

Original article

Vesicular swelling in the cervical region with lymph sac formation in human embryos

Ohga

Short title: Cervical swelling in human embryos

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Abstract

Vesicular swelling in the cervical region (VSC) is occasionally observed among human embryos around Carnegie stage (CS) 21. However, its mechanism and significance in fetal development are unclear. The present study aimed to analyze the relation of development of VSC with jugular lymph sac (JLS) formation. Serial histological sections that were digitalized from 14 embryos at CS20 and CS21 stored at the Kyoto Collection were used for the analysis. Subcutaneous edema and enlargement of the subarachnoid space were found to cause VSC. No obvious abnormalities in cranial regions that may be related to the VSC were detected on histological sections. Three-dimensional reconstructions revealed the following: (1) the JLS was located bilaterally at the levels between the 1st and 4th cervical vertebrae; (2) the JLS was pyramidal in shape; and (3) no severe deformity and/or malformation was found in all samples. The JLS was not connected to the subcutaneous tissue and subarachnoid space in all samples. The mean volume of the JLS increased nine-times from CS20 (0.02 mm³ in VSC (-) group) to CS21 (0.18 mm³ in VSC (-) group). The mean volume of the JLS was comparable between the VSC (-) and VSC (+) groups at both CS20 and CS21. A moderate correlation was observed between VSCd and the mean volume of the JLS in both

groups at CS20 ($R^2=0.75$) and CS21 ($R^2= 0.56$). In conclusion, the dynamics of the lymphatic system at the cervical region may contribute to VSC observed around CS21.

Key words: external vesicular swelling, Carnegie stage 21, jugular lymph sac, development of lymphatic system, 3D reconstruction

Introduction

Vesicular swelling in the cervical region (VSC) found among human embryos around Carnegie stage (CS) 21 has been described in previous studies through the observation of the Kyoto collection¹⁻⁴. Its mechanism and significance in fetal development are unclear. VSC seems to be a transient change in most embryos and has been found to be histologically a simple subcutaneous edema. Such an embryo may follow a normal course of development afterwards. However, embryos with anomaly related to cranial structure including the central nervous system may also develop VSC⁴. Töndury et al.⁵ found VSC in 12 embryo samples with 23-36 mm crown-rump length. VSC was originally described as a nuchal bleb (NB) in previous studies, but the term was later registered to describe fetus with cystic hygroma (OMIM 257350; <http://www.omim.org/entry/257350>). Thus, this abnormality was referred to as VSC in the present study.

As for the swelling at the cervical region, nuchal translucency (NT) and NB in cystic hygroma are well-known phenomenon. NT is a transient phenomenon observed at 10-14 weeks gestation, which is a diagnostic term used in fetal ultrasound scan⁶. NT enlargement is strongly associated with fetal chromosomal abnormalities such as

trisomy 21; therefore, NT measurement is included as part of prenatal ultrasonography screening during the first trimester⁷. Meanwhile, cystic hygromas, which are marked malformations of the lymphatic system in both the main lymphatic trunks and the peripheral lymphatic system, were found in aborted fetuses and postnatal babies with prominent swelling in the nuchal region^{8, 9}. Although the exact pathogenesis of the swelling at the cervical region in NT and NB is still unknown, changes in the lymphatic system dynamics at the cervical region during fetal development may be involved⁷⁻⁹. The lymphatic system is developed through the fusion of several separate primordia, all derived from the venous walls¹⁰⁻¹³. As for the cranial-cervical region, the bilateral jugular lymph sac (JLS) arises from the jugular vein (JV) after CS18¹⁰. This study aimed to describe the formation of VSC and its relation to JLS as a reference for the formation of the lymphatic system in the jugular region in human embryo around CS21. The dynamics of the lymphatic system at the cervical region in CS21 embryos were discussed by comparing them with NB in cystic hygroma and animal models.

Materials & Methods

Human embryo specimens

Fourteen Japanese human embryos at CS20 (n=4) and CS21 (n=10) (Crown-Rump length (CRL): 16.5-24.1 mm) were selected from the Kyoto Collection at the Congenital Anomaly Research Center of Kyoto University^{14,15}. The Kyoto Collection is one of the largest working collections of human embryos. While the field of human embryology started in the 19th century, today the field has grown, with a global focus on sharing the histological data of human embryos¹⁵. All embryos were obtained when a pregnancy was terminated during the first trimester for socioeconomic reasons under the Maternity Protection Law of Japan. All selected samples exhibited no overt damage. VSC was described as an external finding when the embryo was first observed externally. Samples were divided into two groups by external observation of VSC: VSC (+), VSC externally observed and VSC (-), VSC not observed externally.

Whole embryonic samples were fixed with formalin, embedded in paraffin, and serially sectioned. These samples were stained with hematoxylin-eosin staining. The

ethics committee of the Kyoto University Graduate School and Faculty of Medicine approved this study (Approval no.: E986, R0316).

Digitalization of histological sections and 3D reconstruction of lymphatic sac

The sections were scanned and stored as digital data using a film scanner (CanoScan 9000F; Canon, Tokyo, Japan) at 4800 dpi and digitalized with an Olympus virtual slide system (VS120-S5-J; Olympus, Japan). Sequential 2-D images were trimmed digitally using ImageJ 64-bit (ver. 1.46, National Institutes of Health, Bethesda, MD, USA). The JLS, JV, and several anatomical structures adjacent to the JLS (i.e., the cricoid, hyoid, epiglottis, and clavicle) were segmented on serial digital sections. Three-dimensional images were computationally reconstructed by piling the serial images with each positional relationship visually confirmed individually using Amira software (ver. 5.5.0, Visage Imaging, Berlin, Germany).

The VSC depth (VSCd) was measured at the midline on the re-sliced plane after 3D reconstruction (Fig. 1A). The plane was determined as that which included the cranial border of the hyoid and intervertebral space between the 2nd and 3rd cervical vertebrae (Fig. 1B). The VSCd was divided into the subarachnoid space depth (SAd) and the

subcutaneous tissue depth (SCd) as shown in Figure 1A.

JLS in the embryos were irregular in shape and this continued to the axillary parts and the internal thoracic lymph plexus. To define the border plane for the measurements, the XY-plane was set as the plane connecting the ventral-cranial edge of the cricoid and the cranial border of the 4th cervical vertebrae on the mid-sagittal plane (Fig. 1B). The height of the lymph sac was measured from the XY-plane to the most cranial point along the z-axis, while the volume of the lymph sac located at the cranial side of the XY-plane was measured as the lymph sac volume. We extracted both JLC and JV because it was difficult to separate both structures.

Results

Evaluation of the vesicular swelling in the region of the cervical region

VSC was described positive in 2 of the 4 samples, whereas it was negative in the remaining 2 samples at CS20. VSC was described positive in 3 of the 10 samples, whereas it was negative in the remaining 7 samples at CS21 (Table 1). VSCd in each embryo was measured, which revealed that the external observation well matched with

VSCd measurements. Namely, the VSCd ranged from 0.54 mm to 2.07 mm in the VSC (+) group, whereas it ranged from 0.20 mm to 0.48 mm in the VSC (-) group (Fig. 2, Table 1). In the VSC (+) group, increases in SCd were prominent in most of the samples and occasional increases in SAd were also detected (see sample ID 28835 in Fig. 3). No obvious abnormalities causing the VSC were noted in the cranial, cardiac, and vascular regions of the histological sections.

Evaluation of the JLS

JLS was observed bilaterally on transverse histological sections at the levels between the caudal side of 1st cervical vertebra and the cranial side of the 4th cervical vertebra (Fig 3, 4). The jugular vein was detected to be adjacent to the lymph sac, which was connected to the JLS in all samples. JLS was irregular in shape and extended to the axillary sac and internal thoracic lymph plexus. Meanwhile, the JLS was not connected to any subcutaneous tissue in all samples, even in those in which edema was obviously detected.

Three-dimensional reconstructions revealed that pyramidal shape of the lymph sac and absence of severe deformity and/or malformation in all samples (Fig. 4; Supplementary

Videos 1-3).

The mean volume of the JLS increased nine-times from CS20 (0.02 mm³ in the VSC (-) group) to CS21 (0.18 mm³ in the VSC (-) group) (Table 1). The mean volume of the JLS between the VSC (-) and VSC (+) groups was comparable at CS20 (0.02 mm³ and 0.04 mm³, respectively) and CS21 (0.18 mm³ and 0.31 mm³, respectively, $p=0.25$) (Fig. 5A). A moderate correlation was observed between VSCd and the mean volume of the JLS in both groups at CS20 ($R^2=0.75$) and CS21 ($R^2=0.56$) (Fig. 5B).

Discussion

The development of the lymphatic system is established by the fusion of many separate primordial lymph sacs¹⁰⁻¹³. The bilateral JLS arises from the JV after CS18¹⁰. The JLS is thereafter isolated and subsequently reorganized into the lymph nodes and then reconnected to the JV¹⁰⁻¹³. This process is usually completed in human embryos at 10 weeks' gestational age. The present study demonstrated the rapid enlargement of the JLS at CS20 and CS21 by both 3D reconstruction and morphometry, which was previously described in limited studies¹⁰⁻¹³.

The relation between VSC and JLS enlargement was observed in several different

conditions. In knockout mice models, the correlation between nuchal edema and lymphatic distention was controversial as both enlargement of JLS and no formation of JLS were observed¹⁶. In trisomy 16 mouse, the animal model for human Down syndrome, extremely enlarged JLS was observed at an earlier developmental stage than the occurrence of nuchal edema. These studies indicate that enlarged JLS could be the result of a delay in reconnection with the venous system¹⁷. As for the correlation between VSC and JLS enlargement in cystic hygroma, a delay in JLS drainage into the JV is indicated as the possible mechanism¹⁸.

VSC observed around CS21 may also be related to the dynamic changes in the lymphatic system at the cranial cervical region. In the present study, a moderate correlation between VSCd and the mean volume of the JLS was observed, irrespective of external findings of VSC (-) and VSC (+). It might be possible that such rapid growth of the lymphatic system might cause a transient distended JLS and formation of VSC, which indicates the accumulation of lymph fluid within the lymphatic system at the cervical region.

The prosencephalon exponentially increases the volume after CS21¹⁹, which may accelerate the blood supply to the brain. The circle of Willis, which consists of the main arteries supplying blood from the internal carotid arteries and vertebral - basilar artery

to the prosencephalon, is formed at CS21²⁰⁻²². As for the region of the external carotid arteries, a subcutaneous capillary plexus is first detected at CS20, spreads upward at CS21, and reaches the vertex of the head at CS23²³. Similar to the development of the subcutaneous capillary plexus, the return of blood flow through the subcutaneous vein should increase. We speculate that the increase in blood flow in the cranial region around CS21 might also contribute to the pathogenesis of VSC. Furthermore, the choroid plexus in the lateral ventricle, which produces cerebral fluid, is prominent after CS20²⁴. The production of cerebral fluid might affect the increase in SAd detected in several samples.

The present study has some limitations. First, our data were not very clear in that the mean volume of the JLS was comparable between the VSC (-) and VSC (+) groups at both CS20 and CS21. The correlation between VSCs and the volume of JLS is merely an association and does not indicate a causal relationship. Anatomically speaking, JLSs are not directly connected to the VSC. Second, VSC seems to be histologically heterogeneous. At least two different causes, namely, enlarged SA and SC, were observed in the present study. We could not provide any data explaining the mechanism of SA enlargement observed in several samples and whether SA enlargement itself is a normal or abnormal phenomenon. Previous studies have

indicated several other causes, such as a combination of delayed or disturbed lymphangiogenesis, cardiac and vascular abnormalities, and abnormal extracellular matrix components⁶⁻⁹. Further analytical studies are warranted to clarify the developmental mechanism of VSC and the correlation of VSC with JLS as well as to further analyze whether VSC is indeed a normal or abnormal phenomenon. Third, it still remains unclear whether VSC is really a transient event around CS21 or whether it continues after CS22. An analysis using the samples from CS22 and higher is required to resolve this in addition to analysis using the samples from CS20 and CS21. Other approaches apart from that (use of serial histological sections) utilized in the present study are necessary for evaluating the VSC in large samples at CS22 and later. The use of MR imaging data and PXCT may be suitable for the evaluation of VSC in larger samples.

In conclusion, the dynamics of the lymphatic system in the cranial and cervical region may contribute to the formation of VSC around CS21. However, whether such dynamics are due to normal physiological changes during development or potentially abnormal causes remain unclear. Further research on the formation of the lymphatic system and fluid regulation during embryonic period will allow for better understanding of the causes and pathogenesis of the VSC around CS21, which can ultimately help to discriminate

between normal and abnormal VSC.

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Disclosure

None

References

1. Nishimura H, Uwabe C, Shiota K. Study of human post-implantation conceptuses, normal and abnormal. *Okajimas Folia Anat Jpn* 1987;63:337-357.
2. Nishimura H, Okamoto N. Abnormalities of the central nervous system. In Nishimura H, Okamoto N, eds. Sequential atlas of human congenital malformations: observations of embryos, fetuses, and newborns.. Baltimore, MD: University Park Press;1976: 34-75.
3. Nishimura H. Prenatal versus postnatal malformations based on the Japanese experience on induced abortions in the human being. In Blandau RJ, ed. Aging Gametes. Basel: S. Karger;1975:349-368.
4. Shiota K, Uwabe C, Tanimura T, Nishimura H. Nuchal blebs occasionally found in human seven week embryos. *Teratology* 1973;8:105.
5. Töndury G, Schenk R, Morger R. Menschliche Keimlinge mit Nackenblasen. *Biol Neonat* 1959;1:68-105.
6. Nafziger E, Vilensky JA. The anatomy of nuchal translucency at 10-14 weeks gestation in fetuses with trisomy 21: An incredible medical mystery. *Clin Anat* 2014;27:353-359.
7. Haak MC, van Vugt JMG. Pathophysiology of increased nuchal translucency: A

review of the literature. *Hum Reprod Update* 2003;9:175-184.

8. Chervenak FA, Isaacson G, Blakemore KJ, et al. Fetal cystic hygroma. Cause and natural history. *N Engl J Med* 1983;309:822-825.
9. Byrne J, Blanc WA, Warburton D, Wigger J. The significance of cystic hygroma in fetuses. *Hum Pathol* 1984;15:61-67.
10. O'Rahilly R, Müller F. The cardiovascular and lymphatic system. In: O'Rahilly R, Müller F, eds. *Human Embryology & Teratology*. 3rd ed. New York: Wiley-Liss; 2001:175-228.
11. van der Putte SCJ, van Limborgh J. The embryonic development of the main lymphatics in man. *Acta Morphol Neerl Scand* 1980;18:323-335.
12. Sabin FR. The lymphatic system in human embryos, with a consideration of the morphology of the system as a whole. *Am J Anat* 1909;9:43-91.
13. Nishimura H, Takano K, Tanimura T, Yasuda M. Normal and abnormal development of human embryos: first report of the analysis of 1,213 intact embryos. *Teratology* 1968;1:281-290.
14. Yamaguchi Y, Yamada S. Cells Tissues Organs. The Kyoto Collection of Human Embryos and Fetuses: History and Recent Advancements in Modern Methods. 2018;205:314-319.

15. Burger NB, Bekker MN, Kok E, et al. Increased nuchal translucency origins from abnormal lymphatic development and is independent of the presence of a cardiac defect. *Prenat Diagn* 2015;35:1278-1286.
16. Haak MC, Bartelings MM, Jackson DG, Webb S, Vugt JM, Groot AC. Increased nuchal translucency is associated with jugular lymphatic distension. *Hum Reprod* 2002;17:1086-1092.
17. Gilbert-Barness E, Debich-Spicer D. *Fetal hydrops and cystic hygroma in Embryo and Fetal Pathology: color atlas with ultrasound correlation*. Cambridge: Cambridge University Press; 2004:321-334.
18. Van der Putte SCJ. *The Development of the Lymphatic System in Man (Advances in Anatomy, Embryology and Cell Biology)*. New York, NY: Springer-Verlag; 1975.
19. Shiraishi N, Katayama A, Nakashima T, et al. Morphology and morphometry of the human embryonic brain: A three-dimensional analysis. *NeuroImage* 2015;115:96-103.
20. Padget DH. The development of the cranial arteries in the human embryo. *Contr Embryol* 1948;12:205-261.

21. Takakuwa T, Koike T, Muranaka T, et al. Formation of the circle of Willis during human embryonic development. *Congenit Anom (Kyoto)* 2016;56:233-236.
22. Furuichi K, Ishikawa A, Uwabe C, Makishima H, Yamada S, Takakuwa T. Variations of the circle of Willis at the end of the human embryonic period. *Anat Rec* 2018;301:1312-1319.
23. O'Rahilly R, Müller F. Developmental stages in human embryos: including a revision of Streeter's Horizons and a survey of the Carnegie Collection. Washington: Carnegie Inst of Washington; 1987.
24. Shiraishi N, Nakashima T, Yamada S, Uwabe C, Kose K, Takakuwa T. Morphogenesis of lateral choroid plexus during human embryonic period. *Anat Rec (Hoboken)* 2013;296:692-700.

Figure legends

Figure 1. Morphometrical landmark and definition of the measurements

A) Measurement of the vesicular swelling in the cervical region (VSC). The depth of VSC (VSCd) was measured on the re-sliced plane after three-dimensional reconstruction. The plane was determined as the plane that included the cranial border of the hyoid and the intervertebral space between the 2nd and 3rd cervical vertebrae.

SAd, depth of subarachnoid space; SCd, depth of subcutaneous tissue

B) Left lateral view image indicating the relative position of the jugular lymph sac (JLS) and adjacent anatomical structures.

The XY-plane and Z-axis used for morphometry are shown. The red line indicates the plane in which the vesicular swelling in the cervical region (VSC) was evaluated. The purple, yellow, dark green, and light green colors indicate the jugular lymph sac, epiglottis, hyoid, and cricoid, respectively. C1-C4: 1st to 4th cervical vertebrae

Fig. 2 Depth of vesicular swelling in the cervical region, subcutaneous tissue, and subarachnoid space measured in each sample

VSC (+), VSC externally observed; VSC (-), VSC not observed externally; CS, Carnegie

stage

Fig. 3 Representative gross pictures of the samples and histological sections showing vesicular swelling in the cervical region (VSC).

The depth of VSC (VSCd) was minimal in the sample (ID 1071). The depth of the subarachnoid space (SAd) was prominent in the sample (ID 28835). The depth of subcutaneous tissue (SCd) was prominent in the sample (ID 21231).

VSC (+), VSC externally observed; VSC (-), VSC not observed externally.

Fig. 4 Histology of the cervical region of the sample (ID 19846) showing vesicular swelling and a jugular lymph sac.

A) Histology of the cervical region

SC; subcutaneous tissue, SA; subarachnoid space, R; right lymph sac, L; left lymph sac

B) Histology of the right lymph sac (high magnification) and 3D reconstruction

C) Histology of the left lymph sac (high magnification) and 3D reconstruction

Histological image and 3D reconstruction in subpanel C) were inverted for comparison with those in subpanel B). In this sample, the volume was different between the right and left sacs (depth of vesicular swelling in the cervical region (VSCd)=2.07 mm, depth

of subcutaneous tissue (SCd) =1.67 mm).

Fig. 5 Evaluation of the jugular lymph sac

A) Left lateral view of the three-dimensional reconstruction. The left lymph sac is shown.

The purple, yellow, dark green, and light green colors indicate the jugular lymph sac, epiglottis, hyoid, and cricoid, respectively.

See Supplementary Videos 1-3

B) Volume of the jugular lymph sac of the VSC (+) and VSC (-) groups at CS20 and CS21.

VSC (+), VSC externally observed; VSC (-), VSC not observed externally.

C) Volume of the jugular lymph sac according to the depth of vesicular swelling in the cervical region (VSCd)

Supporting information

Supplementary Table 1. Morphometry of the vesicular swelling in the cervical region and cervical lymph sac

Supplementary Video 1: Three-dimensional reconstruction of the jugular lymph sac and adjacent organs [sample ID 1071, VSC externally not observed (VSC (-)), depth of vesicular swelling in the cervical region (VSCd) =0.37 mm].

The purple, yellow, dark green, light green, light blue, and red colors indicate the jugular lymph sac, epiglottis, hyoid, cricoid, clavícula, and Jugular artery, respectively.

Supplementary Video 2: Three-dimensional reconstruction of the jugular lymph sac and adjacent organs [sample ID.28835, VSC externally observed (VSC (+)), depth of vesicular swelling in the cervical region (VSCd) =0.69 mm, depth of subarachnoid space (SAd) = 0.43 mm]

The purple, yellow, dark green, light green, light blue, and red colors indicate the jugular lymph sac, epiglottis, hyoid, cricoid, clavícula, and jugular artery, respectively.

Supplementary Video 3: Three-dimensional reconstruction of the jugular lymph sac and adjacent organs [sample ID 21231, VSC externally observed (VSC (+)), depth of vesicular swelling in the cervical region (VSCd) =1.33 mm]

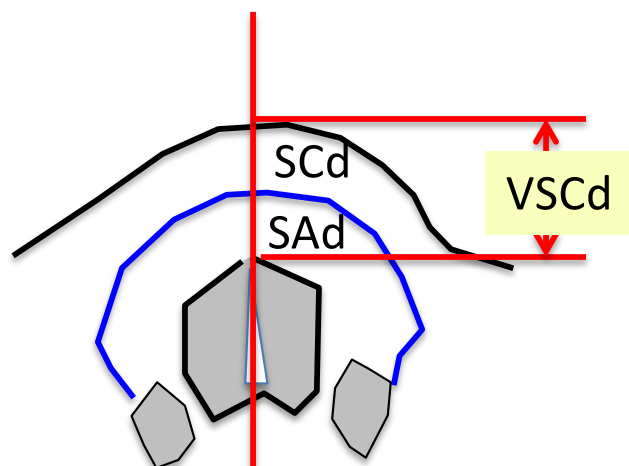
The purple, yellow, dark green, light green, light blue, and red colors indicate the jugular lymph sac, epiglottis, hyoid, cricoid, clavícula, and jugular artery, respectively.

Table 1 Morphometry of the VSC and jugular lymph sac

CS	External	(n)	CRL	VSC depth (mm)		JLS Volume (mm ³)	
	VSC		mean	mean	range	mean	Range
20		4	17.3	0.48		0.03	
	(-)	2	16.7	0.23	0.20-0.27	0.02	0.01-0.03
	(+)	2	18.6	0.73	0.54-0.92	0.04	0.02-0.06
21		10	20.2	1.13		0.27	
	(-)	3	19	0.40	0.35-0.48	0.18	0.12-0.28
	(+)	7	21.1	1.45	0.69-2.07	0.31	0.12-0.57

CRL, Crown-Rump length; VSC, vesicular swelling in the cervical region; JLS, jugular lymph sac

A



Larynx at the level of
cranial border of cricoid

B

