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CLINICAL RESEARCH

Impact of lymph node micrometastasis in hilar bile duct carcinoma patients

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Abstract

AIM: To immunohistochemically examine micrometastasis and VEGF-C expression in hilar bile duct carcinoma (HBDC) and to evaluate the clinical significance of the results.

METHODS: A total of 361 regional lymph nodes from 25 patients with node-negative HBDC were immunostained with an antibody against cytokeratins 8 and 18 (CAM 5.2), and immunohistochemical staining of VEGF-C was performed in 34 primary resected tumors.

RESULTS: Lymph node micrometastasis was detected in 6 (24%) of the 25 patients and 10 (2.8%) of the 361 lymph nodes. Patients with micrometastasis showed significantly poorer survival rates than those without ($P = 0.025$). VEGF-C expression was positive in 17 (50%) of 34 HBDC, and significantly correlated with lymph node metastasis ($P = 0.042$) and microscopic venous invasion ($P = 0.035$).

CONCLUSIONS: It is suggested that immunohistochemically detected lymph node micrometastasis has an impact on the outcome of HBDC. VEGF-C expression is highly correlated with lymph node metastasis in HBDC and might therefore be a useful predictor.

Key words: Hilar bile duct carcinoma; Lymph node metastasis; Micrometastasis; Vascular endothelial growth factor-C

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INTRODUCTION

Hilar bile duct carcinomas (HBDC) are one of the most difficult to cure malignant gastroenterological tumors[1-4] and curative resection is essential for long-term survival. Because hilar bile duct tumors are in close proximity to vital structures in the hepatic hilum, such as the hepatic artery and portal vein, and since they tend to spread to the proximal biliary tract and perineural and perilymphatic spaces, hepatectomy with thorough systematic extended lymph node dissection is frequently required for curative resection. However, even with margin-negative resection, the prognosis after curative resection remains poor. One possible reason for the poor outcome is existence of occult lymph node metastasis that cannot be detected by conventional hematoxylin and eosin (HE) staining at the time of surgical resection. Immunohistochemical and molecular techniques have, however, made it possible to identify lymph node micrometastasis missed by traditional methods. Recently, immunohistochemical and/or genetic detection of lymph node micrometastases of various tumors, including carcinomas of the breast[5,6], lung[7,8], esophagus[9,10], stomach[11-14], colorectum[15,16] and gallbladder[17-19], has been reported. However, we were able to find only one report documenting this in HBDC[20].

Vascular endothelial growth factor C (VEGF-C) is a member of the highly glycosylated vascular endothelial growth factor (VEGF) family that regulates vasculogenesis, hematopoiesis, angiogenesis, lymphangiogenesis and vascular permeability, and has been implicated in many physiological and pathological processes[21,22]. Overexpression of VEGF-C cDNA in the skin of transgenic mice has been shown to selectively induce lymphatic endothelial cell proliferation and hyperplasia of the lymphatic vasculature[23]. It was also recently reported that a VEGF-C-transfected tumor cell line implanted into the stomach of nude mice gave rise to numerous lymph node metastases[24]. The most prominent VEGF-C expression has been detected in the human heart, placenta, muscle, ovary, and small intestine[25], and a positive correlation between expression and various clinicopathological factors, especially lymph node metastasis, has been reported in a number of tumors, including carcinomas of the thyroid[26], breast[27], lung[28], esophagus[29], stomach[30,31], colorectum[32], prostate[33] and pancreas[34]. However, no investigations have been conducted with regard to VEGF-C expression in HBDC and possible clinicopathological associations. In this study, we examined lymph node micrometastasis and VEGF-C expression in patients with HBDC and evaluated the clinical significance of the results.

MATERIALS AND METHODS

Patients and specimens

From January 1981 to August 2000, 61 patients with HBDC underwent surgical resection plus systematic lymph node dissection in the First Department of Surgery, Mie University School of Medicine. Of these patients, 34 underwent macroscopic and microscopic margin-negative resection. Patients consisted of 21 males and 13 females with a mean age of 64.4 ± 11.0 years (range: 37-89 years). The median follow-up period was 31.8 mo (minimum: 1.0 mon). No lymph node metastases were detected in 25 (73.5%) of the 34 patients by traditional pathologic examinations with HE staining.

Hepatectomy was performed in 29 (85.3%) of the 34 patients: extended right hepatectomy in 9 patients, left hepatectomy in 8, resection of segments 4a and 5 in 5, hilar resection in 4, extended left hepatectomy in 2, and caudate lobectomy only in 1. All 29 patients underwent combined resection of the caudate lobe. Two patients underwent combined resection of the portal vein, and 3 underwent pancreatoduodenectomy (PD) or pylorus-preserving pancreatoduodenectomy. Another 5 patients were treated with bile duct resection alone, including 2 patients who underwent combined PD.

A total of 361 lymph nodes dissected from 25 node-negative patients were examined immunohistochemically by staining with an antibody against cytokeratins 8 and 18, and all 34 primary tumors were immunohistochemically stained for VEGF-C. Tumor specimens and lymph nodes were collected from pathology files after obtaining informed consent from all patients in accordance with institutional guidelines.

Lymph node groups and resected margin status

Identification of the sites of lymph node metastasis were performed in accordance with the TNM Classification of Malignant Tumors proposed by the International Union Against Cancer (UICC)[35], which defines regional lymph nodes as the cystic duct, pericholedochal, hilar and peripancreatic (head only), periduodenal, periportal, celiac and superior mesenteric nodes, N0 as no regional lymph node metastasis and N1 as regional lymph node metastasis.

Evaluation of resected margin status was performed in accordance with the General Rules for Surgical and Pathological Studies on Cancer of the Biliary Tract (The 5th Edition) proposed by the Japanese Society of Biliary Surgery (JSBS)[36], which defines pEM0 as no tumor invasion within 5 mm of the resected margin, pEM1 as tumor invasion within 5 mm of the resected margin and pEM2 as distinct tumor invasion of the resected margin. pEM0 and pEM1 resections were defined as margin-negative in this study.

Immunohistochemical staining

Tissue samples were fixed in 10% formaldehyde with phosphate-buffered saline (PBS) and embedded in paraffin. Lymph node tissue was cut into six 5-μm thick sections, and primary tumor tissue was cut into a single 5-μm thick section. Briefly, the sections were deparaffinized with xylene and rehydrated through graded concentrations of ethanol. For antigen retrieval, sections were placed in 0.1*mol*/L citrate buffer (pH 6.0) and heated three times for 3 min each in a microwave oven (500 W). Lymph node sections were then incubated with a mouse monoclonal antibody (CAM 5.2; Becton Dickinson, San Jose, CA) specific for cytokeratins 8 and 18, and tumor sections were incubated with affinity-purified goat polyclonal antibodies (IBL, Fujioka, Japan) to VEGF-C at 1:30 dilution. Immunohistochemical detection of CAM 5.2 and VEGF-C was performed by a standard avidin-biotin method on an automated Ventana ES immunostainer (Ventana Medical Systems, Tucson, AZ) according to the manufacturer's instructions[37].

We examined 6 sections per lymph node and diagnosed micrometastasis when tumor cells were detected immunohistochemically, after being missed by routine histologic examinations with HE staining. VEGF-C immunoreactivity was mainly present in the cytoplasm of cancer cells and/or in the connective tissue around cancer cells. For evaluation of VEGF-C immunostaining, we examined at least 200 cancer cells per case. Cases in which at least 10% of the cancer cells were immunoreactive were defined as VEGF-C positive. All immunohistochemical evaluations were performed by an experienced histopathologist unaware of the clinicopathological features of the patients.

Statistical analysis

All statistical calculations were carried out using StatView-J 5.0 statistical software (SAS Institute, USA). Results are expressed as the means ± SD. Statistical analysis for comparisons of VEGF-C expression and clinicopathological factors (age, gender, lymphatic vessel invasion, microscopic venous invasion, perineural invasion and lymph node metastasis) were performed using the chi-square test and Fisher's exact probability test. Analysis for comparisons of VEGF-C expression and other factors (pT classification and histopathological grading) was performed using the Mann-Whitney *U*-test. The Kaplan-Meier method was used to estimate postoperative survival rates, and the generalized log-rank test was used to compare differences in survival rates. All P values were two-sided and $P < 0.05$ was considered statistically significant.

RESULTS

Patient outcomes

Of the 34 patients with margin-free resected HBDC, 4 died of other causes; three of multiple organ failure including 1 postoperative death (within 1 mo), and 1 of unknown causes. In addition, 15 (50.0%) of the remaining 30 patients died of disease. Recurrence sites were the

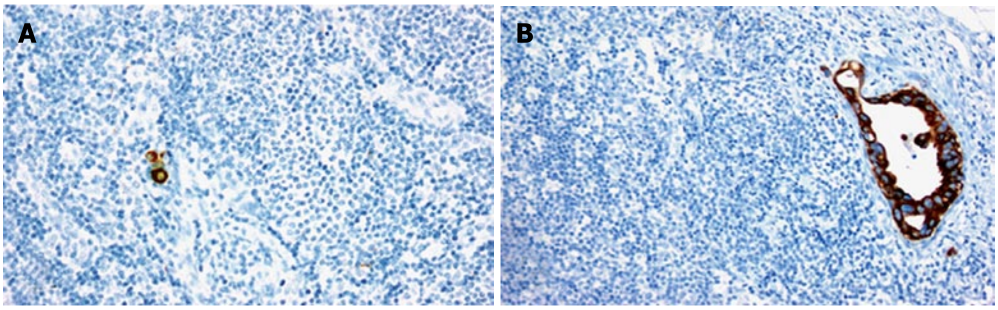

Figure 1 Immunohistochemical staining of lymph node micrometastasis with the monoclonal antibody CAM 5.2. **A:** Micrometastasis consisting of a single cell (original magnification, ×200). **B:** Micrometastasis consisting of a small cluster of tumor cells (original magnification, ×100).

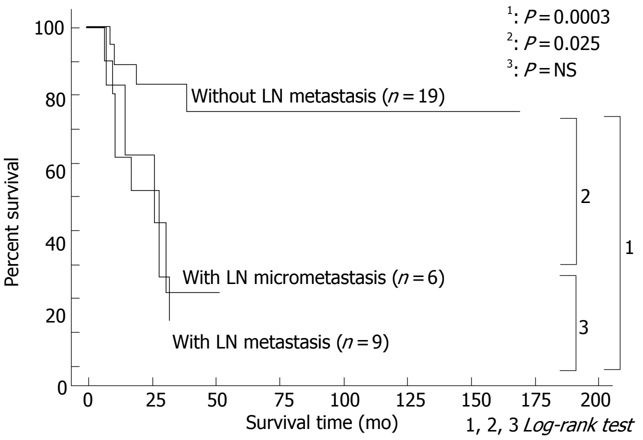

Figure 2 Survival curves after resection for hilar bile duct carcinoma according to the presence of lymph node metastasis, including micrometastasis.

liver in 2 patients including 1 patient with combined lung recurrence, the peritoneum in 1 patient, and local regions in 11 patients. Of these 11 patients, 3 showed combined recurrence in other sites; 1 showed combined liver metastasis, 1 showed combined lung metastasis, and 1 showed combined peritoneum recurrence.

Detection of lymph node micrometastasis

Micrometastasis was detected in 6 (24.0%) of the 25 node-negative patients and 10 (2.8%) of the 361 lymph nodes by immunohistochemical examination with CAM5.2. Lymph node micrometastasis was present in the form of a single-cell metastasis (Figure 1A) or a small cluster of tumor cells (Figure 1B). Of the 6 patients with lymph node micrometastasis, 5 had regional lymph node micrometastasis and 1 had regional lymph node with para-aortic lymph node micrometastases.

Impact of lymph node micrometastasis on survival

Cumulative survival rates were compared according to nodal status: the without lymph node metastasis group versus lymph node micrometastasis group versus HE diagnosed (overt) lymph node metastasis group (Figure 2). The 3- and 5-year survival rates of the 19 patients without lymph node metastasis were 81.6 and 72.5%, respectively, as opposed to 20.8 and 20.8%, respectively, in the 6 patients with micrometastasis and 29.6% and 0.0%, respectively, in the 9 patients with overt lymph node metastasis. Patients with lymph node micrometastasis showed significantly worse survival rates than those without ($P = 0.025$), and moreover, patients with overt lymph node metastasis showed worse survival rates than those without ($P = 0.0003$). There were no statistical differences between patients with lymph node micrometastasis and those with overt lymph node metastasis ($P = 0.469$). Five patients died of disease without overt lymph node or micrometastasis. Of these, 4 died of local recurrence, including 1 patient with combined liver metastasis. The remaining patient died of liver and lung metastasis. Follow-up revealed that 3 patients with lymph node micrometastasis survived with no evidence of disease for 11.7 and 36.7 and 60.3 mon after surgical resection, respectively.

To further evaluate the impact of lymph node micrometastasis on survival, survival rates were compared according to two groups: patients without lymph node metastasis versus those with overt lymph node and micrometastasis (Figure 3), and patients without lymph node metastasis and those with lymph node micrometastasis versus those with overt lymph node metastasis (Figure 4). The 3- and 5-year survival rates of the 19 patients without lymph node metastasis were 81.6 and 72.5%, respectively, as opposed to 25.5 and 8.5%, respectively, in the 15 patients with overt lymph node and micro metastasis ($P = 0.0004$). On the other hand, the 3- and 5- year survival rates of the 25 patients without lymph node metastasis and those with lymph node micrometastasis were 66.9 and 60.2%, respectively, as opposed to 14.8 and 0.0%, respectively, in the 9 patients with overt lymph node metastasis ($P = 0.0015$).

VEGF-C expression and clinicopathological factors

VEGF-C expression was observed in 17 (50.0%) of the 34 primary tumors (Figure 5). The correlations between VEGF-C expression and clinicopathological factors are shown in Table 1. Microscopic venous invasion ($P = 0.035$) and lymph node metastasis ($P = 0.042$) were significantly correlated with VEGF-C expression.

Prognostic factors for hilar bile duct carcinoma

To identify useful prognostic factors, we performed univariate analysis of the following possible independent prognostic factors: age (above 60 years versus 60 years or less), gender, operative procedure (hepatectomy versus bile duct resection), histopathological grading (well differentiated versus moderately or poorly differentiated), lymphatic vessel invasion, microscopic venous invasion, perineural invasion, microscopic resection margin (em0 versus em1), VEGF-C expression, lymph node metastasis (including micrometastasis) and lymph node metastasis (excluding micrometastasis) (Table 2). Ultimately, 4 independent variables (microscopic resection margin ($P = 0.040$), VEGF-C

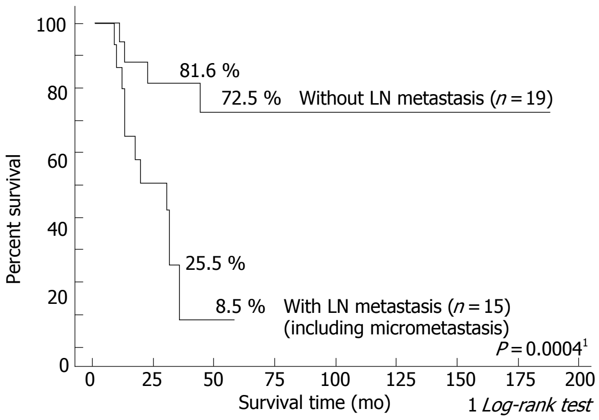

Figure 3 Survival curves after resection for hilar bile duct carcinoma according to the presence of lymph node metastasis: patients without lymph node metastasis versus those with overt lymph node and micro metastasis.

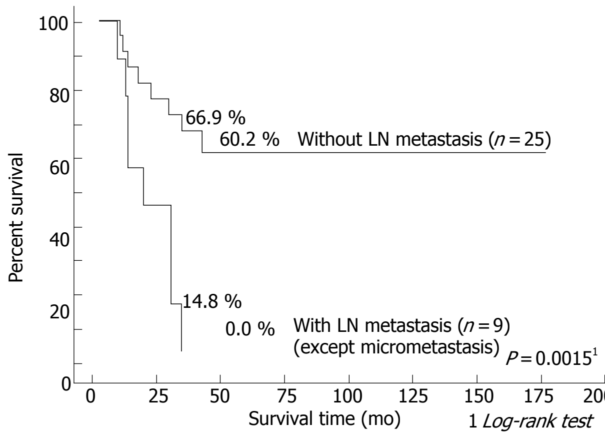

Figure 4 Survival curves after resection for hilar bile duct carcinoma according to the presence of lymph node metastasis: patients without lymph node metastasis and those with lymph node micrometastasis versus those with overt lymph node metastasis.

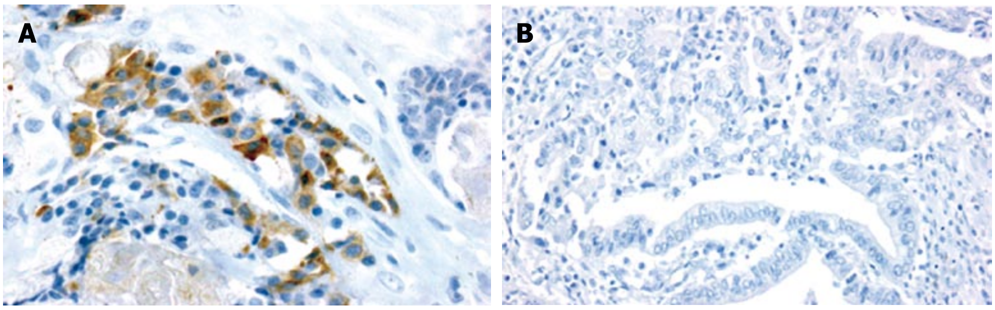

Figure 5 Immunohistochemical staining of primary tumors with VEGF-C polyclonal antibody. **A:** VEGF-C positive (original magnification, ×400). **B:** VEGF-C-negative (original magnification, ×200).

Table 1 Clinicopathological factors and VEGF-C expression

		VEGF-C expression		
		Positive (n = 16)	Negative (n = 18)	P Value
Age		64.7±11.6	64.1±10.8	0.870
Gender (M / F)		10 / 6	11 / 17	0.999
pT classification[1]	pT1	1	4	0.56
	pT2	8	11	
	pT3	7	3	
Histopathological Grading[1]	G1	10	15	0.225
	G2	6	2	
	G3	0	1	
Lymphatic vessel invasion	(presence)	14 (87.5 %)	13 (72.2 %)	0.405
Venous invasion	(presence)	10 (62.5 %)	4 (22.2 %)	0.035
Perineural invasion	(presence)	14 (87.5 %)	10 (55.6 %)	0.063
Lymph nodes metastasis	Metastasis (-)	6	13	0.042
	Metastasis (+) (including micrometastasis)	10	5	

[1]According to the TNM staging system. pT classification: pT1: Tumor confined the bile duct; pT2: Tumor invades beyond the wall of the bile duct; pT3: Tumor invades the liver, gallbladder, pancreas, and/or unilateral tributaries of the portal vein (right or left) or hepatic artery (right or left); pT4: Tumor invades any of follwing: main portalvein or its tributaries bilatellary, common hepatic artery, or other adjacent structures, e.g., colon, stomach, duodenum, abdominal wall. Histopathological Grading: G1: Well differentiated; G2: Moderately differentiated; G3: Poorly differentiated.

expression ($P = 0.036$), lymph node metastasis (including micrometastasis) ($P = 0.0004$) and lymph node metastasis (excluding micrometastasis) ($P = 0.0017$)) were identified as statistically significant predictors of survival.

DISCUSSION

Lymph node metastasis is a well known important predictor of prognosis with a wide variety of malignant tumors, and some studies have reported a significant relationship

Table 2 Univariate analysis of survival

Variable		5-yr survival (%)	*P* Value
Age	< 60 *vs* ≥ 60	40.0 *vs* 44.9	0.912
Gender	male *vs* female	42.3 *vs* 45.5	0.872
Operative procedure	hepatectomy *vs* bile duct resection	42.2 *vs* 60.0	0.430
Histopathologic Grading[1]	G2, G3 *vs* G1	37.5 *vs* 46.0	0.393
Lymphatic vessel invasion	present *vs* absent	37.4 *vs* 75.0	0.076
Venous invasion	present *vs* absent	36.4 *vs* 49.7	0.185
Perineural invasion	present *vs* absent	36.3 *vs* 68.6	0.064
Microscopic resection margin[2]	em1 *vs* em0	31.7 *vs* 83.3	0.040
VEGF-C expression	positive *vs* negative	23.3 *vs* 70.5	0.036
Lymph node metastasis (Including micrometastasis)	positive *vs* negative	8.5 *vs* 72.5	0.0004
Lymph node metastasis (except micrometastasis)	positive *vs* negative	0.0 *vs* 60.2	0.0017

[1] According to the TNM staging system. Histopathological Grading: G1 Well differentiated, G2 Moderately differentiated, G3 Poorly differentiated.
[2] According to the Japanese Society of Biliary Surgery. General Rules for Surgical and Pathological Studies on Cancer of the Biliary Tract. em0: no tumor invades within 5 mm from resected margin; em1: tumor invades within 5mm from resected margin.

between lymph node metastasis and prognosis of HBDC patients[38-40]. However, patients with early stage carcinoma and no apparent lymph node metastasis sometimes die of metastasis after surgery despite complete resection of the primary lesion. One of the possible reason for the poor outcome in these patients is occult lymph node metastasis not identified by conventional HE staining at the time of surgical resection.

Numerous studies on the incidence and significance of lymph node micrometastasis in cancer patients have been conducted in recent years. A number of investigators have proposed the prognostic significance of lymph node micrometastasis for various tumors including lesions of the lung, esophagus, stomach and colon, while others have suggested that lymph node micrometastasis is not significant for patient outcome. Thus, there is no consensus on the clinical significance of lymph node micrometastasis. However, we were able to find only one report documenting this in HBDC. Tojima *et al*[20] investigated 954 nodes from 45 patients with pN0 hilar cholangiocarcinoma after curative resection, and found micrometastasis in 13 (1.4%) nodes from 11 (24.4%) patients. Their data yielded similar survival curves for patients with and without lymph node micrometastasis (5-year survival rates: 43.6% *vs* 42.1%, respectively).

In this study, we demonstrated significant differences between outcomes of HBDC patients with and without lymph node micrometastases. Interestingly, a stronger correlation was recognized when patients with lymph node micrometastasis were treated as lymph node metastasis positive, compared to when they were treated as lymph node metastasis negative ($P = 0.0004$ versus $P = 0.0017$) (Figures 3 and 4). This might suggest the need to consider lymph node micrometastasis as overt lymph node metastasis.

One possible reason for the above-mentioned differing results is the number of sections examined. The number of sections immunohistochemically stained is considered an important factor in the diagnosis of lymph node micrometastasis. Many investigators examine lymph node micrometastasis using various sections of different thickness for immunohistochemical staining; however, the total thickness examined tends to range from 3 to 30 μm[6,8,10,13-20]. Sasaki *et al* examined the correlation between the number of CAM 5.2 sections and cumulative positive rate of lymph node metastasis[41]. They found that positive metastasis detection reached a plateau when over 9 sections (total thickness 27 μm) were examined. In this study, to identify lymph node micrometastasis, we examined six 5-μm sections (total thickness 30 μm) per lymph node by immunohistochemical staining. When we examined only one to four sections per lymph node, we found fewer lymph node micrometastases (data not shown).

Another possible reason for the differing results is the criteria of lymph node micrometastasis. In many studies, including ours, micrometastasis is defined as tumor cells detected only by immunohistochemical staining. However, some authors set size criteria for micrometastasis, such as deposits less than 2[42] or 0.5 mm in diameter[20,43]. Recent progress in molecular biological techniques has led to the development of genetic methods for detecting micrometastasis, including RT-PCR. RT-PCR is capable of detecting more micrometastasis foci than immunohistochemical staining[44]. Five patients without overt lymph node or micro metastasis died of disease recurrence in this study. If we use RT-PCR to detect lymph node micrometastasis, we will be able to evaluate lymph node micrometastasis in more detail, and the significance of lymph node micrometastasis will potentially increase. Therefore, further examinations using RT-PCR appear necessary.

VEGF-C is a specific ligand of VEGFR-3 and VEGFR-2, and has been shown to stimulate lymphangiogenesis and angiogenesis both *in vitro* and *in vivo*. Nakashima *et al*[45] investigated VEGF-C expression in 52 patients with gallbladder carcinoma and found that expression was significantly stronger ($P < 0.001$) in patients with lymph node metastasis than those without, and that the VEGF-C-positive group showed poorer outcomes than the negative group ($P < 0.001$).

Our study revealed a significant correlation between VEGF-C expression and both the presence of lymph node metastasis (HE detected and micrometastasis) and outcome of HBDC. These results suggest that VEGF-C expression might play an important role in causing lymph node metastasis in HBDC, consistent with the findings of previous studies regarding other malignant tumors.

In conclusion, our findings suggest that immunohistochemical detection of lymph node micrometastasis provides very useful information of survival rates after surgery for HBDC. However, considering that 1 patient with lymph node micrometastasis survived for more than 5-years with no evidence of tumor recurrence, long-term survival is thus possible for some patients with lymph node micrometastasis; therefore, extended lymph node dissection is necessary in HBDC patients. Although further study is needed, VEGF-C seems to be a useful predictor of overt and micro lymph node metastasis.

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