Clinical Implications of the LPN During Palatoplasty

All surgical operations including palatoplasty are performed based on clinical anatomy. Now, some researchers (Broomhead, 1951; Fujino, 1977) have suggested that the LPN is at risk of division during operation, but the LPN is not a focus of attention during the operation because it has been popularly assumed that the LPN is not a motor nerve. The LPN is a branch of the maxillary nerve, but it also innervates the palatal glands by the parasympathetic pathway [facial nerve (intermediate nerve) > greater petrosal nerve > pterygopalatine ganglion > lesser palatine nerve]. We hypothesize that the pathway includes a motor nerve. Identifying the LPN during palatoplasty and fully preserving it may lead to improvements in speech disorder persisting after palatoplasty. From now on, we intend to clarify the LVP innervation by stimulating the LPN during palatoplasty.

Experimental Procedures

Kyoto Collection of Human Embryos and Fetuses

Since 1961, a large number of human conceptuses have been collected in the Congenital Anomaly Research Center at Kyoto University and is called the Kyoto Collection (Nishimura et al., 1966, 1968). It is one of the four most famous human embryo collections in the world. The embryo collection now comprises more

TABLE 2. Summary of Observations of the LVP in Previous Reports

	CRL19 mm	20 mm	25 mm	28 mm
Domenech-Ratto (Domenech-Ratto, 1977)	Not observed	Observed	No data	Observed
Iwase & Kitamura (Iwase and Kitamura, 1985)	No data	Not observed	Observed	Observed

TABLE 3. Corresponding Ranges of CRL in CS 18 to 23^a

	CRL (mm)
CS 18	11.7~18
CS 19	15.5~21.5
CS 20	18~25
CS 21	19~26.4
CS 22	23~27.5
CS 23	23~32.2

^aAdapted from O'Rahilly and Müller (1987).

than 45,000 specimens. In the great majority of cases, pregnancy was terminated for social reasons during the first trimester of the pregnancy under the Maternity Protection Law of Japan (Nishimura et al., 1966; Yamada et al., 2004). It is important to note that the donation of embryos for research purposes was requested after surgical intervention at the clinic and did not, therefore, affect the mother's decision for termination. Neither the obstetricians nor the researchers coerced the mother into donating her embryo for research. Therefore, these embryos were obtained both legally and ethically (Nishimura et al., 1966; Nishimura, 1975). Further details on the embryo collection were reported previously (Nishimura et al., 1968; Iizuka, 1973; Shiota, 1991; Yamada et al., 2004; Yamada and Takakuwa, 2012).

Histological Sections

In the Kyoto Collection, parts of the normal human embryos and fetal tissue have been serially sectioned in 10- μ m-thick slices and stained with hematoxylin and eosin. In this study, a total of 32 embryos ranging from CS 19 to CS 23 (CS 19, n = 2; CS 20, n = 10; CS 21, n = 10; CS 22, n = 5; CS 23, n = 5) and 6 fetuses were selected and examined microscopically, and images were captured using a BIOREVO BZ-9000 (Keyence Corp., Japan).

3D Imaging

Phase-contrast X-ray computed tomography.

The 3D phase-contrast X-ray computed tomography (PCT) images were acquired using the imaging system at the Photon Factory, Institute of Materials Structure Science, High Energy Accelerator Research Organization (KEK, Tsukuba, Japan). In this system, X-rays are used as waves and phase-shifts of the waves are imaged, in contrast to conventional X-ray imaging, which is based on absorption-contrast. The specimens were embedded in 0.7% agarose gel. Additional principles and conditions required when

using PCT have been described in previous studies (Takeda et al., 1995; Yamada et al., 2012; Yoneyama et al., 2013). Two specimens of human embryos (1 case at CS 21 [#28066], and another at CS 23 [#22761]) were subjected to PCT. PCT has much higher resolution than MRI, but it has an upper limit of sample size. The maximum size is approximately 15 mm $\times$ 15 mm $\times$ 20 mm, so the fetal sample (which is larger than 30 mm) could not be scanned by PCT.

MRI

The MRI images were obtained using a 7 Tesla MR system (Biospec 70/20 USR, Bruker Biospin MRI GmbH, Ettlingen, Germany) with a 35 mm-i.d. ¹H quadrature transmit-receive volume coil. Two specimens of human embryos (1 at CS 21 [#21875] and CS 23 [#15919], respectively) and four specimens of human fetuses (CRL: 50 mm [#F1354], 57 mm, 61 mm [#36113], and 65 mm [#F1289]) were subjected to MR scanning. The head region of the embryos and fetuses was imaged using MRI for a detailed examination of the palatal muscles.

Data Processing

First, the images were opened using ImageJ software (ver.1.44, National Institutes of Health, Bethesda, MD), and examined in the original plane. Then, to facilitate comprehension of the 3D relationship, we constructed 3D images using the software AmiraTM (ver.5.4.5, Visage Imaging, Berlin, Germany).

The study was approved by the Ethics Committee at the Kyoto University Graduate School and Faculty of Medicine, Kyoto, Japan (E986). This work was conducted under the approval of the Photon Factory Program Advisory Committee at the Institute of Materials Structure Science, High Energy Accelerator Research Organization (Proposal No. 2009S2-006, 2012G138, 2013G514, 2014G018).

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References

- Arey LB. 1966. Development anatomy. Philadelphia: Saunders.
- Beevor CE, Horsley V. 1888. Note on some of the motor functions of certain cranial nerves (V, VII, IX, X, XI, XII), and of the three first cervical nerves, in the monkey (*Macacus sinicus*). *Proc R Soc Lond* 44:269–277.
- Broomhead IW. 1951. The nerve supply of the muscles of the soft palate. *Br J Plast Surg* 4:1–15.
- Cords E. 1910. Zur Morphologie des Gaumensegels. *Anatomischer Anzeiger* 37:305–318.
- De la Cuadra Blanco C, Peces Pena MD, Rodriguez-Vazquez JF, Merida-Velasco JA, Merida-Velasco JR. 2012. Development of the human tensor veli palatini: specimens measuring 13.6–137 mm greatest length; weeks 6–16 of development. *Cells Tissues Organs* 195:392–399.
- Dellon AL. 1989. The levator veli palatini muscle arises from the second branchial arch: a hypothesis. *Ann Plast Surg* 23:317–319.
- Dickson DR. 1972. Normal and cleft palate anatomy. *Cleft Palate J* 9:280–293.
- Domenech-Ratto G. 1977. Development and peripheral innervation of the palatal muscles. *Acta Anat (Basel)* 97:4–14.
- Edgeworth FH. 1916. On the development and morphology of the pharyngeal, laryngeal and hypobranchial muscles of mammals. *Q J Microsc Sci* 61:383–432.
- Edgeworth FH. 1923. On the development of the cranial muscles of tatusia and manis. *J Anat* 57:313–335.
- Edgeworth FH. 1935. The cranial muscles in vertebrate. Cambridge: Cambridge University Press.
- Fara M, Dvorak J. 1970. Abnormal anatomy of the muscles of palatopharyngeal closure in cleft palates: anatomical and surgical considerations based on the autopsies of 18 unoperated cleft palates. *Plast Reconstr Surg* 46:488–497.
- Foley JO. 1947. Functional components of the greater superficial petrosal nerve. *Proc Soc Exp Biol Med* 64:158–162.
- Fujino T. 1977. Microsurgical repair of the soft cleft palate. *Keio J Med* 26:91–98.
- Futamura R. 1906. Über die Entwicklung der Facialismuskulatur des Menschen. *Anatomische Hefte* 30:433–516.
- Gasser RF. 1967. The development of the facial nerve in man. *Ann Otol Rhinol Laryngol* 76:37–56.
- Huang MH, Lee ST, Rajendran K. 1998. Anatomic basis of cleft palate and velopharyngeal surgery: implications from a fresh cadaveric study. *Plast Reconstr Surg* 101:613–627; discussion 628–619.
- Ibuki K, Matsuya T, Nishio J, Hamamura Y, Miyazaki T. 1978. The course of facial nerve innervation for the levator veli palatini muscle. *Cleft Palate J* 15:209–214.
- Iizuka T. 1973. High incidence of cleft lip and cleft palate in the human embryos and early fetuses. *Okajimas Folia Anat Jpn* 50:259–271.
- Ishijima K, Sando I, Balaban CD, Miura M, Takasaki K. 2002. Functional anatomy of levator veli palatini muscle and tensor veli palatini muscle in association with eustachian tube cartilage. *Ann Otol Rhinol Laryngol* 111:530–536.
- Iwase M, Kitamura H. 1985. [Palatal muscles in human fetuses]. *Kaibogaku Zasshi* 60:711–727.
- Keller JT, Saunders MC, van Loveren H, Shipley MT. 1984. Neuroanatomical considerations of palatal muscles: tensor and levator veli palatini. *Cleft Palate J* 21:70–75.
- Kitamura R. 1993. The treatment plan of children with cleft lip and/or cleft palate with other anomalies. *J Jpn Cleft Palate Assoc* 18:117–122.
- Klueber K, Langdon HL. 1979. Anatomy of musculus levator veli palatini in the 15-week human fetus. *Acta Anat (Basel)* 105:94–105.
- Kochi S. 2007. Prevalence of cleft lip/palate and assessment of treatment in Japan: for enhancement of quality of life of cleft patients. *J Jpn Cleft Palate Assoc* 32:1–9.
- Kriens OB. 1969. An anatomical approach to veloplasty. *Plast Reconstr Surg* 43:29–41.
- Masuoka H, Kawai K, Mitsugochi A, Takeuchi M, Suzuki S. 2011. Results after primary palatoplasties for cleft palate; push back closure technique. *Jpn J Plast Surg* 54:965–973.
- Moll KL. 1964. Cineradiography in research and clinical studies of the velopharyngeal mechanism. *Cleft Palate J* 30:391–397.
- Nishimura H. 1975. Prenatal versus postnatal malformations based on the Japanese experience on induced abortions in the human being. In: Blandau R, editor. *Aging gametes: their biology and pathology*. Basel: Karger AG. p 349–368.
- Nishimura H, Takano K, Tanimura T, Yasuda M. 1968. Normal and abnormal development of human embryos: first report of the analysis of 1,213 intact embryos. *Teratology* 1:281–290.
- Nishimura H, Takano K, Tanimura T, Yasuda M, Uchida T. 1966. High incidence of several malformations in the early human embryos as compared with infants. *Biol Neonat* 10:93–107.
- Nishio J, Matsuya T, Ibuki K, Miyazaki T. 1976. Roles of the facial, glossopharyngeal and vagus nerves in velopharyngeal movement. *Cleft Palate J* 13:201–214.
- O’Rahilly R, Müller F. 2010. Developmental stages in human embryos: revised and new measurements. *Cells Tissues Organs* 192:73–84.
- O’Rahilly R, Müller F. 1987. Developmental stages in human embryos: including a revision of Streeter’s “Horizons” and a survey of the Carnegie collection. Washington, DC: Carnegie Institution of Washington Publication.
- Patten BM. 1953. Human embryology. New York: Blakiston.
- Podvinec S. 1952. The physiology and pathology of the soft palate. *J Laryngol Otol* 66:452–461.
- Rich AR. 1920. The innervation of the tensor veli palatini and levator veli palatini muscles. *Johns Hopkins Hosp Bull* 31:305–310.
- Ross MA. 1971. Functional anatomy of the tensor palati. Its relevance in cleft palate surgery. *Arch Otolaryngol* 93:1–3 passim.
- Seif S, Dellon AL. 1978. Anatomic relationships between the human levator and tensor veli palatini and the eustachian tube. *Cleft Palate J* 15:329–336.
- Shimokawa T, Yi S, Tanaka S. 2005. Nerve supply to the soft palate muscles with special reference to the distribution of the lesser palatine nerve. *Cleft Palate Craniofac J* 42:495–500.
- Shiota K. 1991. Development and intrauterine fate of normal and abnormal human conceptuses. *Congenit Anom* 31:67–80.
- Sonobe M. 1982. Studies on the development and innervation of the rat palatal muscles. *Jpn J Oral Biol* 24:864–892.
- Streeter GL. 1920. Weight, sitting height, head size, foot length, and menstrual age of the human embryos. *Contrib Embryol* 11:143–170.
- Streeter GL. 1948. Developmental horizons in human embryos. Description of age groups XV, XVI, XVII, XVIII, being the third issue of a survey of the Carnegie Collection. *Contrib Embryol* 32:133–203.
- Streeter GL. 1951. Developmental horizons in human embryos. Description of age groups XIX, XX, XXI, XXII and XXIII, being the fifth issue of a survey of the Carnegie Collection. *Contrib Embryol* 34:165–196.
- Takeda T, Momose A, Itai Y, Wu J, Hirano K. 1995. Phase-contrast imaging with synchrotron X-rays for detecting cancer lesions. *Acad Radiol* 2:799–803.
- Toyoda K, Tachibana M. 1986. Sixteen cases of soft palate paralysis. *Pract Otorhinolaryngologica* 79:421–426.
- van Loveren H, Saunders MC, Keller JT. 1983. Localization of motoneurons innervating the levator veli palatini muscle in the cat. *Brain Res Bull* 11:303–307.
- Vanderas AP. 1987. Incidence of cleft lip, cleft palate, and cleft lip and palate among races: a review. *Cleft Palate J* 24:216–225.
- Yamada S, Nakashima T, Hirose A, Yoneyama A, Tadedo T, Takakuwa T. 2012. Developmental anatomy of human embryo. In: Yamada S, Takakuwa T, editors. *The human embryo*. Rijeka, Croatia: Intech Publisher. pp 113–126.
- Yamada S, Takakuwa T. 2012. Introduction: overview of development in human embryos. In: Yamada S, Takakuwa T, editors. *The human embryo*. Rijeka, Croatia: Intech Publisher. p 3–20.
- Yamada S, Uwabe C, Fujii S, Shiota K. 2004. Phenotypic variability in human embryonic holoprosencephaly in the Kyoto Collection. *Birth Defects Res A Clin Mol Teratol* 70:495–508.
- Yamaoka M. 1973. [Fiberscopic studies of velopharyngeal movements of cleft palate patients (author’s trans)]. *Nihon Koku Geka Gakkai Zasshi* 19:29–43.
- Yoneyama A, Nambu A, Ueda K, Yamada S, Takeya S, Hyodo K, Takeda T. 2013. Phase-contrast X-ray imaging system with sub-mg/cm3 density resolution. *J Phys Conf Ser* 425.