Korean J Oral Anatomy Vol. 37, no. 1 (2016) pp.1~8

Localization patterns of LYVE1 and CD31 in mice tongue development

Sanjiv Neupane[1], Jaemin Cho[1], Sung Won Lee[1], Hyuk Moon Son[1], Yong Hyun Bae[1], Sunghyun Kim[1], Wern-Joo Sohn[1], Youngkyun Lee[1], Jae-Kwang Jung[2], Jae-Young Kim[1,*]

[1]*Department of Biochemistry,* [2]*Department of Oral Medicine, School of Dentistry, IHBR, Kyungpook National University, Daegu, 41940, Korea*

접수: 2016년 10월 15일/ 수정접수: 2016년 12월 14일/ 게재 승인: 2016년 12월 19일/ 출간: 2016년 12월 31일

Lymphatic vessel endothelial hyaluronan receptor-1 (LYVE1) is a cell surface receptor on lymphatic endothelial cells which is the earliest known marker of lymphatic endothelial commitment. Platelet endothelial cell adhesion molecule also known as cluster of differentiation 31 (CD31) is a well-known marker for blood vessel formation. In this study, we examined the localization patterns of LYVE1 and CD31 in the developing tongue at E14, E16, E17 and PN0, PN5, PN10, PN15 and PN20 to understand the detailed developmental processes of lymphangiogenesis along with vasculogenesis by immunostaining examinations. Our observations revealed that there were specific patterns of proteins localizations through developmental stages. At E14, LYVE1 was localized mainly in the ventral region along the median fibrous septum and laterally in between the genioglossus and hyoglossus muscle forming regions. The localization of CD31 was more ubiquitous in the developing tongue at E14. The localization patterns of LYVE1 and CD31 showed more specified in the dorsal and ventral region at E16. In addition, branching formation of the lymphatic and blood vessels were more evident. At E17, more distinct localization patterns of LYVE1 and CD31 were observed in the intrinsic tongue muscles forming region. At later stages, the LYVE-1 and CD31 were mostly localized in the interface between the muscle fibers. Our results suggest that there would be relationships between lymphangiogenesis and vasculogenesis in developing mouse tongue. These normal developmental processes in lymphangiogenesis and vasculogenesis would be an excellent foundation for further studies in pathological conditions and tongue development malformations.

Keywords: Lymphangiogenesis, Vasculogenesis, Tongue development, Organogenesis

***** Corresponding author: Jae-Young Kim.

Department of Biochemistry, School of Dentistry, IHBR, Kyungpook National University, 2177, Dalgubeol-daero, Jung-gu, Daegu, 41940, Korea

Tel: 82-53-420-4998, Fax: 82-53-421-4276, E-mail: jykim91@knu.ac.kr

INTRODUCTION

The tongue has important role in speaking, swallowing, mastication and degustation, because it is a highly flexible organ[1]. Most of the reports on tongue development have been studied using mice model systems, effective for molecular and genetic tools, since they provide various useful experimental approaches with its musculature, derived from occipital somites[2,3]. In mice tongue starts to develop at E11 from lateral lingual swellings[4] which merge together forming tongue primordia at E11.5[3,5]. Tongue increases in size following proliferation and differentiation in the epithelium and mesenchyme[6]. Some cells in the mesenchyme become myoblast by E13[7] and give rise to myotubes at around E15[8]. Similarly, the differentiation of tongue epithelium begins at E13 and forms circumvallate, fungiform and foliate papilla[4]. The mobility is high in the anterior two-third of tongue and has crucial role in speaking, and mastication[9]. However, the posterior third has less mobility which is known as the pharyngeal area and plays a prominent role in upper airway maintenance[9]. In human, the tongue has eight skeletal muscles (four extrinsic and four intrinsic) in different locations due to this the tongue can show movements in all directions[3]. For these dynamic movements of tongue, there is necessary complex system of nerve innervations. Except palatoglossus, an extrinsic muscle (a vagus innervated muscle), all seven muscles are innervated by hypoglossal nerve which is primary motor nerve important for superficial sensation and movement of tongue[10]. According to these systemic architectures in tongue functions, the tongue showed very dense loops of the vessels beneath the epithelium in leap pattern, which enables systemic circulation[11]. Although the lymphatic vessel of the tongue is considered to play prominent role in drainage, and it is also can be assumed that crucial relation is found in tongue development between lymphatic and blood vessels, but there are still no studies about lymphatic vessel developmental pattern.

The lymphatic system is an open-ended, one-way transit system having wide network of capillaries, collecting vessels and ducts which invade most of the organs[12]. Initial lymphatic vessels have non-fenestrated single layer of endothelial cells having attenuated cytoplasm and poorly developed basal lamina[13]. Moreover, lymphatic vessels are devoid of pericytes and major intercellular junctional elements are fewer in numbers than in blood vessels[14]. The lymphatic endothelial cells are anchored to surrounding connective tissue by fine anchoring filaments[15] which are made of fibrillin[16]. Merging of initial lymphatic vessels results formation of larger vessels called precollectors[17] and serves as the initial drainage routes of lymph. Precollectors are similar with the initial lymphatic vessel structurally[17]. In addition, the precollectors have well developed muscular coat and thin and well- formed valves to prevent retrograde flow of the lymph[17]. The transdifferentiation of venous endothelial cells towards the lymphatic endothelial cells, separation of lymphatic and blood vessels, sprouting and maturation of lymphatic vessels are essential for lymphatic vascular development[18]. The progenitors of lymphatic endothelial cells (LECs) originate from venous endothelial cells[19,20], intersomitic veins[21] and superficial venous plexus[22]. The LEC progenitor expresses Prox1 transcription factor and a Lymphatic vessel hyaluronan receptor-1 (LYVE-1) in the embryonic cardinal vein at around

E9.5[21,23]. The lymphangiogenesis in mouse encompass lymphatic endothelial cell fate commitment marked by LYVE-1 expression[24], lymphatic sprouting marked by expression of VEGFR-3, VEFG-C and VEGF-D[25,26], separation of lymphatic and blood vasculature mediated by tyrosine kinase SYK and adaptor protein Slp76[27] and maturation of lymphatic vessel marked by persistent expression of LYVE-1[28]. Moreover, LYVE-1 (lymphatic vessel hyaluronan receptor-1), is one of the most widely used markers of lymphatic endothelial cells both in normal and tumor tissues[29].

In this study, we carefully examined the developmental processes and localization patterns of lymphatic vessels in mice tongue development along with blood vessel formation to understand the developmental mechanisms of lymphangiogenesis.

MATERIALS AND METHODS

Animal

Adult Institute of Cancer Research (ICR) mice were housed in a temperature-controlled room (22°C) under artificial illumination (lights on from 05:00 to 17:00), at 55% relative humidity, with free access to food and water. Mouse embryos were obtained from time-mated pregnant mice. The day on which a vaginal plug was confirmed was designated as embryonic day 0 (E0). We used tongues from embryonic mice at E14, E16 and E17 and postnatal mice at day 0, 5, 10 and 20.

Immunochemistry

Primary antibodies were used against LYVE1, 1:100 (Rabbit anti-Mouse) (Cat. no. ab14917, Abcam, UK) and CD31, 1:100 (Rat anti-Mouse) (Cat. no. 550274, BD Pharmingen, USA). The secondary antibodies were used alexa-flour 488 tagged polyclonal goat-anti-rabbit, 1:1000 (Cat. no. RSA1241, Bioacts, Korea). The nuclear staining was performed with Vectashield mounting media with DAPI (Cat. no. H-1200, Vector Laboratories, Inc. Burlingame, CA, USA). The immunostaining was visualized by detecting fluorescence under the fluorescent microscope *(Leica DM2500, Leica Microsystems, Germany*).

Methanol dehydrated embryonic tongues were rehydrated using methanol and phosphate buffered saline and tongues were embedded in 2% low melting agarose gel (Cat. no. V2111, Promega, Madison, WI, USA). We prepared 250 μm frontal slice sections using a vibratome (Leica VT1000s, Leica Microsystems, Germany) and processed for immunostaining. Briefly, slice sections were permeabilized with 0.1% TritonX-100 in PBS for 1 hour at 4 ^{0}C and blocked for 1 hour. Primary antibodies were added (1:400; LYVE1 and 1:200; CD31) and incubated at 4 ^{0}C for 20 hours. Lymphatic and blood vessels were detected with alexa-flour 488 tagged polyclonal goat-anti-rabbit, 1:1000 (Cat. no. RSA1241, Bioacts, Korea) and alexa-flour 647 tagged goat-anti-rat (1:1000) (Cat. no. ab150159, Abcam, UK) respectively. Slides were examined under a confocal microscope (Leica TCS, SP8, Leica, Germany). By scanning 20 thin sections (7-8 μm distance) of each sample, three-dimensional images of each tissue sample were assembled.

RESULSTS AND DISCUSSION

In this study, we examined the localization pat-

terns of LYVE-1 and CD31 during mice tongue development to understand the lymphangiogenesis and its relationship with vasculogenesis. We performed whole mount immunohistochemistry in the frontal sections of tongue at embryonic day 14, 16 and 17 (Fig.1). At earlier stage of E14, the LYVE-1 was sporadically localized in proximity with the lingual artery forming region and along the region between the genioglossus muscle and inferior longitudinal muscle forming region (Fig. 1a). Moreover, LYVE-1 was localized along the median fibrous septum forming region along the CD31 localization (Fig. 1a). The onset of LYVE-1 localization in tongue tissue follows the localization of CD31 which is coinciding with the previous reports[30,31].This result suggests that LYVE-1 positive LECs might be migrated from the blood vessels from other parts and populated in the tongue region which later forms the lymphatic vessels.

The localizations of LYVE1 and CD31 show more specified patterns in the dorsal and ventral regions at E16 tongue (Fig. 1b). In addition, LYVE1 and CD31 positive reactions reveal that there is branching formation of lymphatic and blood vessels. At E16, as the CD31 localization was observed in the mesh like structure throughout the tongue muscles, LYVE-1 Localization was more patterned in the vertical and transverse muscle forming region showing the duct-like structure (Fig. 1b). Similarly, the styloglossus and superior longitudinal muscle forming region also showed the localization of LYVE-1 in the mesh like structure suggesting that the network of capillaries formed from lateral side of tongue and progresses to the centre (Fig. 1b), which is more evident at E17 (Fig. 1b). LYVE-1 was expressed along with CD31 which is the evidence that the lymph sacs are originated along blood capillary.

At E17, the patterned localization of LYVE-1 and CD31 in the the network of lymphatic and vascular cappillaries respectively throughout the tongue were observed (Fig. 1c). More distinct localization patterns of LYVE-1 and CD31 are observed in the intrinsic tongue muscle forming regions showing longitudinal, vertical and transverse patterns (Fig. 1c). The strong positive localization pattern of LYVE-1 is even increased when compared with that of E16. LYVE-1 and CD31 are localized at the constant distance from the tongue surface (Fig. 1c).

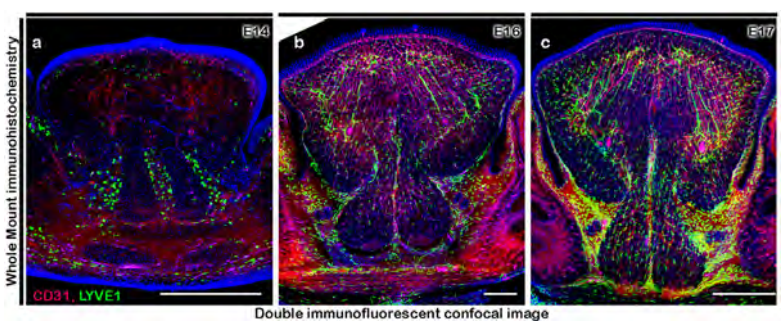

Figure 1. Whole-mount immunohistochemistry showing localization patterns of CD31 and LYVE-1 by double immunofluorescent staining. Confocal images showing localization patterns of LYVE-1 (Green) and CD31 (Azalea purple) at E14 (a), at E16 (b) and at E17 (c). Scale bars: 500 μm (a, c), 250 μm (b).

At later stage of PN0, Lymph sacs are concentrated at the dorsal surface of tongue, and are also scattered throughout the base of tongue (Fig. 2a). Similarly, the dorsal surface of tongue is distinctly appeared to be LYVE-1 positive, and there is a lining of LYVE-1 on the surface of tongue at PN5 (Fig. 2b). LYVE-1 and CD31 are localized in longitudinal, vertical, and transverse patterns at PN10 (Fig. 2c-d) which show LYVE-1 was localized in the interface between intrinsic muscle fibers along with CD31 localization and is especially concentrated along the lining of dorsal surface of tongue and its intrinsic muscle (Fig. 2c-d).

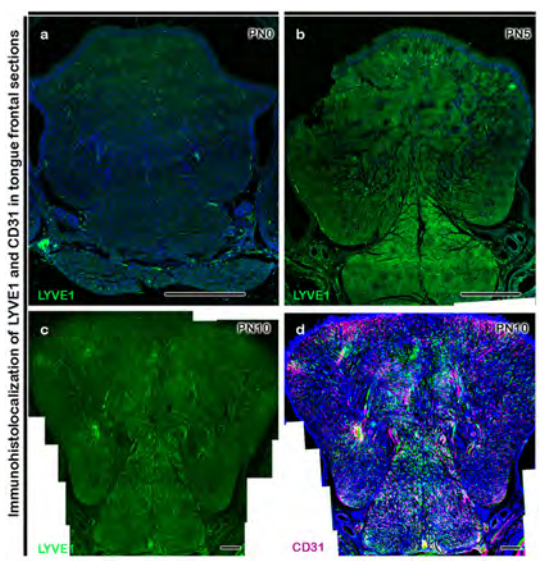

Figure 2. Immunolocalizations of LYVE-1 and CD31 in developing tongue. Localization of LYVE-1 at PN0 (a) PN5 (b) and PN10 (c). Double fluorescent immunostaining of developing tongue at PN10 showing specific localization patterns of LYVE-1 and CD31. Scale bars: 500 μm (a, b), 50 μm (c, d).

The localization of LYVE-1 was similar with that of PN10 (Fig. 3a) whereas at PN20 it was significantly increased, especially at the central part of tongue. These results of localization patterns at PN0, 5, 10, 15 and 20 suggest that the network of lymphatic and blood vascular capillaries was observed in the interface of muscle fibres suggesting that lymphangiogenesis and vasculogenesis progress simultaneously in the tongue along with the differentiation of muscle fibres.

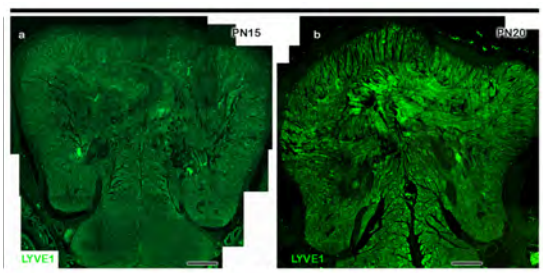

Figure 3. Immunolocalization of LYVE-1 at PN15 (a) and PN20 (b). Scale bars: 50 μm (a, b).

Our study would provide the basic knowledge of lymphangiogenesis during mouse tongue formation which would be helpful for understanding the further characterization of the underlying molecular mechanisms for lymphangiogenesis and may provide therapeutic approaches to suppress diseases such as cancer by selective inhibition, lymphademas and inflammation-related diseases and stimulation of lymphangiogenesis in lymphatic insufficiency.

REFERENCES

1. Noden DM, Francis-West P: The differentiation and morphogenesis of craniofacial muscles. Dev Dyn Off Publ Am Assoc Anat 235:1194-1218, 2006.
2. Shuler CF, Dalrymple KR: Molecular regulation of

tongue and craniofacial muscle differentiation. Crit Rev Oral Biol Med 12(1):3-17, 2001.

3. Parada C, Han D, Chai Y: Molecular and cellular regulatory mechanisms of tongue myogenesis. J Dent Res 91(6):528-535, 2012.
4. Paulson RB, Hayes TG, Sucheston ME: Scanning electron microscope study of tongue development in the CD-1 mouse fetus. J Craniofac Genet Dev Biol 5:59-73, 1985.
5. Nagata J, Yamane A: Progress of cell proliferation in striated muscle tissues during development of the mouse tongue. J Dent Res 83:926-929, 2004
6. Nie X: Apoptosis, proliferation and gene expression patterns in mouse developing tongue. Anat Embryol 210:125-132, 2005.
7. Buckingham M, Bajard L, Chang T, Daubas P, Hadchouel J, Meilhac S, Montarras D, Rocancourt D, Relaix F: The formation of skeletal muscle: from somite to limb. J Anat 202: 59-68, 2003.
8. Yamane A, Mayo M, Shuler C, Crowe D, Ohnuki Y, Dalrymple K, Saeki Y: Expression of myogenic regulatory factors during the development of mouse tongue striated muscle. Arch. Oral Biol 45:71-78, 2000.
9. Zaidi FN, Meadows P, Jacobowitz O, Dsvidson TM: Tongue anatomy and physiology, the scientific basis for a novel targeted neurostimulation system designed for the treatment of obstructive sleep Apnea. Neuromodulation 16:376-386, 2013.
10. Moore KL, Persaud TV: The developing human. Philadelphia: Saunders, 2008.
11. Naumova EA, Dierkes T, Sprang T, Arnold WH: The oral mucosal surface and blood vessels. Head Face Med 9:8, 2013.
12. Ryan TJ, Mortimer PS, Jones RL: Lymphatics of the skin. Int J Dermatol 25:411-419, 1986.
13. Leak LV: Electron microscopic observations on lymphatic capillaries and the structural components of the connective tissue–lymph interface. Microvasc Res 2:361-391, 1970.
14. Schmelz M, Moll R, Kuhn C, Franke WW: Complexus adhaerentes, a new group of desmoplakin-containing junctions in endothelial cells. II. Different types of lymphatic vessels. Differentiation 57:97-117, 1994.
15. Gerli R, Solito R, Weber E, Aglianò M: Specific adhesion molecules bind anchoring filaments and endothelial cells in human skin initial lymphatics. Lymphology 33, 148-157, 2000.
16. Solito R, Alessandrini C, Fruschelli M, Pucci AM, Gerli R: An immunological correlation between the anchoring filaments of initial lymph vessels and the neighboring elastic fibers: a unified morphofunctional concept. Lymphology 30:194-202, 1997.
17. Sacchi G, Weber E, Aglianò M, Raffaelli N, Comparini L: The structure of superficial lymphatics in the human thigh: precollectors. Anat Rec 247:53-62, 1997.
18. Yang Y, Oliver G: Development of the mammalian lymphatic vasculature. J Clin Invest 124(3):888-897, 2014.
19. Srinivasan RS, Dillard ME, Lagutin OV, Lin FJ, Tsai S, Tsai MJ, Samokhvalov IM, Oliver G: Lineage tracing demonstrates the venous origin of the mammalian lymphatic vasculature. Genes Dev 21(19):2422-2432, 2007.
20. Yaniv K, Isogai S, Castranova D, Dye L, Hitomi J, Weinstein BM: Live imaging of lymphatic development in the zebrafish. Nat Med 12(6):711-716, 2006.
21. Yang Y, García-Verdugo JM, Soriano-Navarro M, Srinivasan RS, Scallan JP, Singh MK, Epstein JA, Oliver G: Lymphatic endothelial progenitors bud from the cardinal vein and intersomitic vessels in mammalian embryos. Blood: 120(11):2340-2348, 2012.
22. Hägerling R, Pollmann C, Andreas M, Schmidt C, Nurmi H, Adams RH, Alitalo K, Andresen V, Schulte-Merker S, Kiefer F: A novel multistep mechanism for initial lymphangiogenesis in mouse embryos based on ultramicroscopy. EMBOJ 32(5):629–644, 2013.

23. Wigle JT, Oliver G: Prox1 function is required for the development of the murine lymphatic system. Cell 98(6):769-778, 1999.

24. Oliver G: Lymphatic vasculature development. Nat Rev Immunol 4(1):35-45, 2004.

25. Hamada K, Oike Y, Takakura N, Ito Y, Jussila L, Dumont DJ, Alitalo K, Suda T (2000) VEGF-C signaling pathways through VEGFR-2 and VEGFR-3 in vasculoangiogenesis and hematopoiesis: Blood 96:3793-3800, 2000.

26. Veikkola T, Jussila L, Makinen T, Karpanen T, Jeltsch M, Petrova TV, Kubo H, Thurston G, McDonald DM, Achen MG, Stacker SA, Alitalo K: Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice: EMBOJ 20:1223-1231, 2001.

27. Sebzda E, Hibbard C, Sweeney S, Abtahian F, Bezman N, Clemens G, Maltzman JS, Cheng L, Liu F, Turner M, Tybulewicz V, Koretzky GA, Kahn ML: Syk and Slp-76 Mutant Mice Reveal a Cell-Autonomous Hematopoietic Cell Contribution to Vascular Development. Dev Cell 11:349-361, 2006.

28. Mäkinen T, Veikkola T, Mustjoki S, Karpanen T, Catimel B, Nice EC,Wise L, Mercer A, Kowalski H, Kerjaschki D, Stacker SA, Achen MG, Alitalo K: Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3. EMBOJ 20:4762-4773, 2001

29. Jackson DG: The lymphatics revisited: new perspectives from the hyaluronan receptor LYVE-1. Trends Cardiovasc. Med 13:1-7, 2003.

30. Kaipainen A, Korhonen J, Mustonen T, van Hinsbergh VM, Fang GH, Dumont D, Breitman M, Alitalo K: Expression of the fms-like tyrosine kinase FLT4 gene becomes restricted to lymphatic endothelium during development. Proc Natl Acad Sci USA 92; 3566-3570, 1995.

31. Wigle JT, Oliver G: Prox1 function is required for the development of the murine lymphatic system. Cell 98:769–778, 1999.

한글초록

생쥐 혀 발생 동안의 LYVE1과 CD31의 발현양상에 관한 연구

산지브뉴페인, 조재민, 이성원, 손혁문, 배용현, 김승현, 손원주, 이영균, 정재광[1], 김재영[#]

경북대학교 치의학전문대학원, 생화학교실, 구강내과학교실[1]

혀는 그 주된 구조와 기능을 이해하기 위하여 신경의 분지, 근육의 형성 그리고, 혈관의 분포에 관한 연구가 주로 진행되어졌다. 그러나 많은 장기들에서 정상적인 발생과 기능 유지를 위해 중요한 역할을 하는 것으로 알려진 림프관 형성 및 분포에 관한 연구는 부족한 상황이다. 림프관 내피의 hyaluronan 수용체-1(LYVE1)은 림프관 내피의 최초의 알려진 림프관 내피세포 특이적인 세포표면수용체이다. CD31(cluster of differentiation 31)로도 알려진 혈소판 내피세포 부착분자(PECAM-1)는 혈관 형성의 잘 알려진 표지자이다. 본 연구에서는 면역조직화학염색기법을 활용하여 혈관과 림프관의 형성과정과 분포양상을 확인하기 위해 LYVE1과 CD31 항체의 발현양상을 배아시기(Embryonic day; E) 14일, 16일, 17일 그리고 태어난 후(Postnatal day; PN) 0일, 5일, 10일, 15일, 그리고 20일의 생쥐 배아 및 신생자를 희생한 후 조직절편을 제작하여 확인하였다. LYVE1과 CD31의 경우, 생쥐 혀 발생단계 동안, 특징적인 단백질 발현양상이 관찰되었다. E14에서는 LYVE1이 주로 정중섬유중격(median fibrous septum)을 따라 배측(ventral region)에 분포하고 측부에는 이설근(genioglossus muscle)과 부대설근(hyoglossus muscle) 형성 부위에서 발현되었다. 같은 시기 CD31의 발현양상은 발생중인 혀의 전반에 걸쳐 확인되었다. E16에서 LYVE1과 CD31의 발현양상은 혀의 등측(dorsal region)와 배측(ventral region)에서 더욱 특징적으로 확인할 수 있었으며, E17에서는 이러한 특징적인 발현이 혀의 근육 형성과 관련하여 분포하는 것을 확인할 수 있었다. PN0, PN5, PN10, PN15 그리고 PN20 시기에서는 LYVE1과 CD31(PN10)의 발현이 대부분 근섬유 사이의 공간에 분포되어 그 분포양상이 보다 복잡해지는 것을 확인할 수 있었다. 본 면역조직화학방법을 통해 확인한 혈관 및 림프관 형성인자의 발현양상은 매우 유사한 양상을 확인할 수 있었으며, 발생단계에 따라 특이적인 양상을 확인할 수 있었다. 이러한 림프관과 혈관 형성 및 분포의 정상 발생과정은 암과 같은 병리학적인 상태의 변화양상을 이해하는데 도움이 될 것으로 기대된다.

주제어: 림프관형성, 혈관형성, 혀 발생, 기관발생