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ORIGINAL ARTICLE

Clinicopathologic significance of the delta-like ligand 4, vascular endothelial growth factor, and hypoxia-inducible factor-2α in gallbladder cancer

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Background: Gallbladder cancer (GBC) is usually detected in advanced stages with a low 5-year survival rate. Delta-like ligand 4 (DLL4), vascular endothelial growth factor (VEGF), and hypoxia-inducible factor-2alpha (HIF2α) have been studied for their role in tumorigenesis and potential for therapeutic target, and multiple clinical trials of the agents targeting them are ongoing. We investigated the expression of these markers in surgically resected GBC and tried to reveal their association with the clinicopathologic features, mutual correlation of their expression, and prognosis of the GBC patients by their expression. **Methods:** We constructed the tissue microarray blocks of 99 surgically resected GBC specimens and performed immunohistochemistry of DLL4, VEGF, and HIF2α. We used the quantitative digital image analysis to evaluate DLL4 and VEGF expression, while the expression of HIF2α was scored manually. **Results:** The expression of VEGF and HIF2α showed a significant trend with tumor differentiation ($p=.028$ and $p=.006$, respectively). We found that the high DLL4 and VEGF expression were significantly correlated with lymph node metastasis ($p=.047$, both). The expression of VEGF and HIF2α were significantly correlated ($p<.001$). The GBC patients with low HIF2α expression showed shorter recurrence-free survival than those with high HIF2α expression. **Conclusions:** This study suggested the possibility of the usage of DLL4 and VEGF to predict the lymph node metastasis and the possibility of VEGF and HIF2α to predict the expression level mutually. Further studies may be needed to validate our study results and eventually accelerate the introduction of the targeted therapy in GBC.

Key Words: Gallbladder neoplasms; DLL4; Vascular endothelial growth factor; HIF2α; Targeted therapy

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Gallbladder cancer (GBC) ranks in the top 10 for both incidence and mortality in South Korea [1]. GBC is known as a deadly disease because only 21.8% of the patients are diagnosed at a localized stage, while near half (41.0%) present distant metastasis at the time of diagnosis in Korea [2,3]. The recently reported 5-year survival rate of total Korean GBC patients was 28.7%, and in cases with distant metastasis, it was even lower as 2.5% [2]. The poor prognosis of Korean GBC patients suggests the limited efficacy of conventional systemic treatments such as gemcitabine plus cisplatin, 5-fluorouracil, etc. It implicates the need to develop other treatment modalities for advanced-stage patients [4]. In the era of molecular pathology, there have been many attempts to discover actionable targets in GBC, and there are several ongoing clinical trials with novel therapeutic agents including tyrosine kinase inhibitors and immunotherapeutic agents [5,6].

Delta-like ligand 4 (DLL4) is one of the transmembrane agonistic ligands of Notch receptors [7,8] which is induced by vascular endothelial growth factor (VEGF). It plays a role as a negative feedback regulator to prevent over-exuberant angiogenesis and promote the proper formation of vascular structures [9]. Furthermore, DLL4 in vasculature cells is also involved in tumor angiogenesis through interactions with the VEGF pathway [9,10]. Previous studies demonstrated that high DLL4 expression was correlated with or predicted poor prognosis in gastric cancer and pancreatic cancer patients [11,12]. In other cancer types, includ-

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ing breast cancer and head and neck squamous cell carcinoma, several studies were executed regarding DLL4 as a potential therapeutic target [13-15]. In more recent years, there have been multiple clinical trials for the efficacy of anti-DLL4 antibodies and anti-DLL4/anti-VEGF bispecific antibodies in advanced solid tumors [16,17].

Hypoxia-inducible factor-2alpha (HIF2α) is a transcription factor that is stabilized in hypoxic conditions and activates multiple downstream genes, including VEGF [18,19]. Hypoxia, in turn, increases the expression of Notch ligands, including DLL4, whereas Notch signaling regulates the response for hypoxia in multiple cancers by controlling the expression of HIF2α [18]. It is well known that the VHL-HIF2α-VEGF axis is involved in tumor development and progression of conventional clear cell type renal cell carcinoma (RCC), and the treatment options that target the molecules in this pathway have progressed in recent years. The target therapies for RCC include: targeting angiogenesis through VEGF inhibitors, anti-proliferative agents targeting the mammalian target of rapamycin pathway, immune-checkpoint inhibitor, and novel HIF2α inhibitors [20,21].

Based on the accumulated data about the interactions and associations of DLL4, VEGF, and HIF2α, we assumed that it is worthy of analyzing the association between the expression of these markers in GBC. We aimed first to find out whether the expression levels are associated with the clinicopathologic characteristics and prognosis of the patients and whether there is an association between the expressions of the three markers. On the therapeutic aspect, if substitution between an anti-DLL4 antibody or anti-DLL4/anti-VEGF bispecific antibody and HIF2α-inhibitor would be proved possible in the future based on our results, it would be a benefit for patients considering possible adverse effects or resistance [16,20].

MATERIALS AND METHODS

Patient selection and tissue samples

We collected the tissues of the GBC patients that underwent surgical resection between January 2010 and December 2017 from the surgical pathology database of Samsung Medical Center (Seoul, Korea). Initially, 101 cases were found, but one was excluded because the tumor was a metastatic tumor from the liver, and another one was excluded due to pre-operative chemotherapy. Finally, we enrolled a total study population of 99 GBC cases. Clinical data, including age, sex, date of surgery, history of post-operative chemotherapy, recurrence-free survival (RFS), overall survival, and duration of follow-up, were extracted from electronic medical records. As all hematoxylin and eosin (H&E)-stained slides were reviewed by two pathologists (K.T.J. and S.P.), the histologic type and differentiation were reviewed for all tumor tissues. We checked the tumor staging according to the American Joint Committee on Cancer staging system (8th edition) [22].

Tissue microarray construction and immunohistochemistry

Representative tumor areas confirmed for the absence of hemorrhage or necrosis were marked on the formalin-fixed paraffin-embedded blocks. Two tissue cores with a diameter of 2.0 mm were acquired from each donor block and were arranged in the recipient paraffin blocks. Each tissue microarray (TMA) block contained up to 40 tumor tissue cores and two control tissue cores. One normal pancreas tissue core and one normal tonsil tissue core were used as control cores.

Immunohistochemistry (IHC) was performed on 4-μm-thick tissue sections obtained from TMA blocks. For detection of DLL4 and HIF2α, automated Ventana BenchMark Ultra instrument (Ventana Medical Systems, Tucson, AZ, USA) was used for antigen retrieval and primary antibody reaction. After the antigen retrieval for 92 minutes with CC1 in Ventana BenchMark Ultra, the sections were incubated with anti-DLL4 antibody (HPA023392, 1:50, Sigma-Aldrich, St. Louis, MO, USA) for 60 minutes in 37°C and with EPAS-1 (sc-46691, 1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for HIF2α detection for 120 minutes in 37°C, respectively. For EPAS-1, the chromogenic reactions were carried out for 12 minutes with OptiView Amplification Kit (860-099, Ventana Medical Systems) and OptiView DAB IHC Detection kit (760-700, Ventana Medical Systems), but for anti-DLL4 antibody, with OptiView DAB IHC Detection kit (760-700, Ventana Medical Systems) only. For VEGF protein detection, the sections were incubated with a mouse monoclonal anti-VEGF antibody (sc-7269, 1:500, Santa Cruz Biotechnology) for 20 minutes in a Bond-max autoimmunostainer (Leica Biosystems, Melbourne, Australia) after antigen retrieval with ER1 buffer (pH 6.0, Leica Biosystems) in 100°C. Antigen-antibody chromogenic reactions were developed for 10 minutes using the Bond Polymer refine detection kit, DS9800 (Vision Biosystems, Melbourne, Australia).

Quantitative digital image analysis and manual scoring

The TMA slide stained with H&E and IHC was digitized by Aperio AT2 scanner (Leica Biosystems, Buffalo Grove, IL, USA) at 20× magnification. For DLL4 and VEGF expression analysis, Aperio ImageScope software (ver. 12.4.2, Leica Biosystems, Buffalo Grove, IL, USA) was used. All tumor cells except those

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in lymphocyte-rich areas were exclusively annotated in each TMA core. According to the expression patterns of the antibodies, DLL4 expression and VEGF expression were analyzed with membrane v9 algorithm and cytoplasm v2 algorithm (Fig. 1), respectively. The algorithms were available as a component in the commercial version of Aperio ImageScope software. Both algorithms automatically counted the VEGF- or DLL4-positive cells based on their staining intensity (0, 1+, 2+, and 3+). The annotation was performed by one pathologist (S.P.), and reviewed by an additional pathologist (K.T.J.) before the automatic analysis. Two pathologists (S.P. and K.T.J.) jointly reviewed the digitally scanned slides and results of the automatic analysis to confirm its performance. The H-scores could be automatically derived from the results (cytoplasm v2), or be calculated from the values of the results (membrane v9).

Due to the extensive background staining of HIF2α compared to DLL4 and VEGF (Fig. 2), the expression of HIF2α was manually scored according to the staining intensity (0, 1+, 2+, and 3+) (Fig. 3) to calculate the H-scores, by one pathologist (S.P.). As an additional pathologist (K.T.J.) reviewed, consensus was achieved between two pathologists for any discrepancy.

Statistical analysis

As appropriate, Pearson's chi-square test or Cochran-Armitage test was used to analyze the correlation between DLL4, VEGF, and HIF2α expression and clinicopathologic parameters. Pearson's chi-square test and Spearman's ρ rank correlation test were used to analyze the correlations between the expression levels of three markers. The Kaplan-Meier survival method was used to analyze survival rates. The Cox proportional hazard regression was used to describe the effects of one or more predictors on survival time or time-to-event outcomes. In all statistical analyses, IBM SPSS ver. 27.0 for Windows (IBM Corp., Armonk, NY, USA) and R software (R Foundation for Statistical Computing, Vienna, Austria) were used.

RESULTS

Clinicopathologic features of the GBC patients

The clinicopathologic features of the GBC patients and the

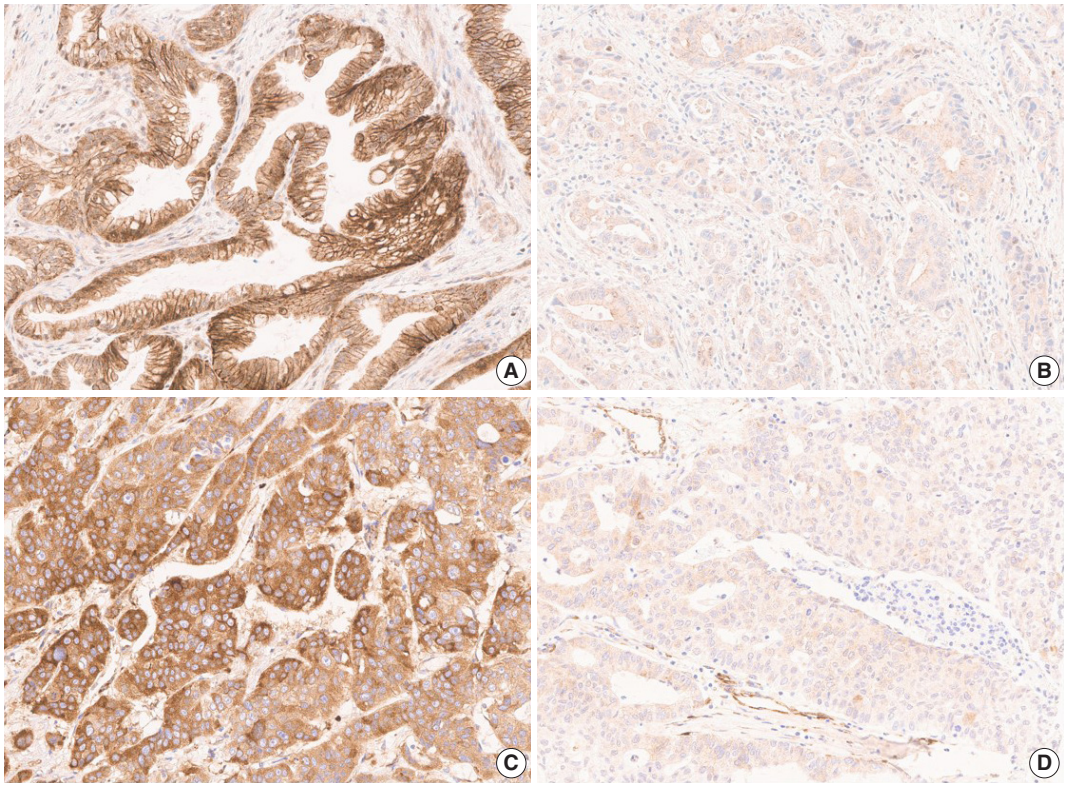

Fig. 1. Representative images of expression of anti-DLL4 and anti-VEGF antibodies in gallbladder cancer of each group, according to the H-score calculated via Aperio ImageScope software. (A) High membranous expression of anti-DLL4 antibody (H-score 208.43). (B) Low membranous expression of anti-DLL4 antibody (H-score 40.28). (C) High cytoplasmic expression of anti-VEGF antibody (H-score 214.01). (D) Low cytoplasmic expression of anti-VEGF antibody (H-score 34.36). DLL4, delta-like ligand 4; VEGF, vascular endothelial growth factor.

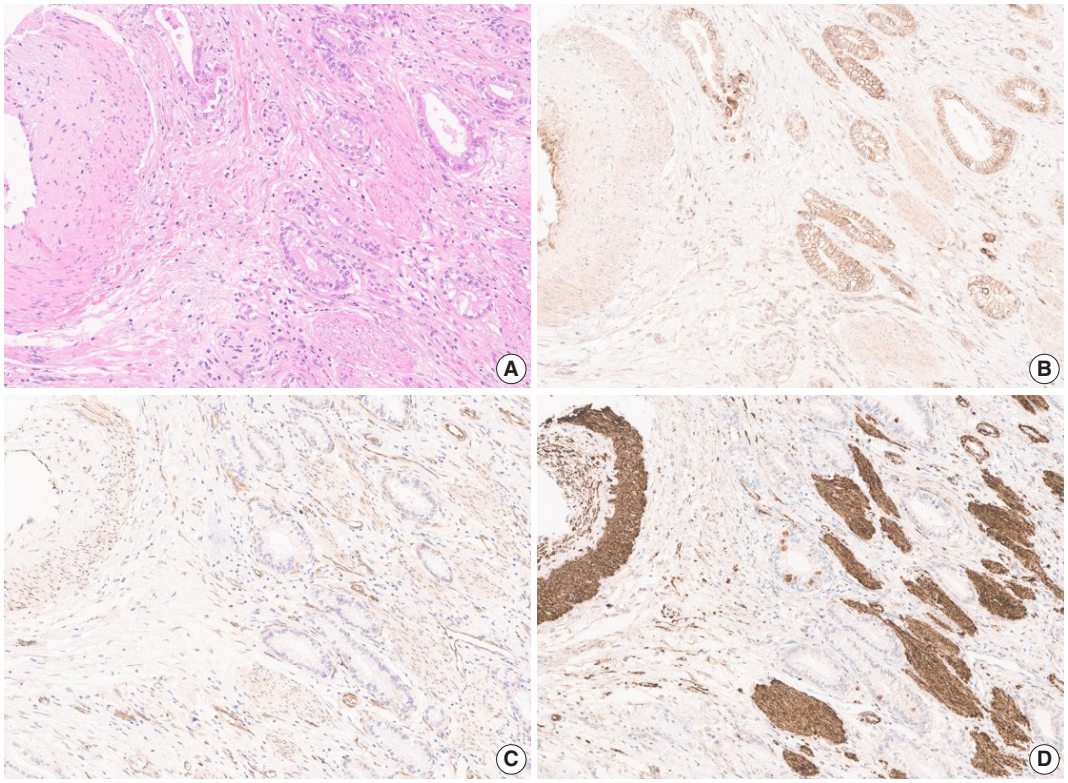

Fig. 2. Images of hematoxylin and eosin stain (A) and expression of anti-DLL4 (B), anti-VEGF (C), and anti-HIF2α (D) antibodies in the corresponding area, showing tumor and surrounding soft tissue, including vessel, muscle, and nerve. Expression of anti-HIF2α antibody shows extensive background stain compared to other markers, which led to manual scoring of H-score. Digital image analysis would mistake the background staining and overestimate the H-score of such cases. DLL4, delta-like ligand 4; VEGF, vascular endothelial growth factor; HIF2α, hypoxia-inducible factor-2α.

association of these features with DLL4, VEGF, and HIF2α expression are summarized in Table 1. For the chi-square test, the expression levels of the markers were divided into two groups: high when the H-score ≥120, low when <120 (Fig. 1). Among the 99 patients, 40 patients (40.4%) were male, and 59 patients (59.6%) were female, and their age at the time of diagnosis ranged widely from 31 to 85 years (median age, 63 years). All tumors were primary GBCs, consist of conventional adenocarcinoma (81 cases, 81.8%), adenosquamous carcinoma (8 cases, 8.1%), neuroendocrine carcinoma (5 cases, 5.1%), mixed neuroendocrine carcinoma and adenocarcinoma, hepatoid adenocarcinoma (1 case, 1.0%), and undifferentiated carcinoma (3 cases, 3.0%, respectively). About half of the cases were well, moderately, or well to moderately differentiated tumors (56 cases, 56.6%). Forty cases (40.4%) had any proportion of poor differentiation, and the remaining three cases (3.0%) were classified as undifferentiated tumors. As a result of surgical resection, most cases were T3 (88 cases, 88.9%). Lymph node metastasis was present in 68 cases (68.7%), and distant metastasis was present in 11 cases (11.1%). Recurrence occurred in 53 cases (53.5%) during the follow-up period (median, 11.6 months; range, 0.7 to 126.9 months) and 36 patients (36.4%) died during the follow-up period (median, 20.0 months; range, 0.7 to 126.9 months).

The chi-square test showed that lower DLL4 expression and VEGF expression was associated with lymph node metastasis ($p = .047$, both). The Cochran-Amitage test revealed that there is a statistically significant linear-trend between the degree of differentiation and VEGF or HIF2α expression ($p = .028$ and $p = .006$, respectively). The test suggests strong evidence of a linearity between the expression of these markers and the tumor differentiation.

Expression of DLL4, VEGF, and HIF2α

When compared by the chi-square test as described above, DLL4 expression did not correlate with the expression of other markers. VEGF and HIF2α expression, however, was significantly correlated ($p < .001$), tumors with high VEGF expression would display higher expression of HIF2α, vice versa.

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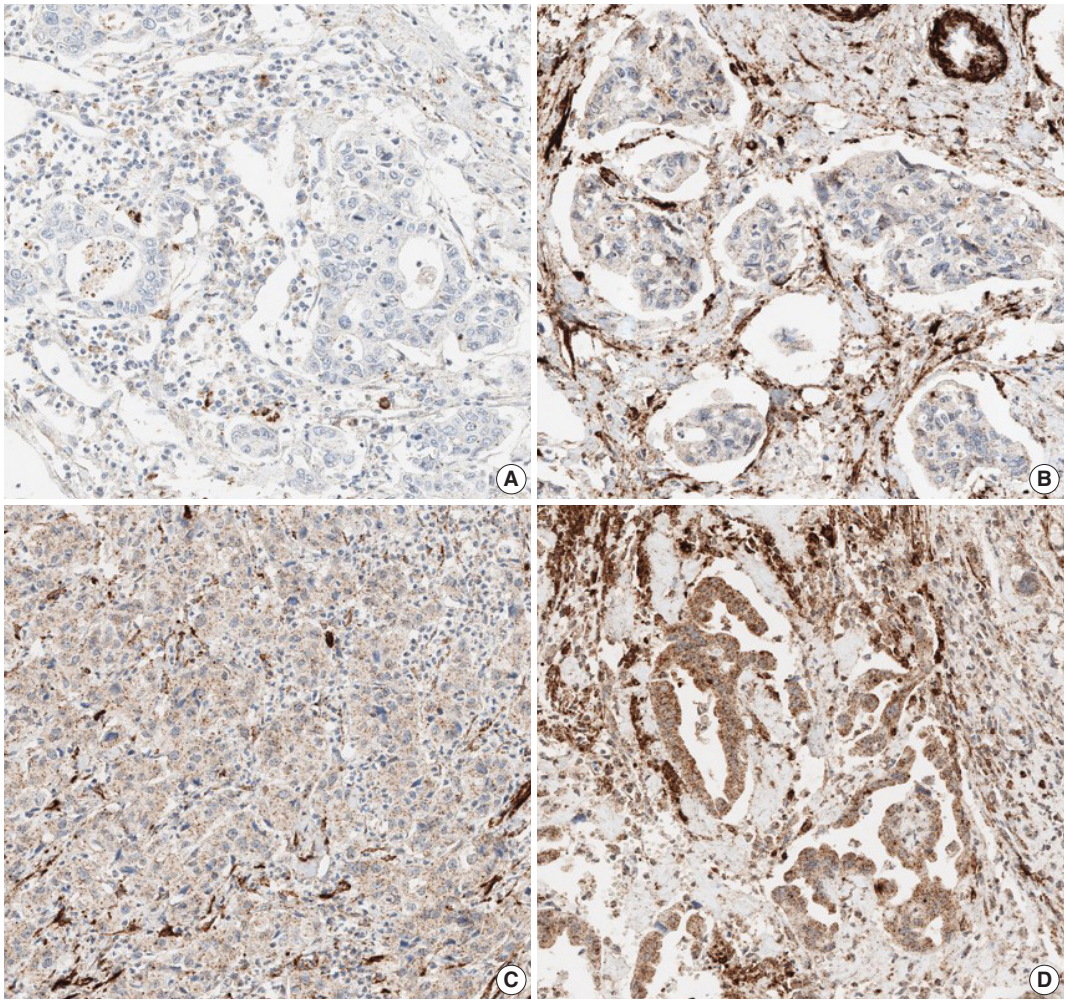

Fig. 3. Representative images of HIF2α immunohistochemistry according to intensity score: (A) score 0, (B) score 1, (C) score 2, and (D) score 3. Background staining was intense in smooth muscles and lymphocytes. HIF2α, hypoxia-inducible factor-2α.

To further confirm the statistical significance of the correlation between expressions of the three markers, we performed an additional statistical analysis. By correlation matrix, the correlations were visualized, showing weak correlations between DLL4 vs. VEGF, and DLL4 vs. HIF2α, but a relatively strong correlation between VEGF vs. HIF2α (Fig. 4).

Considering that the H-score values of three makers are not normally distributed, we used Spearman's ρ rank correlation coefficient to test the significances of correlations between the H-score values of three makers. Between DLL4 and VEGF, and between DLL4 and HIF2α, the Spearman's rank correlation coefficients are 0.09 ($p = .356$) and 0.34 ($p < .001$), respectively, which is consistent with the insignificant results obtained with previous chi-square test. Between H-score values of VEGF and HIF2α, there was a statistically significant positive correlation, with correlation coefficient of 0.57 ($p < .001$). The Spearman's rank correlation coefficient values between each marker are reflected in Fig. 4.

Impact of DLL4, VEGF, and HIF2α expression on the prognosis

According to the Kaplan-Meier survival analysis result, expression of DLL4 and VEGF did not affect recurrence or death. According to HIF2α expression, however, recurrence rates showed a statistically significant difference. When the cutoff for high or low expression was set as H-score = 150, patients with low HIF2α expression (n = 74) showed shorter RFS than the patients with higher HIF2α expression (n = 25) ($p = .048$). When the high expression group was defined as the tumor with more than 30% of 2+ and 3+ cells, patients with low HIF2α expression (n = 70), again, showed shorter RFS than those with higher HIF2α expression (n = 29) ($p = .011$) (Fig. 5). By performing Cox proportional

Table 1. The clinicopathologic features and association with DLL4, VEGF, and HIF2α expression

	Total (n=99)	DLL4 expression			VEGF expression			HIF2α expression		
		Low (n=65, 65.7%)	High (n=34, 34.3%)	p-value	Low (n=56, 56.6%)	High (n=43, 43.4%)	p-value	Low (n=65, 65.7%)	High (n=34, 34.3%)	p-value
Age (yr)				.654			.410			.648
≥ 60	67 (67.7)	43 (64.2)	24 (35.8)		36 (53.7)	31 (46.3)		45 (67.2)	22 (32.8)	
< 60	32 (32.3)	22 (68.8)	10 (31.3)		20 (62.5)	12 (37.5)		20 (62.5)	12 (37.5)	
Sex				.159			.796			.023
Female	59 (59.6)	42 (71.2)	17 (28.8)		34 (57.6)	25 (42.4)		44 (74.6)	15 (25.4)	
Male	40 (40.4)	23 (57.5)	17 (42.5)		22 (55.0)	18 (45.0)		21 (52.5)	19 (47.5)	
Diagnosis				NA			NA			NA
Adenocarcinoma	81 (81.8)	48 (59.3)	33 (40.7)		51 (63.0)	30 (37.0)		52 (64.2)	29 (35.8)	
Adenosquamous carcinoma	8 (8.1)	7 (87.5)	1 (12.5)		2 (25.0)	6 (75.0)		6 (75.0)	2 (25.0)	
NEC	5 (5.1)	5 (100)	0		1 (20.0)	4 (80.0)		4 (80.0)	1 (20.0)	
Mixed NEC and adenocarcinoma	1 (1.0)	1 (100)	0		0	1 (100)		1 (100)	0	
Hepatoid adenocarcinoma	1 (1.0)	1 (100)	0		1 (100)	0		1 (100)	0	
Undifferentiated carcinoma	3 (3.0)	3 (100)	0		1 (33.3)	2 (66.7)		1 (33.3)	2 (66.7)	
Differentiation[a]				.068			.028			.006
WD, MD	56 (56.6)	33 (58.9)	23 (41.1)		37 (66.1)	19 (33.9)		43 (76.8)	13 (23.2)	
PD	40 (40.4)	29 (72.5)	11 (27.5)		18 (45.0)	22 (55.0)		21 (52.5)	19 (47.5)	
UD	3 (3.0)	3 (100)	0		1 (33.3)	2 (66.7)		1 (33.3)	2 (66.7)	
T category[a]				.827			.792			.827
1b, 2a, 2b	5 (5.1)	1 (20.0)	4 (80.0)		3 (60.0)	2 (40.0)		4 (80.0)	1 (20.0)	
3	88 (88.9)	62 (70.5)	26 (29.5)		49 (55.7)	39 (44.3)		56 (63.6)	32 (36.4)	
4	6 (6.1)	2 (33.3)	4 (66.7)		4 (66.7)	2 (33.3)		5 (83.3)	1 (16.7)	
N category				.047			.047			.872
N0	31 (31.3)	16 (51.6)	15 (48.4)		13 (41.9)	18 (58.1)		20 (64.5)	11 (35.5)	
N1, N2	68 (68.7)	49 (72.1)	19 (27.9)		43 (63.2)	25 (36.8)		45 (66.2)	23 (33.8)	
M category				.410			.616			.410
M0	88 (88.9)	59 (67.0)	29 (33.0)		49 (55.7)	39 (44.3)		59 (67.0)	29 (33.0)	
M1	11 (11.1)	6 (54.5)	5 (45.5)		7 (63.6)	4 (36.4)		6 (54.5)	5 (45.5)	
AJCC stage				.112			.155			.772
I–III	71 (71.7)	50 (70.4)	21 (29.6)		37 (52.1)	34 (47.9)		46 (64.8)	25 (35.2)	
IV	28 (28.3)	15(53.6)	13 (46.4)		19 (67.9)	9 (32.1)		19 (67.9)	9 (32.1)	
Recurrence				.610			.690			.174
Yes	46 (46.5)	29 (63.0)	17 (37.0)		27 (58.7)	19 (41.3)		27 (58.7)	19 (41.3)	
No	53 (53.5)	36 (67.9)	17 (32.1)		29 (54.7)	24 (45.3)		38 (71.7)	15 (28.3)	
Death				.873			.789			.549
Yes	63 (63.6)	41 (65.1)	22 (34.9)		35 (55.6)	28 (44.4)		40 (63.5)	23 (36.5)	
No	36 (36.4)	24 (66.7)	12 (33.3)		21 (58.3)	15 (41.7)		25 (69.4)	11 (30.6)	
DLL4 expression							.340			.054
Low	65 (65.7)				39 (60.0)	26 (40.0)		47 (72.3)	18 (27.7)	
High	34 (34.3)				17 (50.0)	17 (50.0)		18 (52.9)	16 (47.1)	
VEGF expression				.340						<.001
Low	56 (56.6)	39 (69.6)	17 (30.4)					49 (87.5)	7 (12.5)	
High	43 (43.4)	26 (60.5)	17 (39.5)					16 (37.2)	27 (62.8)	
HIF2α expression				.054			<.001			
Low	34 (34.3)	47 (72.3)	18 (27.7)		49 (75.4)	16 (24.6)				
High	65 (65.7)	18 (52.9)	16 (47.1)		7 (20.6)	27 (79.4)				

Values are presented as number (%).
DLL4, delta-like ligand 4; VEGF, vascular endothelial growth factor; HIF2α, hypoxia-inducible factor-2α; NEC, neuroendocrine carcinoma; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; UD, undifferentiated; AJCC, American Joint Committee on Cancer.
[a]By Cochran-Armitage trend test, otherwise by chi-square test.

hazard regression, HIF2α expression was confirmed to be a significant predictive factor of the time to recurrence (p = .020), along with the presence of nodal metastasis of the patients (p < .001). The result regarding HIF2α expression shows consistency with the result of Kaplan-Meier survival analysis. Overall survival rate did not differ among patients according to HIF2α expression by Kaplan-Meier survival analysis, and all the negative results were consistent with the result of Cox proportional hazard regression model.

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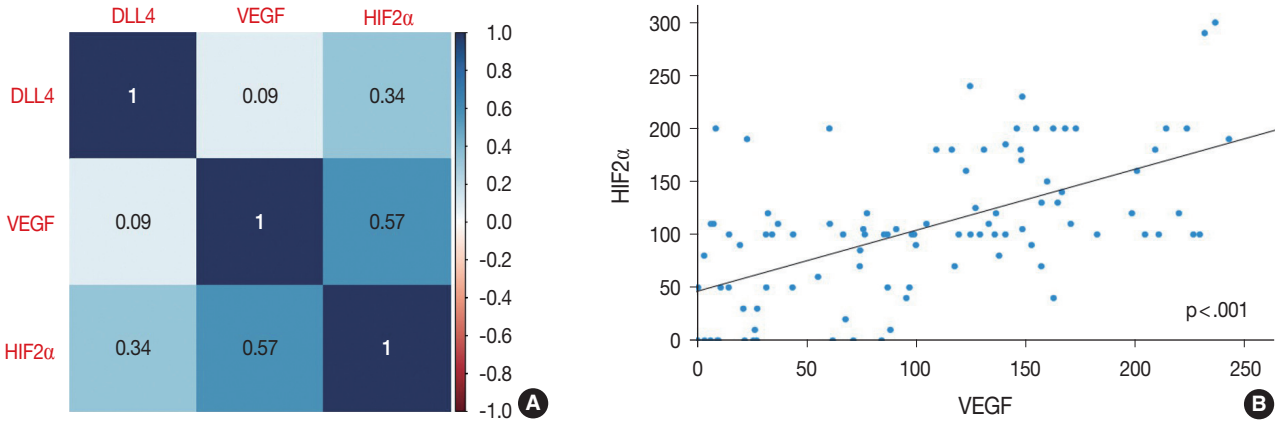

Fig. 4. The correlation matrix visualizing the correlation between the expression levels of DLL4, VEGF, and HIF2α (A). The Spearman correlation coefficients are recorded in the center of each box. The scatter plot showing the positive correlation between VEGF and HIF2α (B). DLL4, delta-like ligand 4; VEGF, vascular endothelial growth factor; HIF2α, hypoxia-inducible factor-2α.

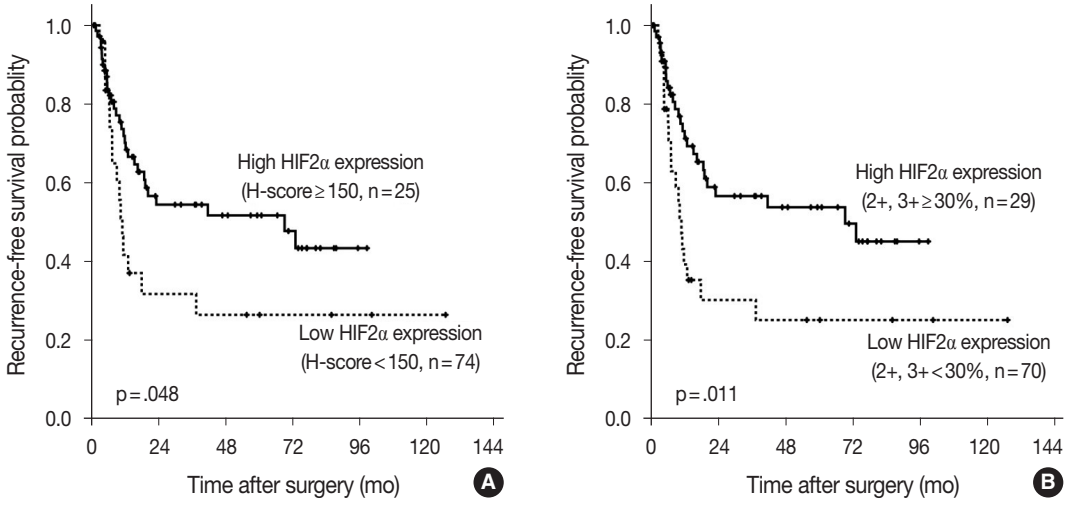

Fig. 5. Kaplan-Meier survival curves according to HIF2α expression. (A, B) Recurrence-free survival curves for differently defined high/low expression groups. HIF2α, hypoxia-inducible factor-2α.

DISCUSSION

Although several previous studies investigated the expression of DLL4, VEGF, or HIF2α in GBC separately [23-27], this is the first study to perform the IHC of these three markers coincidently, attempting to integrate and confirm the results of the previous studies. As the study material, we used the TMA constructed with 99 surgically resected gallbladder cases from a single institution. These cases were followed up for a relatively long period of time – at least 0.7 months to a maximum of 11 years. For analysis of IHC, we digitized the slides and utilized a digital image analysis platform. Complete annotation of tumor area of 99 cases was laborious and time-consuming, but with this tool, we could assure the objectivity of the analysis. Most of the previous studies scored the IHC stain manually with a light microscope, which is inevitably subjective, and this might affect the study results. With digital image analysis, we attempted to overcome such limitations on the aspect of DLL4 and VEGF, but still not for the analysis related to HIF2α, due to the background staining (Fig. 2D). Quantitative digital image analysis has been used in multiple previous studies in various platforms such as Aperio ImageScope, QuPath, ASAP, etc. Since subjectivity of interpretation has long been a hurdle to be overcome and a task for pathologists to conquer, we believe this can be achieved in part by digital image analysis.

The tumors were divided into three groups according to the degree of differentiation. The tumors that consisted of only well-differentiated and/or moderately differentiated portions were clas-

sified as the first group, and the tumors with any poorly differentiated portion were the second group. Only three undifferentiated tumors were classified as the third group. As a result, there was a positive trend between the differentiation and the expression of VEGF or HIF2α; the worse the differentiation tumors showed, the lower expression they exhibited. This trend was not consistent with the results of previous studies: some studies showed that VEGF expression was higher in poorly differentiated tumors compared to more differentiated tumors [25,27], and in other studies, no association was revealed [23,24]. To our knowledge, there have been no previous studies to report the significant association of degree of tumor differentiation and HIF2α expression. Although this study demonstrated a different result than the previous ones because the expression of VEGF and HIF2α are positively correlated, which is consistent with what is stated in other studies. Therefore, the result regarding the trend with differentiation might not be discarded. Instead, such conflicting results should be explained by investigating underlying mechanisms in future studies.

As stated above, VEGF and HIF2α expression were significantly correlated – the tumors with higher VEGF expression tend to show higher HIF2α expression, vice versa. In a study by Giatromanolaki et al. [23], IHC was performed in 60 GBC samples and showed a similar result compared to ours. Since the data showing the association between VEGF and HIF2α expression are being accumulated, the application of these data onto the therapeutic aspects of these markers could be considered. VEGF has long been regarded as a well-established anti-neoplastic therapy. Several anti-VEGF inhibitors have been developed and are currently used, including bevacizumab [28]. Anti-VEGF inhibitor inhibits angiogenesis by reducing endothelial cell proliferation and thus tumor growth. Recently, anti-DLL4/anti-VEGF bispecific monoclonal antibody has been developed to enhance the anti-neoplastic activity and avoid the cardiac toxicity observed in patients when treated with anti-DLL4 inhibitors [16]. HIF2α, on the other hand, playing a pivotal role in tumor progression and metastasis [19], and being the main driver in the development of clear cell RCC [29], is an attractive therapeutic target. Multiple agents have been designed, and some have shown promising results in preclinical level and clinical trials [30,31]. Since the role of both VEGF and HIF2α and drugs that inhibit their action are being vigorously investigated, if VEGF and HIF2α could work as surrogate markers for each other or helps predict the expression level of each other, it might be considerably useful and convenient, in possible future occasions that expression level of these markers may work as a treatment indication.

By chi-square test, we found that DLL4 and VEGF are correlated with lymph node metastasis status. DLL4 and VEGF expression tended to be lower in cases with lymph node metastasis ($p = .047$). Although our result did not reveal any prognostic significance of DLL4 and VEGF, because lymph node metastasis is determining factor for TNM stage that reflects patient survival, this correlation may point to the potential prognostic implication. In the cases that show low expression when stained with DLL4 and VEGF and if it is detected in biopsy sample prior to surgical resections, surgeons should perform meticulous lymph node dissection considering the higher possibility of lymph node metastasis. The pathologists should also spend more time evaluating the presence of tumor cells in dissected lymph nodes. If such a patient is subject to concurrent chemotherapy and radiotherapy, it would provide information for oncologists or radiologists' decision. Low expression groups of DLL4 and VEGF, however, account for more than half of the patients (65.7% and 56.6%, respectively), there is the possibility that they might not work as the effective screening tool. A study by Liu et al. [32] showed a relevant result in non-small cell lung cancer cases, stating that low DLL4 expression was significantly correlated with lymph node metastasis. A study with a conflicting result compared to ours [33] revealed that high DLL4 expression predicted pelvic lymph node metastasis in early cervical cancer patients. Moreover, high DLL4 expression was an independent predictor of poor survival in these cervical cancer patients. Such conflict is possibly due to the bi-functional cellular responses that Notch signaling pathway may induce during tumorigenesis in different tumors [17]. DLL4, working as a ligand in the Notch signal pathway, may either promote or inhibit tumor cell proliferation or survival, and this might be different according to tumor cell origin of tumor cell types. Further studies are necessary to clarify the underlying mechanism of DLL4 activity in GBC to confirm our results on lymph node metastasis.

Except for HIF2α, two other markers did not show any correlation with prognosis. The correlation of HIF2α with recurrence was not clear when the patients were divided into two groups: high as H-score ≥ 120 or low as H-score < 120. When the high group was set with more conservative criteria (higher H-score) or the proportion of 2+ and 3+ cells, patients with lower HIF2α expression showed shorter RFS than those with higher HIF2α expression. By Cox proportional hazard regression model, HIF2α expression was confirmed as a significant predictive factor for recurrence. High HIF2α expression, therefore, may help to expect a better prognosis regarding recurrence, but the threshold for "high" expression should be relatively high to gain

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reliable results. In our study cohort, some of the patients were transferred to different hospitals right after surgery for subsequent treatment and follow-up. The data regarding recurrence, death, and additional treatment could be incomplete, which might have affected our results.

In this study, multiple cutoffs for statistical analysis was used. For example, H-score = 120, H-score = 150 or the tumor with more than 30% of 2+ and 3+ cells, in each analysis. In studies using quantitative measuring of expression level, especially regarding the studies using immunohistochemical stain, a certain cutoff is needed for grouping the patients. The gold standard for setting the cutoff value, however, is not established or even recommended for pathologists. H-score = 120 was helpful in dividing the patients into two groups with adequate population in each group, for all three markers (DLL4: n = 65 in low group, n = 34 in high group; VEGF: n = 56 in low group, n = 43 in high group; HIF2α: n = 65 in low group, n = 34 in high group). For survival analysis, however, with the same cutoff no statistically significant result was yielded, so that other cutoff values were adopted and utilized in this study. Considering that all researchers should report any meaningful data they obtained during the analysis, it was unavoidable to report the statistically significant results with multiple cutoffs.

In a previous study that demonstrated DLL4 expression was a prognostic marker and predicted gemcitabine effect in pancreatic cancer [34], two cohorts of patients, total 154, were enrolled and their clinicopathologic and treatment data for at least 6 years were collect. When a larger number of patients with complete data for survival and post-operative treatment is available in our study, DLL4 expression is worth being re-evaluated for its prognostic significance in association with treatment effect like the study by Drouillard et al. [34]. Our study has other limitations. The study cohort only includes surgically resected cases. Although 25 cases were advanced diseases as to be staged surgically as IVB, including 11 cases with distant metastasis and 14 cases without distant metastasis but with N2 lymph node metastasis, the majority of this cohort was relatively early GBC cases. Because GBC is one of the lately detected cancers, most of the patients are subject to systemic treatment rather than surgical resection at the time of diagnosis. Our cohort, therefore, may not represent the whole GBC patients. Another limitation is that we could not evaluate the HIF2α expression digitally. Objectivity gained by quantitative digital image analysis for DLL4 and VEGF is one of the strengths of our study. However, due to the intensive background stain of HIF2α (Fig. 2), there was no available tool to annotate and evaluate the intensity of staining exactly. Although the consensus was made between two pathologists, manual evaluation might be relatively crude and subjective compared to digitized analysis.

In conclusion, this study studied the expression patterns and levels of DLL4, VEGF, and HIF2α in surgically resected GBC. We demonstrated that VEGF and HIF2α expression intensity is positively correlated, suggesting the possibility for these markers to work as mutually substitutable markers. Low DLL4 and VEGF expression levels were significantly associated with the status of lymph node metastasis, presumably with prognosis, although such a result was not yielded in this study. Lastly, patients with lower HIF2α expression showed shorter RFS in our cohort. The cellular mechanisms of DLL4, VEGF, and HIF2α in GBC are worth further investigating to explain these results, to accelerate the application the target therapy for these molecules to treat GBC patients.

Ethics Statement

The institutional review board of Samsung Medical Center approved this study (2021-10-053) and waived informed consent.

Availability of Data and Material

The datasets generated or analyzed during the study are available from the corresponding author on reasonable request.

Code Availability

Not applicable.

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Conflicts of Interest

The authors declare that they have no potential conflicts of interest.

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