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**Para-aortic lymph node micrometastasis in patients with node-negative biliary cancer**

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**Original article**

**Running head:** Micrometastasis in biliary cancer

**Key words:** Para-aortic lymph node, Micrometastasis, Isolated tumor cells, Biliary cancer

## **ABSTRACT**

**Background/Aims:** The presence of para-aortic lymph node metastasis in biliary cancer negatively impacts prognosis. The present study aims to immunohistochemically identify and evaluate the clinical significance of para-aortic lymph node micrometastases in 66 patients who had undergone curative resection of biliary cancer.

**Methods:** We used an antibody against cytokeratins 7 and 8 (CAM5.2) to immunostain 529 para-aortic lymph nodes that were negative according to conventional analysis, from 66 patients with biliary cancer.

**Results:** We detected CAM5.2-positive occult carcinoma cells in para-aortic lymph nodes from 3 (5%) of the 66 patients and in 3 (0.6%) of the 529 para-aortic lymph nodes. One of the three patients also had micrometastasis in the regional lymph nodes. All three patients with para-aortic lymph node micrometastasis are alive at 45, 48 and 90 months after surgery despite having locally advanced cancer.

**Conclusions:** Occult cancer cells were identified in para-aortic lymph nodes from 5% of patients with node-negative biliary cancer, yet these patients have survived over the

long term. **The presence of para-aortic nodal micrometastasis might not have influence on survival.** However, further studies including a greater number of patients are required to support this notion.

## INTRODUCTION

Immunohistochemical techniques have facilitated the identification of lymph node micrometastases that would be overlooked by conventional histological staining with hematoxylin and eosin (HE). The clinical significance of immunohistochemically detected lymph node micrometastasis for cancers of the breast (1,2), lung (3-5), esophagus (6,7), stomach (8,9) and colon (10-12) has been evaluated, whereas little has been published regarding biliary cancer (13) including hilar bile duct (14-16), gallbladder (17-20), and ampullary (21) carcinoma. However, whether or not the presence of lymph node micrometastasis relates to prognosis is controversial. We previously found that occult cancer cells in regional lymph nodes from 22% of patients with regional node-negative biliary cancer were associated with significantly worse survival (22).

Para-aortic lymph node metastasis is associated with a poor prognosis for patients with biliary cancer (23-25); such lymph nodes represent the final nodes in the abdominal lymphatic system from the biliary tract (26-28). The survival of patients with positive para-aortic lymph nodes without distant metastasis is as poor as that of patients with hepatic or peritoneal involvement (23). Yet, preoperative computed tomography and laparoscopic examinations are not sufficiently sensitive to detect para-aortic lymph node metastasis (29-31). We found that para-aortic lymph node micrometastasis does not impact the survival of patients with regional node-positive and para-aortic node-negative biliary cancer (32) and it also might have little influence on the survival of patients with regional node-positive biliary cancer. Alternatively, micrometastasis might be the earliest stage of para-aortic disease and such patients might benefit from para-aortic lymphadenectomy.

This retrospective study uses immunohistochemistry to determine the incidence and prognostic importance of micrometastasis in para-aortic lymph nodes resected from patients with biliary cancer that is pathologically node-negative. The present study is the first to analyze the relevance of para-aortic lymph node micrometastasis in

node-negative biliary cancer.

## **METHODS**

We immunohistochemically re-examined para-aortic lymph nodes that had been removed between June 1998 and June 2007 from patients during curative resection of biliary cancer that had been diagnosed by HE staining as being node-negative.

We cut eight serial 5- $\mu$ m thick sections from archival, formalin-fixed, paraffin-embedded samples of regional lymph nodes in which metastasis were undetectable by conventional histological staining with HE. The first section was stained with HE to detect metastatic tumor cells.

The second, fifth, and eighth sections were stained using the CAM5.2 monoclonal antibody (Becton Dickinson, San Jose, California, USA). Thus, we examined one and three 5- $\mu$ m sections stained with HE and with CAM5.2, respectively, at 10- $\mu$ m intervals. Sections were deparaffinized with xylene, rehydrated through a graded series of ethanol, immersed in 1 mmol/L ethylenediaminetetraacetic acid (pH 8.0) and heated for 2.5 minutes in a pressure cooker for antigen retrieval. Thereafter, sections were

immunohistochemically stained with the CAM5.2 monoclonal antibody (dilution 1:5) that is specific for cytokeratins 7 and 8 (33), and then visualized using a standard streptavidin biotinylated antibody method on an automated immunostainer (NexES<sup>®</sup>; Ventana Medical Systems, Tucson, Arizona, USA). CAM5.2 stains most epithelium-derived tissues, but not stratified squamous epithelium (34-36).

Single cells or clusters of cells immunostained by CAM5.2 were considered as metastases only when the morphological features of carcinoma cells were apparent. Artfactual contamination of carcinoma cells from outside a lymph node was excluded. Although CAM5.2 occasionally reacted with reticulum and plasma cells, these could be differentiated from carcinoma cells based on staining profile and intensity (10). Experienced histopathologists who were blinded to the clinicopathological features of patients performed all histological and immunohistochemical evaluations.

We defined ‘micrometastasis’ as occult cancer cells that are detectable only by immunostaining, regardless of size.

Biliary cancer was staged according to the seventh edition of the Union Internacional Contra la Cancrum Tumor Node Metastasis (TNM) classification (37). We staged

intrahepatic perihilar cholangiocarcinoma using the classification for perihilar extrahepatic bile duct cancer.

### **Statistical analysis**

Statistical calculations were performed using StatView<sup>®</sup> J 5.0 software (SAS Institute, Cary, North Carolina, USA). Cumulative survival after surgery was calculated using the Kaplan-Meier method with a census date of August 2009; cumulative survival was compared using the log rank test.  $P < 0.050$  was considered statistically significant.

### **RESULTS**

A total of 122 patients underwent resection of biliary cancer with surgical pick-up biopsy or lymphadenectomy of the para-aortic lymph nodes. Sixty-six patients (54%) had no regional and para-aortic lymph node metastasis according to routine HE staining, but of 56 (46%) with confirmed regional lymph node metastasis, 50 (89%) had no para-aortic lymph node metastasis and 6 (11%) had overt metastasis. Surgical resection was abandoned in the other 15 patients, due to para-aortic metastasis being identified by intraoperative frozen section pathology (Fig. 1).

Survival was significantly better for node-negative, than node-positive patients ( $P < 0.0001$ ) and tended to be worse for those with positive regional and positive para-aortic nodes than with positive regional nodes and negative para-aortic nodes ( $P = 0.1912$ ) (Fig. 2).

The immunohistochemical study therefore included 66 node-negative patients (35 bile duct carcinomas, 19 gallbladder carcinomas, 7 intrahepatic cholangiocarcinomas and 5 ampullary carcinomas). Lymph node numbers distributed by each primary lesion are shown in Table 1. A total of 529 para-aortic lymph nodes were removed from the 66 patients, which was a median of 6 (range 1–29) per patient. The median age of the 42 men and 24 women was 71 (range 43–81) years. The median follow-up was 69.2 (range 30.9–135.7) months. Surgical procedures included pancreatoduodenectomy ( $n = 15$ ), extrahepatic bile duct resection and cholecystectomy ( $n = 7$ ), extended cholecystectomy, including partial resection of the liver ( $n = 5$ ), and several types of hepatectomy ( $n = 39$ ) including combined resection of the extrahepatic bile duct ( $n = 35$ ) and pancreatoduodenectomy ( $n = 2$ ).

Twenty-eight of the 66 patients had died from tumor recurrence, six were alive with

recurrent disease, three had died from other causes, and 29 remained disease-free and alive. The first site of detectable recurrence was the liver (12) followed by local (10), peritoneum (8), lung (2) and unknown (2). Although several patients treated by chemotherapy after the diagnosis of tumor recurrence, no adjuvant chemotherapy was administered for patients without recurrence.

Carcinoma cells were CAM5.2-positive in 3 (0.6%) of 529 para-aortic lymph nodes from 3 (5%) of 66 patients. They were identified in one patient each with bile duct carcinoma, gallbladder carcinoma and intrahepatic cholangiocarcinoma (Fig. 3), but not in patients with ampullary carcinomas. The clinicopathological characteristics of patients with CAM5.2-positive para-aortic lymph nodes are shown in Table 2. Case 1 is a female patient with perihilar bile duct carcinoma and main portal vein invasion who underwent right hepatic and caudate lobectomy with bile duct and portal vein resection. She has remained alive for 45 months without recurrence. Case 2 is a female patient with gallbladder carcinoma with extrahepatic bile duct and duodenum invasion. She underwent left hepatic and caudate lobectomy with bile duct resection and has been alive for 48 months with no recurrence after the operation. Case 3 is a female patient

with intrahepatic cholangiocarcinoma and invasion of the left branch of the portal vein. She underwent left hepatic and caudate lobectomy with bile duct resection. However, liver metastasis was resected 9 months later and lung metastasis was resected at 13 and 23 months after the initial operation. Despite multiple liver and lung metastasis, she remains alive 90 months after the initial operation under gemcitabine chemotherapy. Micrometastasis was found in a periduodenal node from Case 2, but not in regional lymph nodes from the other two patients.

## **DISCUSSION**

The para-aortic lymph nodes of 3 (5%) of 66 patients with node-negative biliary cancer according to conventional histological staining harbored occult cancer cells. We previously found occult cancer cells in regional lymph nodes from 33 (22%) of 151 patients with node-negative biliary cancer according to conventional analyses (22), and in the para-aortic lymph nodes of 9 (18%) of 49 patients with regional node-positive and para-aortic node-negative biliary cancer according to conventional analyses (32). The postoperative survival of patients with node-negative biliary cancer was

significantly worse among those with, than without regional lymph node micrometastasis (22), whereas that of patients with regional node-positive biliary cancer and with and without micrometastasis in para-aortic lymph nodes did not significantly differ (32). The present study included data from only a few patients. Nevertheless, the fact that three with node-negative with micrometastasis in para-aortic lymph nodes have survived over the long term is notable. One patient remains alive but with recurrent disease treated by several surgeries and chemotherapy, but the other two patients remain alive without recurrence.

**Sasaki et al. (10) examined 13 consecutive 3- $\mu$ m sections per lymph node from patients with colorectal cancer and found that the positivity rate of metastasis detection reached a plateau after nine sections (total thickness 27  $\mu$ m) had been examined. They suggested that the examination of sections giving 30  $\mu$ m in total might be sufficient. Therefore we performed the examination of three consecutive 5- $\mu$ m sections at intervals of 10  $\mu$ m per node.** The incidence of micrometastasis in para-aortic lymph nodes detected by immunostaining was very low (0.6%). Therefore, conventional pathological examination of para-aortic lymph node might give enough

information for treatment strategy for node-negative biliary cancer.

Sasaki et al. (17) detected lymph node micrometastases in 12 (30.0%) patients and in 16 (1.9%) of 856 lymph nodes obtained from 40 patients with node-negative (pN0) biliary cancer. They identified para-aortic, but not regional lymph node micrometastasis in one patient. Taniguchi et al. (16) detected micrometastasis in 11 (39.2%) of 28 patients and in 14 (3.3%) of 423 lymph nodes obtained from pN0 hilar bile duct carcinoma. Among the 11 patients with lymph node micrometastasis, 10 included the regional lymph nodes, and one included regional and para-aortic lymph nodes. We found micrometastasis in 3 (0.6%) of 529 para-aortic lymph nodes from 3 (5%) of 66 patients. We detected regional lymph node micrometastasis in one of the three patients, but not in the other two. The route of lymphatic drainage from the biliary tract is via the regional nodes to the abdominal para-aortic lymph nodes (26-28), but the present results indicate skip metastasis. However, regional lymph node micrometastasis might have been identified had we cut more sections from more patients with only para-aortic lymph node micrometastasis.

Survival in the present study was significantly worse for patients with overt

para-aortic and lymph node metastasis than for those who were node-negative ( $P < 0.0001$ ) (Fig. 2). However, three patients with para-aortic lymph node micrometastasis with node-negative biliary cancer have remained alive over the long time despite having locally advanced cancer. Isolated tumor cells (ITCs) are single tumor cells or small clusters of cells that are smaller than 0.2 mm, which is the greatest extent that can be detected by routine HE or immunohistochemical staining (37). ITCs do not typically show evidence of metastatic activity, such as proliferation, stromal reaction, or penetration of vascular or lymphatic sinus walls (38). The size of occult tumor cell clusters detected in para-aortic lymph nodes in the present study corresponded to ITCs in two patients (Table 2). Although tumor cells had reached even the para-aortic lymph nodes via the lymphatic drainage route from a primary tumor, they may have lacked the ability to form overt metastases in lymph nodes or they may have been biologically inert. Thus, these tumor cells would be eliminated eventually. Alternatively, another patient with 0.3-mm occult tumor cell nests in a para-aortic lymph node might have benefited from para-aortic lymphadenectomy (Table 2). **In our previous study (32), there was no difference in postoperative survival between patients with and**

**without CAM5.2-positive para-aortic nodes in patients with regional node-positive and para-aortic node-negative biliary cancer, but survival for five patients with micrometastases (larger than 0.2 mm) was significantly worse than that for four patients with only ITCs (0.2 mm or smaller).** Radical surgery including prophylactic dissections or sampling of para-aortic lymph nodes that has been an optional procedure in limited institutes, may improve the prognosis of such selected patients. **If there is an efficient method of intraoperative detection of para-aortic lymph node micrometastases larger than 0.2 mm, it seems that the indication of the extended surgery can be decided. However, because the number of cases is too small in the present study, it is not possible to conclude about prognostic importance of para-aortic micrometastasis in patients with node-negative biliary cancer.**

In conclusion, 5% of patients with node-negative biliary cancer had occult cancer cells in para-aortic lymph nodes, yet they have survived over the long term. **The presence of para-aortic micrometastasis might not have influence on the survival of patients with node-negative biliary cancer.** However, we cannot definitively conclude this because of the limited number of patients analyzed. Further studies

including a greater number of patients are required to confirm this supposition.

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## Figure legends

### **FIGURE 1.** Study population.

We examined specimens from 66 of 122 patients with resected biliary cancer. BDC, bile duct carcinoma; GBC, gallbladder carcinoma; ICC, intrahepatic cholangiocarcinoma; AC, ampullary carcinoma.

**FIGURE 2.** Kaplan-Meier survival curves for patients with biliary cancer with negative nodes and positive regional nodes with or without para-aortic nodes.  $P < 0.0001$  (log-rank test).

**FIGURE 3.** Metastatic cells stained positive for CAM5.2 in para-aortic lymph nodes from patients with various types of carcinoma.

**a.** 0.3-mm bile duct carcinoma (forming gland-like nest in marginal sinus). **b.** 0.01-mm gallbladder carcinoma. **c.** 0.04-mm intrahepatic cholangiocarcinoma. Magnification,  $\times 20$ .

Fig. 1

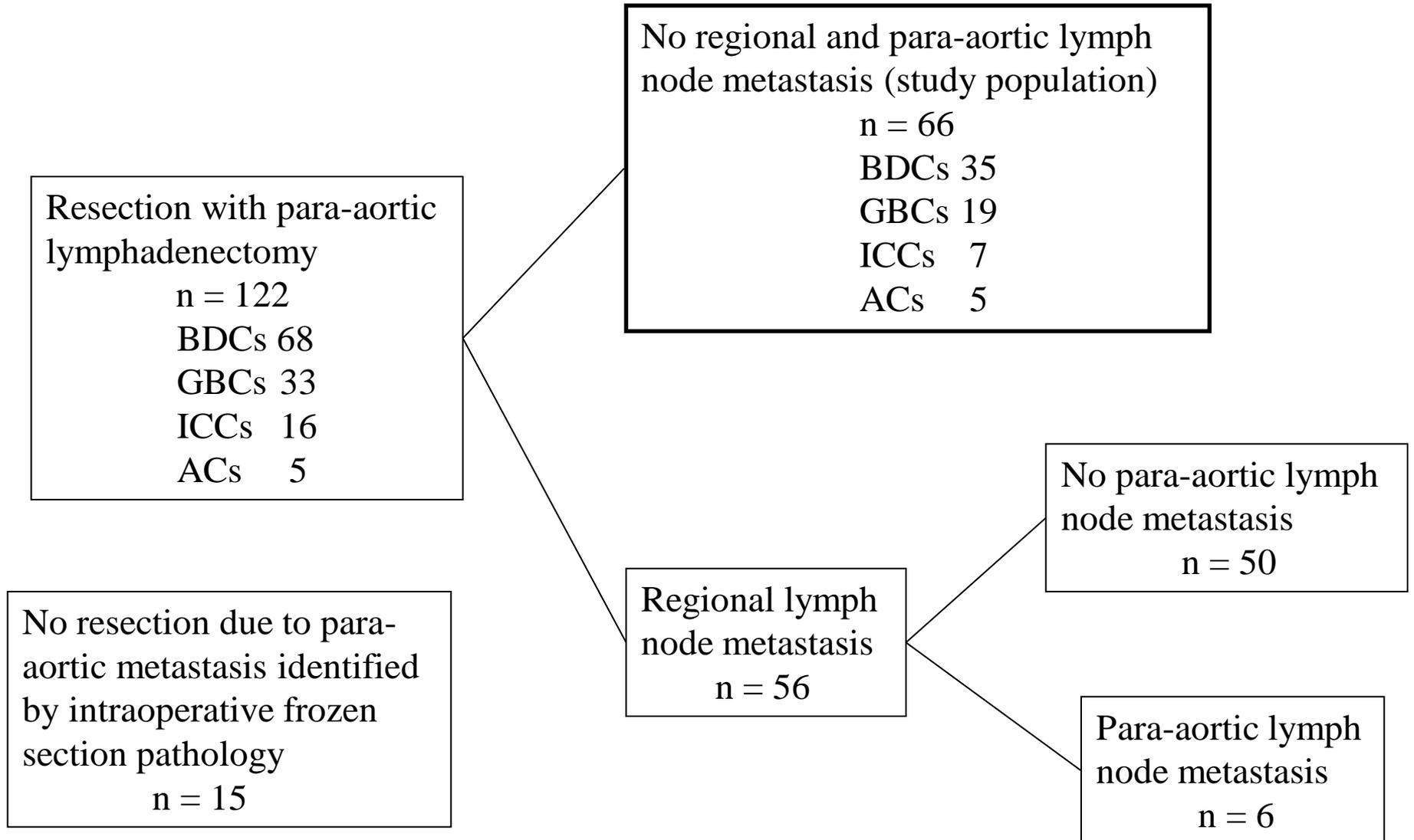
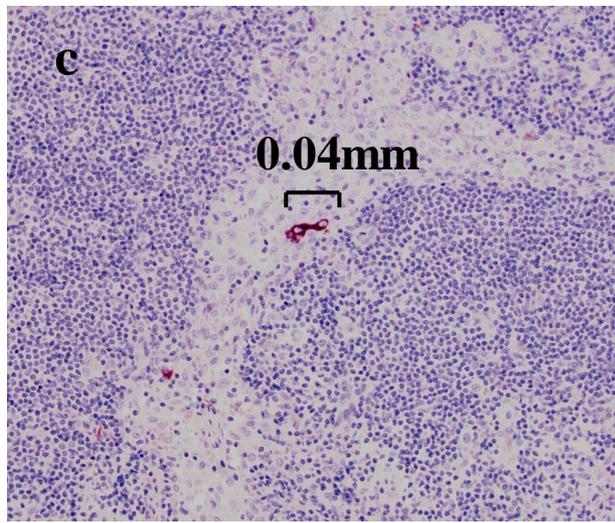
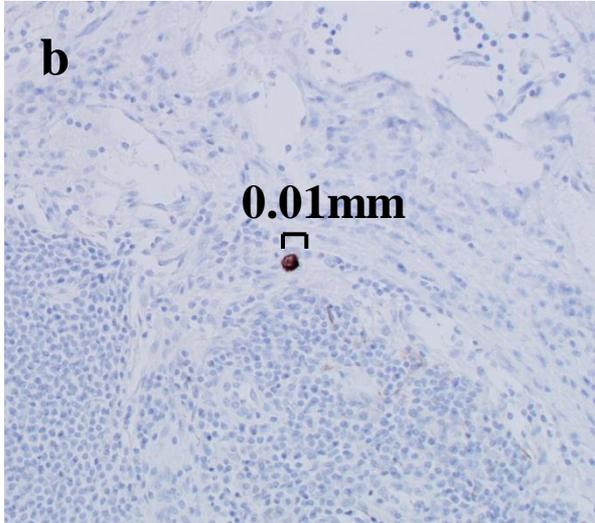
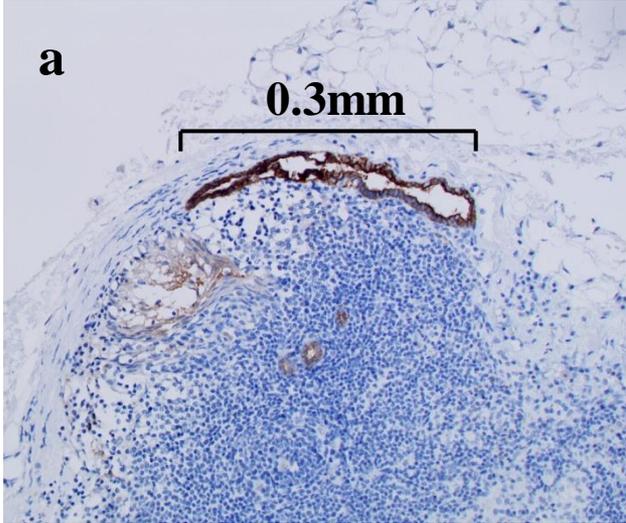




Fig. 3



**Table 1.** Lymph node numbers distributed by each primary lesion.

Primary lesion	BDC	GBC	ICC	AC	Total
No. of patients	35	19	7	5	66
Regional nodes					
Median no. of nodes sampled per patient	9	11	7	16	10
[range]	[1-25]	[5-21]	[1-13]	[7-18]	[1-25]
Para-aortic nodes					
Median no. of nodes sampled per patient	4	8	13	8	6
[range]	[1-28]	[1-24]	[3-29]	[5-15]	[1-29]

BDC, bile duct carcinoma; GBC, gallbladder carcinoma; ICC, intrahepatic cholangiocarcinoma; AC, ampullary carcinoma.

**Table 2.** Clinicopathological characteristics of patients with CAM5.2-positive para-aortic lymph nodes.

	Case 1	Case 2	Case 3
Age (years), Sex	78, F	63, F	67, F
Primary lesion	BDC (perihilar)	GBC	ICC
Tumor size	1.2 × 1.8 cm	7.5 × 6.0 cm	5.0 × 4.0 cm
Surgical procedure	Right hepatic lobectomy	Left hepatic lobectomy	Left hepatic trisegmentectomy
	Caudate lobectomy	Caudate lobectomy	
	Bile duct resection	Bile duct resection	Caudate lobectomy
	with portal vein resection	with duodenum partial resection	Bile duct resection
pT category	pT4 (Main portal vein invasion)	pT4 (Bile duct and duodenum invasion)	pT3 (Left branch of portal vein invasion)
Lymphatic vessel invasion	-	+	+
Venous invasion	+	+	+
Perineural invasion	+	+	+
Histopathological grading	G2	G3	G2
pTNM stage	pStage IVA	pStage IVA	pStage IIIA
Regional lymph node micrometastasis	-	Periduodenal node (0.1mm)	-
Size of para-aortic lymph node micrometastasis	0.3mm	0.01 mm	0.04 mm
Postoperative survival	45 months alive	48 months alive	90 months alive
	No recurrence	No recurrence	Liver/lung recurrence

BDC, bile duct carcinoma; GBC, gallbladder carcinoma; ICC, intrahepatic cholangiocarcinoma; pTNM, pathological tumour node metastasis.