

# Microanatomical profiles on the lymphatic system in the human ampulla of Vater (immunohistochemistry and scanning electron microscopy)

Takuji Kagiya · Hiroshi Shimoda · Hirokazu Narita ·  
Tadashi Odagiri · Seiji Watanabe · Keinosuke Ishido ·  
Daisuke Kudo · Norihisa Kimura · Taiichi Wakiya ·  
Kenichi Hakamada

Published online: 29 September 2017

© 2017 Japanese Society of Hepato-Biliary-Pancreatic Surgery

## Abstract

**Background** Little information is available regarding microanatomy of lymphatic system in the ampulla of Vater, though it is of critical importance for an understanding of tumor progression via the lymphatics and determination of surgical strategy. The present study, therefore, aimed to demonstrate the distribution and microanatomical profiles on the lymphatic system in the ampulla.

**Methods** The fine distribution and structure of the lymphatic vessels were investigated in the ampulla and the stomach by immunohistochemistry for lymphatic-(D2-40) and blood vascular- (CD31) specific markers and scanning electron microscopy. The densities of lymphatic and blood vessels were also compared.

**Results** The duodenal papilla densely developed the lymphatics with distinct aspects of lymphatic capillaries, together with blood vessels. The density of lymphatic capillaries in the extramuscular layer in the ampulla was higher than those of both the other ampullary layers and the gastric extramuscular (subserosal) layer.

**Conclusions** The ampulla of Vater showed widespread lymphatic capillaries throughout the entire wall. The specific vascular system is suited to produce lymph everywhere and drain without via such a large vessel as

lymphatic collector. This suggests that tumor cells invade the lymphatics and metastasize more easily in the ampulla than in the other gastrointestinal regions.

**Keywords** Ampulla of Vater · Immunohistochemistry · Lymphatic system · Lymphatic vessels · Scanning electron microscopy

## Introduction

Carcinoma in the ampulla of Vater (CAV) is a rare disease throughout gastrointestinal tract disorders [1], but its malignant potential is thought to be worse than the other gastrointestinal cancers. Surgically localized resection of CAV is sometimes performed if the tumor is diagnosed as an early stage cancer [2], whereas most cases have undergone pancreaticoduodenectomy because of its high metastatic potential to lymph nodes. Since pancreaticoduodenectomy is highly invasive enough to invite various severe complications, it goes without mentioning that application of lower invasive surgery is better for keeping patients' quality of life [3]. A new strategy governing lymph dynamic metastasis of CAV is desired to resolve the above problem. The elucidation of precise organization of its lymphatic vasculature is referred to be essential to develop such a surgical strategy leading to lower invasiveness as sentinel lymph node theory used in cases of breast cancer [4] and gastric cancer [5] for prediction of lymph node metastasis. However, even the microanatomical structure of the lymphatic system remains to be explained in the case of the ampulla of Vater. Therefore, the present study aimed to demonstrate precise organization of the lymphatic vasculature in the human ampulla of Vater.

T. Kagiya · T. Odagiri · K. Ishido · D. Kudo · N. Kimura ·  
T. Wakiya · K. Hakamada (✉)  
Department of Gastroenterological Surgery, Hirosaki University  
Graduate School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori  
036-8562, Japan  
e-mail: hakamada@hirosaki-u.ac.jp

H. Shimoda · H. Narita · S. Watanabe  
Department of Anatomical Science, Hirosaki University Graduate  
School of Medicine, Hirosaki, Aomori, Japan

## Materials and methods

### Human tissue preparation

The specimens of common bile duct, pancreatic head and duodenum containing the ampulla of Vater were collected from three cases of adult male cadavers with neither gastrointestinal diseases nor abdominal malformations under approval from the Human Research Ethics Committee of the Hirosaki University Graduate School of Medicine (Reference number 2015-227). The tissue pieces of the gastric corpus were also taken from the cadavers to compare the vascular densities between the duodenal papillary region and in the gastric wall. The tissues were immersed in 10% buffered formalin for at least 5 days, transitionally cut from the bile duct to the duodenal papilla at every 1 cm, and the tissue slices were embedded in paraffin. They were cut into several 5  $\mu\text{m}$  thick sections for hematoxylin-eosin (HE) and immunohistochemical staining, and the remaining tissues were processed for scanning electron microscopy.

### Immunohistochemistry

The deparaffinized tissue sections were incubated in a 0.01 M citrate buffer (pH 6.0) at 121°C for 15 min to retrieve antigenicity of the objective proteins before immunostaining. Some sections were immersed in 0.3%  $\text{H}_2\text{O}_2$  in phosphate buffered saline (PBS; 1/15 M, pH 7.4) containing 0.1% sodium azide (Wako Pure Chemical Industries, Osaka, Japan) at room temperature for 20 min to block the endogenous peroxidase activity. They were incubated in 10% normal goat serum (Vector Laboratories, Burlingame, CA, USA), and then with a mixture of antibodies for podoplanin (D2-40; DAKO, Santa Clara, CA, USA) and CD31 (EP3095; Abcam, Tokyo, Japan) at 4°C overnight. Following a rinse in PBS, they were treated with alkaline phosphatase (AP)-conjugated anti-mouse IgG (Histofine Simple Stain MAX-AP; Nichirei Biosciences, Tokyo, Japan) and visualization by AP reaction (Vector blue substrate kit, Vector Laboratories). They were then treated with PO-bounded anti-rabbit IgG (Histofine Simple Stain MAX-PO) and visualized by AEC substrate chromogen (DAKO). In contrast, the tissue sections of human stomach were processed for similar staining procedure, but immunolocalization of D2-40 and CD31 were colored red and blown by AP reaction (Vector red substrate kit, Vector Laboratories) and DAB reaction, respectively. All the stained sections for light microscopy were examined with a BX-60 light microscope equipped with DP72 digital imaging system (Olympus, Tokyo, Japan). Control immunostaining were

carried out using the same procedures, except for the use of a non-immunized serum instead of the corresponding antibodies. Completely negative results were observed.

### Scanning electron microscopy

The deparaffinized tissues were processed for SEM as described previously [6]. Briefly, they were immersed in 1% tannic acid solution and 1% osmium tetroxide solution for 1 h each, dehydrated in a graded ethanol series, freeze-dried by t-butylalcohol, and observed under a JEOL JSM-6510 (JEOL, Tokyo, Japan). Captured images were processed with imaging and photo editing software, Adobe Photoshop CC (Adobe Systems, San Jose, CA, USA).

### Morphological assessment and statistical analyses

The distribution of lymphatics and blood vessels were calculated in the immunostained tissue sections. In particular, numbers of the two vessels were obtained in 10 fields of view, which were set in the area of 500  $\mu\text{m}^2$  and randomly chosen in each region. The vessels with obvious lumens were preferentially calculated. The calculated numbers of the two vessels were compared among the mucosal, intramuscular and extramuscular layers. The densities of lymphatic vessels in the ampulla of Vater and the stomach were compared in each region of the mucosal, intramuscular and extramuscular layers. Statistical analyses were performed as follows. Wilcoxon signed-rank test was carried out for comparison with the number of the two vessels. Mann–Whitney *U*-test was carried out for comparison with the number of lymphatic vessels in the ampulla of Vater and the stomach. Tukey's honestly significant difference test and Steel–Dwass test were carried out for comparison with three groups. These statistical analyses were tested by Statistical package for social sciences (SPSS) version 21.0 (IBM, Armonk, NY, USA) and Statcel 3 software (OMS Publishing Inc., Saitama, Japan), and the threshold for significance was  $P < 0.05$ .

### Schematic illustration of the lymphatic system in the ampulla of Vater

The distribution of lymphatic vessels was illustrated as a model that was reconstructed from the obtained data of immunohistochemistry, SEM and the above morphological analysis. The schematic illustration was generated using an illustration software, Adobe Illustrator CC (Adobe Systems).

## Results

Under a light microscope, HE staining classified the three layers, mucosal (m) layer, intramuscular (im) layer equal to the sphincter of Oddi and extramuscular (em) layer tentatively in this study (Fig. 1a). The immunohistochemistry for podoplanin and CD31 demonstrated lymphatic and blood vessels with significant immunoreactivity on the tissue sections of both the ampulla of Vater and the stomach to allow a clear distinction between lymphatic and blood vessels (Figs 1b–f, 2c–h). SEM examination of the tissue opposite to the section immunostained for podoplanin permitted a detection of lymphatic vessels and observation of their microanatomical structures in the ampulla of Vater (Fig. 3).

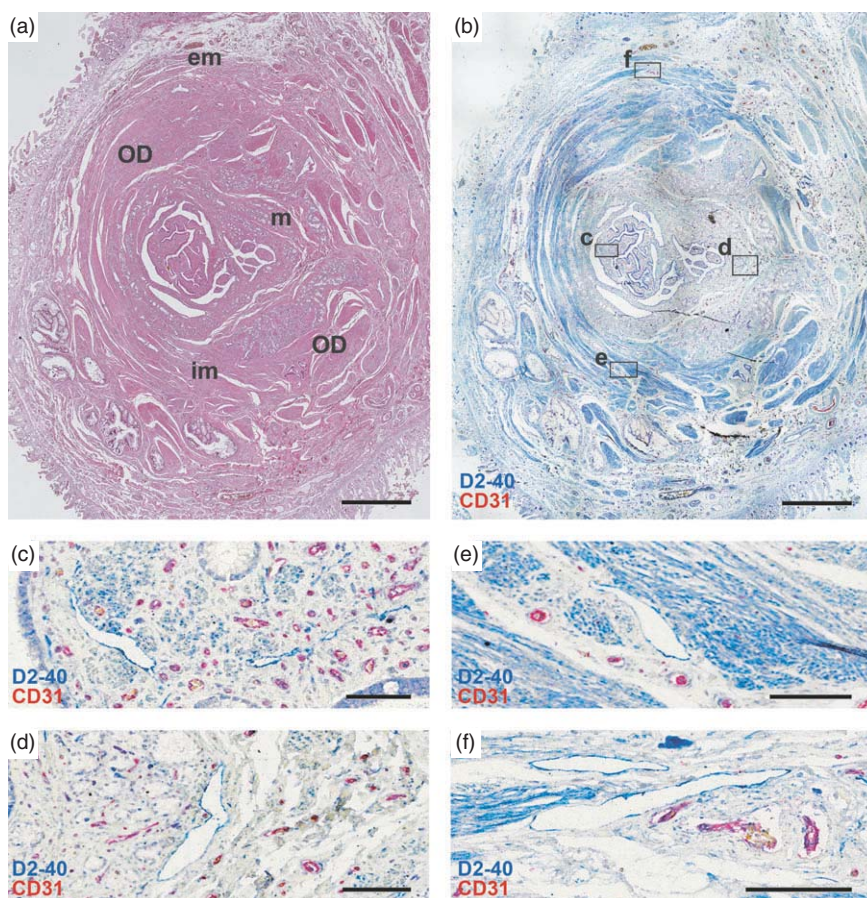
### Structure of lymphatic system in the ampulla of Vater

The podoplanin-immunopositive lymphatic vessels were thoroughly distributed with dense supply of the CD31-positive blood vessels in the mucosal, intramuscular and extramuscular layer and appeared to be isolated from the duodenal lymphatic network (Figs 1,2). The lymphatics

were irregular in shape, approximately 80  $\mu\text{m}$  in diameter and consisted only of thin endothelial cells. They laid flat and smooth-surfaced endothelial cells with irregular contours, the luminal surfaces and contained some biological substances within the lumens (Fig. 3). Therefore, the lymphatic meshwork in the ampulla of Vater was predominantly composed of lymphatic capillaries. The lymphatic connections were seen between the above three layers (m, im and em) (Fig. 4).

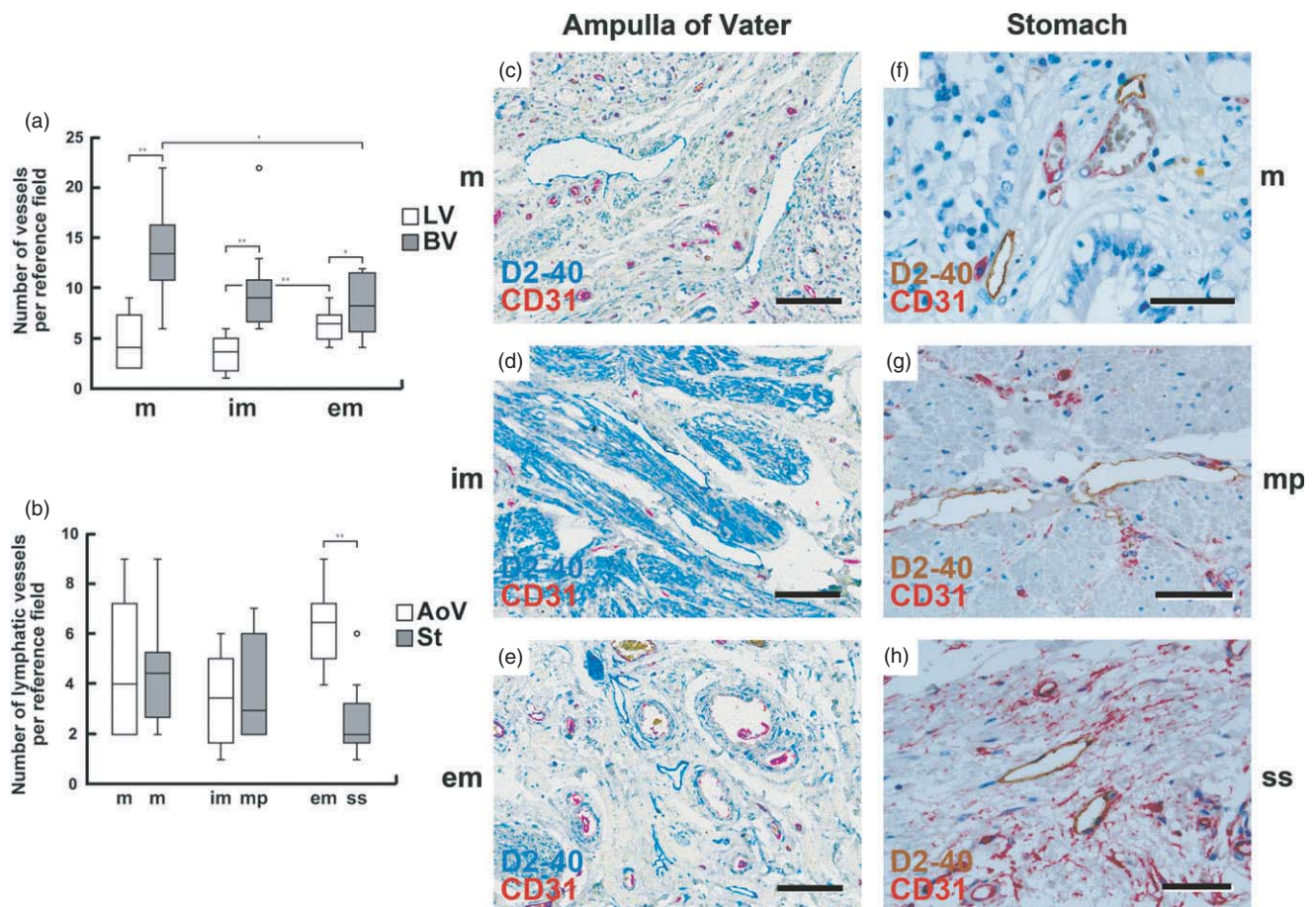
### Statistical analysis

While the density of blood vessels was significantly higher than lymphatic vessels in the mucosal, intramuscular and extramuscular layers ( $P < 0.01$ ,  $P < 0.01$  and  $P = 0.036$ ), the density of lymphatic vessels in extramuscular layer was significantly higher than in intramuscular layer ( $P < 0.01$ ). The blood vessels were significantly greater in number than the lymphatic vessels in all layers of the ampulla of Vater, and the density of blood vessels in the mucosa of the ampulla of Vater was significantly higher than that in the other layers (Fig. 2a). In contrast, the extramuscular layer in the ampulla of Vater showed a



**Fig. 1** Light micrographs of hematoxylin and eosin staining (a) and double immunostaining for D2-40 (blue) and CD31 (red) (b–f) on adjacent tissue sections from the ampulla of Vater. (a) An overview of the ampulla of Vater. The ampulla consists of mucosal (m), intramuscular (im) and extramuscular (em) layers. The intramuscular layer involves the sphincter of Oddi (OD). (b–f) An overview (b) and higher magnifications (c–f) of the boxed areas in b (c, subepithelial lesion of the mucosa; d, deep lesion of the mucosa; e, intramuscular lesion; f, extramuscular lesion). The D2-40-immunopositive lymphatic vessels (blue) and CD31-immunopositive blood vessels (red) are abundantly distributed in each lesion. The smooth muscles in the muscular layer are weakly stained blue. Scale bars = 1 mm (a, b), 50  $\mu\text{m}$  (c), 100  $\mu\text{m}$  (d, e, f)





**Fig. 2** Lymphatic and blood vascular densities in the ampulla of Vater and the stomach. **(a)** Comparison of number of the lymphatic and blood vessels between each layer of the ampulla. While the blood vessel density was higher than that of lymphatic vessels in all layers, lymphatic vascular density in the extramuscular layer was significantly higher than the intramuscular layer. **(b)** Comparison of lymphatic vascular density between in the ampulla of Vater and stomach. The lymphatic vascular density in the extramuscular layer in the ampulla was higher than the other corresponding gastric layer (subserosa), though the other layer in the ampulla showed no significantly in the lymphatic density as compared to those in the stomach. **(c–h)** The representative images of lymphatic (blue in the ampulla; brown in stomach) and blood vessels (red) in each layer of the ampulla and the stomach. *AoV* ampulla of Vater, *BV* blood vessel, *em* extramuscular layer, *im* intramuscular layer, *LV* lymphatic vessel, *m* mucosal layer, *mp* muscularis propria, *ss* subserosal layer, *St* stomach. Scale bars = 100  $\mu$ m (**c, d, e**), 50  $\mu$ m (**f, g, h**), \* $P < 0.05$ , \*\* $P < 0.01$

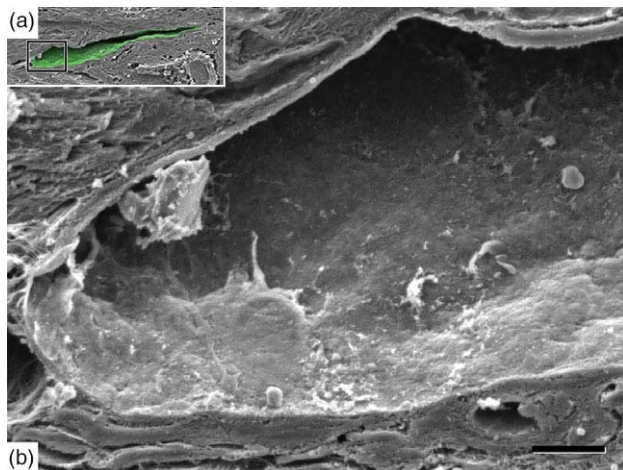
higher lymphatic vascular density than those in both the other papillary layers and in the gastric extramuscular (subserosal) layer (Fig. 2b).

## Discussion

The present study has demonstrated the distribution and microanatomical profiles of the lymphatic system in the human ampulla of Vater by immunohistochemistry and SEM. The ampulla of Vater developed a precise network of lymphatic capillaries independent of the duodenal lymphatic system. The microanatomical organization of the lymphatic system of the ampulla of Vater as demonstrated by the present study is schematically presented in Figure 5.

The CAV is of low malignant potential [2], but the overall 5-year survival rate of CAV after resection is lower than other gastrointestinal cancers such as gastric and colorectal cancers [7, 8]. The pancreaticoduodenectomy has undergone CAV as a standard surgical treatment, whereas such local resection as surgical or endoscopic procedures are conducted in some cases of cancers in early stage [9, 10] instead of pancreaticoduodenectomy involving high morbidity and mortality. However, no distinct criteria have been indicated between local resection and pancreaticoduodenectomy.

Lymph node metastasis and lymphatic invasion on pathological findings have been reported to be poor prognostic factors of CAV after curative resection [11, 12]. Such complete resection as pancreaticoduodenectomy is required to improve these prognostic factors, but cause



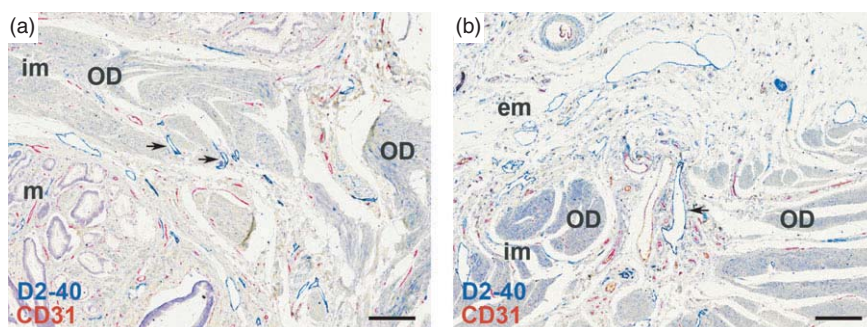
**Fig. 3** Scanning electron microscopy views of a lymphatic vessel (**a**, green; **b**, a higher magnification of the boxed area in **a**) in the extramuscular layer in the ampulla of Vater (a mirror image of Fig. 1f). The lymphatics consists of a single layer of flat and irregularly shaped endothelial cells. Scale bar = 5  $\mu$ m (**b**)

high frequency of complication. In order to resolve this problem, such lymph node dissection procedures meeting curative resection and preservation of quality of life as sentinel lymph node theory [4, 5] for the other cancers should be required, and thus, the present study provides microanatomical information of the lymphatic system of the duodenal papilla. Carcinoma *in situ* in the duodenal papilla clinically caused no lymph node metastasis, but carcinoma limited to the mucosal layer and/or invaded the sphincter of Oddi showed a higher rate of lymph node metastasis in the ampulla of Vater [13, 14]. Yoon et al. [14] have reported that lymph node metastasis less frequently occurred in 9% of patients with early ampullary cancer (pT1 or pTis) (according to the 6th edition of AJCC cancer staging [15]) as compared with 50.8% of pT2, 38.1% of pT3, and 62.5% of pT4 cancer. Furthermore, Winter et al. [16] have reported that the rate of lymph node metastasis significantly increased with T stage (pT1, 28.0%; pT2, 50.9%; pT3, 71.7%; pT4, 77.3%). Therefore, the risk of lymph node metastasis increased

with the depth of tumor invasion on pathological findings in CAV. The lymphatic vessels throughout the entire wall of the duodenal papilla were regarded as lymphatic capillaries by light and electron microscopic examinations (Figs 3,5). The lymphatic capillaries play a pivotal role of fluid absorption and transport of immune cells and tumor cells. This suggested that carcinoma in every mucosal, intramuscular and extramuscular layers might invade to the lymphatic vessels and migrate to the lymph nodes easily. In addition, abundant distribution of blood vessels throughout the three layers (Fig. 2a) may contribute to be favorable situation for tumor metastasis.

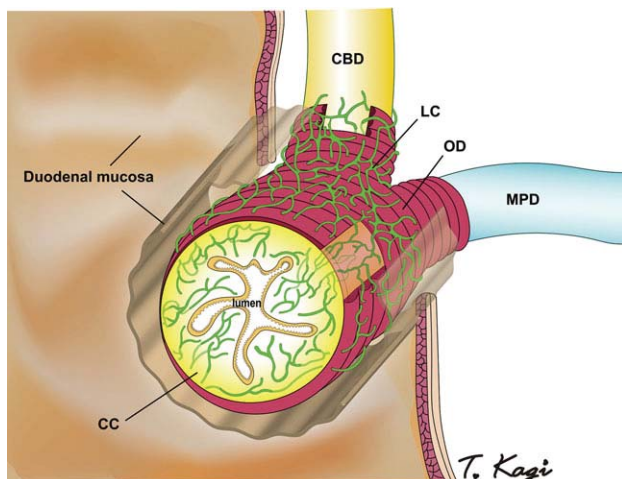
In our investigation into the density of lymphatic vessels between the ampulla of Vater and the gastric wall, the extramuscular layer of duodenal papilla showed a high lymphatic vascular density as compared to the subserosal layer in the stomach (Fig. 2b). While the previous studies [6, 17] described that the mucosal lymphatic capillaries gradually converged to form such large channels as lymphatic pre-collectors in the submucosal and subserosal layers in the mammalian gastrointestinal tract, the extramuscular layer in the duodenal papilla demonstrated numerous lymphatic capillaries. These microanatomical profiles suggested that the cancer limited to the mucosal layer could be resected by local resection, while pancreaticoduodenectomy should be selected against the cancers invaded into the intramuscular layer for response to its high metastatic potential. Furthermore, the distance from the mucosal epithelium to the intramuscular layer of the sphincter of Oddi was shorter than those of the stomach (200–500  $\mu$ m vs. 1,000–2,000  $\mu$ m). This also supported our hypothesis.

Several limitations of the present study should be acknowledged. First, the anatomy of pancreaticobiliary junction is known to be complex and tends to have several anomalies [18]. The present results are applied in cases showing standard anatomical condition of the ampulla of Vater. Second, although the lymphatic vessels observed in the present study ran from the common pancreaticobiliary channel to the common bile duct, it is obscure as to where they drain. We are now trying to



**Fig. 4** Light micrographs of double immunostaining for D2-40 (blue) and CD31 (red) on the part between the layers in the ampulla of Vater. (**a**) The lymphatic vessels entering the intramuscular (im) layer, which involves the sphincter of Oddi (OD), from the mucosal (m) layer are observed (arrows). (**b**) A lymphatic vessel running through the intramuscular (im) layer to the extramuscular (em) layer is observed (arrow). Scale bars = 200  $\mu$ m (**a**, **b**)





**Fig. 5** A schematic presentation of the lymphatic system in the ampulla of Vater based on the present study. Lymphatic capillaries spread continuously from the mucosal layer to the extramucosal layer in the common channel (CC) and the common bile duct (CBD). LC lymphatic capillaries, MPD main pancreatic duct, OD the Sphincter of Oddi

demonstrate the lymphatic system also in the common bile duct and the main pancreatic duct.

In conclusion, the present study is first to provide the lymphatic system in the human ampulla of Vater systematically and implicated those intimate associations with lymphatic metastasis of the carcinoma.

**Acknowledgment** This study was partly supported by the Japan Society for the Promotion of Science KAKENHI Grant Number 17K17574 (Grant-in-Aid for Young Scientists (B)). Funding source has no role. No additional external funding was received for this study.

**Conflict of interest** None declared.

## References

- Carter JT, Grenert JP, Rubenstein L, Stewart L, Way LW. Tumors of the ampulla of Vater: histopathologic classification and predictors of survival. *J Am Coll Surg*. 2008;207:210–8.
- de Castro SM, van Heek NT, Kuhlmann KF, Busch OR, Offerhaus GJ, van Gulik TM, et al. Surgical management of neoplasms of the ampulla of Vater: local resection or pancreatoduodenectomy and prognostic factors for survival. *Surgery*. 2004;136:994–1002.
- Makary MA, Winter JM, Cameron JL, Campbell KA, Chang D, Cunnigham SC, et al. Pancreatoduodenectomy in the very elderly. *J Gastrointest Surg*. 2006;10:347–56.
- Giuliano AE, Kirgan DM, Guenther JM, Morton DL. Lymphatic mapping and sentinel lymphadenectomy for breast cancer. *Ann Surg*. 1994;220:391–8.
- Takeuchi H, Kitagawa Y. Minimally invasive function-preserving surgery based on sentinel node concept in early gastric cancer. *Transl Gastroenterol Hepatol*. 2016;1:23.
- Shimoda H, Kato S, Kudo T, Usui T. Lymphatic network and nerve plexus in the myenteric layer of the monkey jejunum: a topographic study using an enzyme-histochemical method. *Arch Histol Cytol*. 1998;61:65–73.
- Isobe Y, Nashimoto A, Akazawa K, Oda I, Hayashi K, Miyashiro I, et al. Gastric cancer treatment in Japan: 2008 annual report of the JGCA nationwide registry. *Gastric Cancer*. 2011;14:301–16.
- Watanabe T, Itabashi M, Shimada Y, Tanaka S, Ito Y, Ajioka Y, et al. Japanese Society for Cancer of the Colon and Rectum (JSCCR) Guidelines 2014 for treatment of colorectal cancer. *Int J Clin Oncol*. 2015;20:207–39.
- Schneider L, Contin P, Fritz S, Strobel O, Buchler MW, Hackert T. Surgical ampullectomy: an underestimated operation in the era of endoscopy. *HPB (Oxford)*. 2016;18:65–71.
- Alvarez-Sanchez MV, Oria I, Luna OB, Pialat J, Gincul R, Lefort C, et al. Can endoscopic papillectomy be curative for early ampullary adenocarcinoma of the ampulla of Vater? *Surg Endosc*. 2017;31:1564–72.
- Yamaguchi K, Enjoji M. Carcinoma of the ampulla of Vater. A clinicopathologic study and pathologic staging of 109 cases of carcinoma and 5 cases of adenoma. *Cancer*. 1987;59:506–15.
- de Paiva Haddad LB, Patzina RA, Penteado S, Montagnini AL, da Cunha JE, Machado MC, et al. Lymph node involvement and not the histopathologic subtype is correlated with outcome after resection of adenocarcinoma of the ampulla of Vater. *J Gastrointest Surg*. 2010;14:719–28.
- Roggin KK, Yeh JJ, Ferrone CR, Riedel E, Gerdes H, Klimstra DS, et al. Limitations of ampullectomy in the treatment of non-familial ampullary neoplasms. *Ann Surg Oncol*. 2005;12:971–80.
- Yoon YS, Kim SW, Park SJ, Lee HS, Jang JY, Choi MG, et al. Clinicopathologic analysis of early ampullary cancers with a focus on the feasibility of ampullectomy. *Ann Surg*. 2005;242:92–100.
- American Joint Committee on Cancer. *AJCC Cancer Staging Manual*, 6th edn. New York: Springer; 2002. p. 151–6.
- Winter JM, Cameron JL, Olinio K, Herman JM, de Jong MC, Hruban RH, et al. Clinicopathologic analysis of ampullary neoplasms in 450 patients: implications for surgical strategy and long-term prognosis. *J Gastrointest Surg*. 2010;14:379–87.
- Shimoda H. Structural organization of lymphatics in the monkey esophagus as revealed by enzyme-histochemistry. *Arch Histol Cytol*. 1998;61:439–50.
- Hernandez-Jover D, Pernas JC, Gonzalez-Ceballos S, Lupu I, Monill JM, Perez C. Pancreatoduodenal junction: review of anatomy and pathologic conditions. *J Gastrointest Surg*. 2011;15:1269–81.