Prognostic implications of immune classification using IDO1 expression in extrahepatic bile duct carcinoma

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Abstract. Indoleamine 2, 3-dioxygenase 1 (IDO1) is an immunomodulatory enzyme that catalyzes the degradation of tryptophan to kynurenine and induces immune tolerance in tumor cells. The effects of IDO1 on extrahepatic bile duct carcinoma (EHBDC) are poorly understood. Therefore, the present study aimed to investigate the expression and prognostic significance of IDO1 in EHBDC. An immunohistochemical microarray analysis of IDO1 expression was performed for 76 surgically resected cases of EHBDC. $CD8^+$ tumor infiltrating lymphocytes (TILs) were also investigated through a combination analysis with IDO1 expression. IDO1 was highly expressed in 25 of 76 (32.9%) cases. High expression of IDO1 was associated with decreased numbers of $CD8^+$ TILs (P=0.008), a higher pN category (P=0.007), an advanced overall stage (P=0.001) and frequent recurrence (P=0.018). When IDO1 expression was further stratified with $CD8^+$ TIL state, the $IDO1^{high}/CD8^{low}$ subgroup was decreased in terms of overall survival (P=0.025) and disease-free survival (P=0.015) compared with $IDO1^{high}/CD8^{high}$, $IDO1^{low}/CD8^{high}$ and $IDO1^{low}/CD8^{low}$ subgroups. High IDO1 expression was associated with a decreased number of $CD8^+$ TILs and associated with a poor prognosis. As IDO1 may be a new target of immunotherapy applications, IDO1/$CD8^+$ TIL subgrouping can be a useful prognostic and predictive tool in patients with EHBDC.

Introduction

Extrahepatic bile duct carcinoma (EHBDC) is an epithelial tumor that forms in the bile ducts outside the liver, and its incidence ranges from 0.53 to 2.0 per 100,000 in the western world, but can be much higher (0.97 to 85.0) in Asian countries including Thailand, Republic of Korea and China (1). Despite recent advances in diagnostic and therapeutic techniques, complete surgical resection of the tumor remains the best treatment for EHBDC. However, EHBDC patients are often diagnosed at an advanced stage. Even in patients who have undergone therapeutic resection, the prognosis is dismal due to the high recurrence rate of this tumor (2,3). A late diagnosis and frequent recurrence in EHBDC compromise surgical resection, the only potentially curative treatment. Current chemotherapeutic drugs for these EHBDC patients have a limited efficacy because more than 50% have relapsed early (4,5).

Recently, with the advent of immunotherapy, studies for the interactive relationships between tumor cells and the peritumoral immune cells (tumor microenvironment) (TME) have received much attention. However, difficult obstacles remain for deciphering TME because of tumor heterogeneity and immune system complexity, especially in immune suppression mechanisms. Local immune suppression of the tumor microenvironment is crucial for cancer development, metastasis, and even tumor immune escape (6,7). Therefore, elucidation of the immune suppression mechanisms involved in the carcinogenesis, cancer progression, and metastasis can improve the accuracy of predicting its prognosis and provide a target for therapeutic intervention, contributing to the improvement of survival rate.

IDO1 has been emerging as an important biomarker associated with the immunosuppressive mechanism in addition to PD-L1/PD-1 and B7/CTLA-4 interactions. IDO1 is a 403 amino acid and cytosolic heme enzyme encoded by the INDO gene on human chromosome 8p22 that catalyzes the initial and rate-limiting steps in the kynurenine (Kyn) pathway (8). It is expressed ubiquitously in various normal tissues including the small intestine, epididymis, lungs, female genital tract, and placenta as well as various types of solid tumors (8-10). IDO1 is known to be involved in maternal-fetal immune tolerance in the mouse placenta (9) and has been shown to be linked in a mechanism of response to tumor immune evasion through tumor infiltrating lymphocyte (TIL) inhibition (11). To date, contradictory results for prognostic implication of IDO1 expression has been reported

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in various carcinomas (12-19), but there is no study in EHBDC.

In this study, we investigated intratumoral IDO1 expression in epithelial tumor cells in 76 patients with EHBDC who had undergone surgical resection. In addition, we defined the association between $CD8^+$ TILs and IDO1 expression and examined prognostic differences between subgroups by performing stratification combining CD8 TIL status and IDO1 expression.

Materials and methods

Patients and clinicopathological data. This study was approved by the Institutional Review Board of Gangneung Asan Hospital. A total of 76 consecutive patients with bile duct carcinoma between January 2001 and December 2017 were collected at Gangneung Asan Hospital. In detail, inclusion criteria were as follows: i) surgical resection specimen with enough tumor cells, ii) obtaining each patient's informed consent for use of their clinical records and pathological specimen, and iii) without previous history of a cancer other than bile duct carcinoma and chemo-or radiotherapy. Exclusion criteria were as follows: i) histological diagnosis of a tumor type other than bile duct carcinoma, ii) inappropriate amount of tumor sample, iii) insufficient preservation of paraffin blocks for tissue microarray (TMA) construction, iv) Follow-up loss, and v) failure to obtain informed consent.

The patients' medical records were reviewed for clinical information, and histological parameters were evaluated on hematoxylin & eosin (H&E)-stained slides. Clinical data included the patient's sex and age, tumor location, operation date, American Joint Committee on Cancer (AJCC) TNM stage (20), most recent follow-up date, recurrence, and survival status. Pathological data included the tumor size, depth of invasion, tumor location, histologic subtype, tumor differentiation, nodal metastasis, and perineural or lymphovascular invasion.

Tissue microarray (TMA). Tumor samples collected from clinical cases were fixed with 10% formalin at room temperature for 24 h. Formalin-fixed, paraffin-embedded tissue samples of randomly selected bile duct adenocarcinoma (n=76) were obtained and arrayed using a tissue-arraying instrument (Quick-Ray, Unitma Co., Ltd.). Briefly, areas with invasive adenocarcinomas were identified on the corresponding H&E-stained slides, and sections were indicated as representative tumors. Three cores were sampled from the center and border of invasive areas in each representative tumor block using a 2.0-mm punch. Four-μm-thick slides were cut from the TMA blocks for immunohistochemical and H&E staining. H&E stains for TMA blocks were performed by automated DAKO CoverStainer (Dako Korea Co., Ltd.; temperature, 4˚C; duration, 46 min) to correlate with the immunohistochemical TMA slides.

Immunohistochemical staining and interpretation. Five-μm thick sections were cut from the formalin-fixed, paraffin-embedded tumor samples and stained with Leica auto-stainer Bond Max using the Bond Polymer Refine Detection System (Leica Biosystems Newcastle Ltd.) according to the manufacturer's protocols. Briefly, the sections were deparaffinized by Bond Dewax Solution (Leica Biosystems Newcastle Ltd.), followed by heat-induced antigen retrieval using Bond Epitope retrieval solution (Leica Biosystems Newcastle Ltd.) for 20 min at 100˚C. The endogenous peroxidase was quenched by incubation with hydrogen peroxide for 15 min. Sections were incubated for 15 min at ambient temperature with primary mouse monoclonal anti-IDO1 antibody (Abcam; Ab13248, 1:100) and CD8 (SP16; Thermo Fisher Scientific; 1:100). Bound primary antibodies were visualized using a biotin-free polymeric horseradish peroxidase-linker antibody conjugate system (Bond Polymer Refine Detection; ready-to-use dilution; cat. no. DS9800; Leica Biosystems, Inc.) in a Bond-Max automatic slide stainer (Leica Biosystems Melbourne Pty. Ltd.). The nuclei of these sections were counterstained with hematoxylin (Biocare, cat: NM-HEM-M) for 4 min at 25˚C in the Bond Polymer detection kit (Leica Biosystems).

The tumor epithelial immunoreactivity for IDO1 was semi quantitatively interpreted by means of light microscopic examination and evaluated without prior knowledge of the clinicopathological data. Cytoplasmic immunostaining was evaluated as the percentage and intensity of positive epithelial cells, as previously described (21). Staining intensity was graded as follows: 0, negative; 1, weak; 2, moderate; and 3, strong. The percentage of positively stained cells was graded as 1 (1-24%), 2 (25-49%), 3 (50-74%), or 4 (≥75%). The final immunohistochemical score was calculated by multiplying these 2 grades to yield a score ranging from 0 to 12. We defined high IDO1 expression as a total score >3. The cut-point value is determined using the AUC (area under the ROC (receiver operating characteristic) curve) and Youden's index for patient's survival (22).

CD8 was estimated for TILs in the tumor bed including the tumor epithelium and intratumoral stroma using an automatic image analysis software (https://oncoimmunoquantifier.com). The images of each case were captured using a digital camera (Jenoptik ProgRes speedXT) attached to an Olympus light microscope (BX51; Olympus). To reduce the effect of sampling error, whole areas of 2-mm TMA cores were included in analysis using a low power view (x4) according to recommendations of the software guide. CD8-positive cells were automatically detected, segmented, and counted. The cut-off numbers (176.5/mm^2) were used to determine high expression of CD8 in TILs. This cut-off point is referred to as the optimal value that is calculated using AUC and Youden's index for patient's survival (23).

Statistical analysis. Statistical analyses were performed with SPSS software (version 23.0; SPSS Inc., Chicago, IL, USA). The average values were utilized as cut-off points for age and tumor size. Categorical data were assessed using Pearson's χ^2 or Fisher's exact tests. The mean number of $CD8^+$ TILs was examined using an unpaired Student's t-test. Overall survival (OS) was measured by the amount of time the patient is alive after the primary treatment. Disease-free survival (DFS) was referred to as the length of time that the patient survives without any signs or symptoms of that cancer after primary treatment for a cancer ends. In univariate analyses for survival, survival curves were illustrated and the relationship between survival rates and various clinicopathological factors were

Table I. Patient demographics.

Parameters	Grouping	N (%)
Age, years	<70	37 (48.7)
	>70	39 (51.3)
Sex	Male	53 (69.7)
	Female	23 (30.3)
Size, cm	<2.3	40 (52.6)
	>2.3	36 (47.4)
Location	Distal	45 (59.2)
	Proximal	31 (40.8)
Differentiation[a]	Well	21 (27.6)
	Moderately	47 (61.8)
	Poorly	6 (7.9)
LVI[a]	Absent	49 (64.5)
	Present	23 (30.3)
PNI[a]	Absent	15 (19.7)
	Present	53 (69.7)
LN metastasis	Absent	47 (61.8)
	Present	29 (38.2)
pT stage[b]	pT1	12 (15.8)
	pT2	48 (63.2)
	pT3	16 (21.1)
pN stage[b]	pN0	47 (61.8)
	pN1	22 (28.9)
	pN2	7 (9.2)
pTNM stage[b]	I	9 (11.8)
	II	60 (78.9)
	III	7 (9.2)
Recurrence	Negative	23 (30.3)
	Positive	53 (69.7)
Death	Alive	24 (31.6)
	Dead	52 (68.4)

[a]Clinicopathological data that can be not obtained were omitted; [b]AJCC Stage grouping, 8th edition (20). LVI, lymphovascular invasion; PNI, perineural invasion; LN, lymph node; TNM, tumor node metastasis; TIL, tumor-infiltrating lymphocyte.

compared using the Kaplan-Meier method with the log-rank test. In multivariate analyses for survival, we investigated the prognostic significance using Cox proportional hazards modelling. P-values less than 0.05 were considered to denote statistical significance.

Results

Clinicopathological characteristics. The demographics and clinicopathological correlation of IDO1 expression are summarized in Tables I and II, respectively. The series consisted of 54 men and 23 women with an average age of 70 years (range, 40-87 years). The carcinomas were located in the proximal bile duct (26 cases), including 6 cases in the perihilar area, and in the distal (intrapancreatic head) bile duct (45 cases). Histologically, 73 cases were adenocarcinoma, not otherwise specified (NOS), and four cases were classified as adenocarcinoma arising in intraductal papillary neoplasm of the bile duct. Two cases were associated with a congenital biliary abnormality. Surgical resection and regional lymph node dissection were dependent on the location of the primary tumor. Pancreaticoduodenectomy or pylorus-preserving pancreaticoduodenectomy was performed in 45 patients, bile duct resection in 26 patients, and combined hepatectomy with bile duct resection in six patients. Forty-seven patients (61.0%) showed clear resection margins. In 18 patients (23.4%), resection margins were involved by carcinoma. In addition, low-and high-grade dysplasia at resection margins were identified in 5 (6.5%) and 7 (9.1%) patients, respectively. Thirty-one patients received postoperative chemotherapy. The range of follow-up was from 0.3 months to 96.0 months (median: 20.9 months). No follow-up loss is identified.

IDO1 expression was evaluated in 76 cases except for one case with no assessable staining due to loss of the cores in the TMA block. IDO1 was expressed in the cytoplasm and/or nuclei of the tumor epithelium and stromal mononuclear immune cells. The epithelial IDO1-expressing tumors were grouped into two categories of high and low expression according to the proportion and intensity score (Fig. 1). IDO1 was highly expressed in 25 of 76 (32.9%) tumor specimens. In 51 of 76 (67.1%) cases, IDO1 expression is low. IDO1-high expressing tumors (Fig. 1A) exhibited a significantly low proportion of intratumoral $CD8^+$ cells (mean ± standard error (SE), 98.38/mm2±100.26; Fig. 1B), whereas IDO1-low expressing tissue samples (Fig. 1C) demonstrated high CD8+ cells (214.78/mm2±268.26; Fig. 1D) (P=0.008). Comparing IDO1 expression with CD8 expression in TILs, IDO1 expression was inversely associated with the number of $CD8^+$ TILs.

High IDO1 expression was associated with a higher pN category (P=0.007), an advanced overall TNM stage (P=0.001), and more frequent lymph node metastasis with marginal statistical significance (P=0.082). Patients with high IDO1 expression experienced more frequent recurrence (P=0.018). There was non-significant relationship between IDO1 overexpression and disease-specific death (P=0.065) (Table II).

CD8 expression in TILs was evaluated in all 76 cases. Although the difference was not statistically significant, high CD8 expression in TILs tended to be associated with less frequent recurrence (61.5%) and a lower disease-specific death rate (64.1%) than low CD8 expression (78.4 and 73.0%, respectively).

Univariate and multivariate analyses for overall Survival and disease-free survival. High IDO1 expression was significantly associated with shorter disease-free survival (median time: 16.30 months vs. 26.30 months, P=0.026; Fig. 2A) and had a non-significant trend to decreased overall survival (median time: 21.20 months vs. 26.30 months, P=0.097; Fig. 2B). Patients with high $CD8^+$ TILs showed longer disease-free survival (median survival time: 47.30 months vs. 16.30 months, P=0.023; Fig. 2C) than those with low $CD8^+$ TILs. No significant relationship between high $CD8^+$ TILs and overall survival (median survival time: 36.00 months vs. 19.4 months, P=0.135; Fig. 2D) was illustrated.

Table II. Clinicopathological correlation of IDO1 expression.

Parameters	Grouping	IDO1 expression (%)		P-value
		Low	High	
Age, years	<70	25 (67.6)	12 (32.4)	0.933
	>70	26 (66.7)	13 (33.3)	
Sex	Male	34 (64.2)	19 (35.8)	0.441
	Female	17 (73.9)	6 (26.1)	
Size, cm	<2.3	28 (70.0)	12 (30.0)	0.571
	>2.3	23 (63.9)	13 (36.1)	
Location	Distal	28 (62.2)	17 (37.8)	0.200
	Proximal	23 (74.2)	8 (25.8)	
Differentiation	Well	15 (71.4)	6 (28.6)	0.618
	Moderately	31 (66.0)	16 (34.0)	
	Poorly	3 (50.0)	3 (50.0)	
LVI	Absent	35 (71.4)	14 (28.6)	0.422
	Present	14 (60.9)	9 (39.1)	
PNI	Absent	9 (60.0)	6 (40.0)	0.555
	Present	36 (67.9)	17 (32.1)	
LN metastasis	Absent	35 (74.5)	12 (25.5)	0.082
	Present	16 (55.2)	13 (44.8)	
pT stage[a]	pT1	10 (83.3)	2 (16.7)	0.384
	pT2	30 (62.5)	18 (37.5)	
	pT3	11 (68.8)	5 (31.3)	
pN stage[a]	pN0	35 (74.5)	12 (25.5)	0.007
	pN1	15 (68.2)	7 (31.8)	
	pN2	1 (14.3)	6 (85.7)	
pTNM stage[a]	I	9 (100)	0 (0)	0.001
	II	41 (68.3)	19 (31.9)	
	III	1 (14.3)	6 (85.7)	
$CD8^+$ TIL/mm^2	Mean	214.78	98.38	0.008
Recurrence	Negative	20 (87.0)	3 (13.0)	0.018
	Positive	31 (58.5)	22 (41.5)	
Death	Alive	20 (83.3)	4 (16.7)	0.065
	Death	31 (59.6)	21 (40.4)	

[a]AJCC Stage grouping, 8th edition (20). LVI, lymphovascular invasion; PNI, perineural invasion; LN, lymph node; TNM, tumor node metastasis; TIL, tumor-infiltrating lymphocyte.

Patient survival was further stratified according to four expression combinations of IDO1 and CD8 arranged in order of adverse prognostic value as follows (Fig. 3): $IDO1^{high}/CD8^{low}$ (18.4%, 14/76); $IDO1^{low}/CD8^{low}$ (32.9%, 25/76); $IDO1^{high}/CD8^{high}$ (13.2%, 10/76); $IDO1^{low}/CD8^{high}$ (35.5%, 27/76). The patients whose carcinomas were $IDO1^{high}/CD8^{low}$ showed the worst disease-free survival times (Fig. 3A and C) and median overall (Fig. 3B and D) (8.10 and 16.30 months, respectively). The median overall survival time of patients with $IDO1^{low}/CD8^{low}$ and $IDO1^{high}/CD8^{high}$ expression were 19.40 months and 33.70 months, respectively. The median disease-free survival time of patients with $IDO1^{low}/CD8^{low}$ and $IDO1^{high}/CD8^{high}$ expression were 18.80 months and 26.1 months, respectively. Finally, patients with $IDO1^{low}/CD8^{high}$ expression revealed the best survival (medial overall survival, 47.50 months; disease-free survival, 47.50 months) (Table III).

In univariate analyses (Table III), larger tumor size (P=0.030), proximal location (P=0.007), perineural invasion (P=0.026), and the $IDO1^{high}/CD8^{low}$ subgroup (P=0.013) revealed significantly shorter overall survival. Similarly, worse disease-free survival was significantly related to proximal location (P=0.007), perineural invasion (P=0.005), lymph node metastasis (P=0.012), higher pN stage (P=0.017), high IDO1 expression (P=0.026), low $CD8^+$ TILs (P=0.023), and the $IDO1^{high}/CD8^{low}$ expression subgroup (P<0.001). Multivariate statistical analyses of the $IDO1^{high}/CD8^{low}$ expression subgroup were performed with the significant prognostic valuables examined by univariate analyses. As shown in Table IV, $IDO1^{high}/CD8^{low}$ expression (P=0.025,

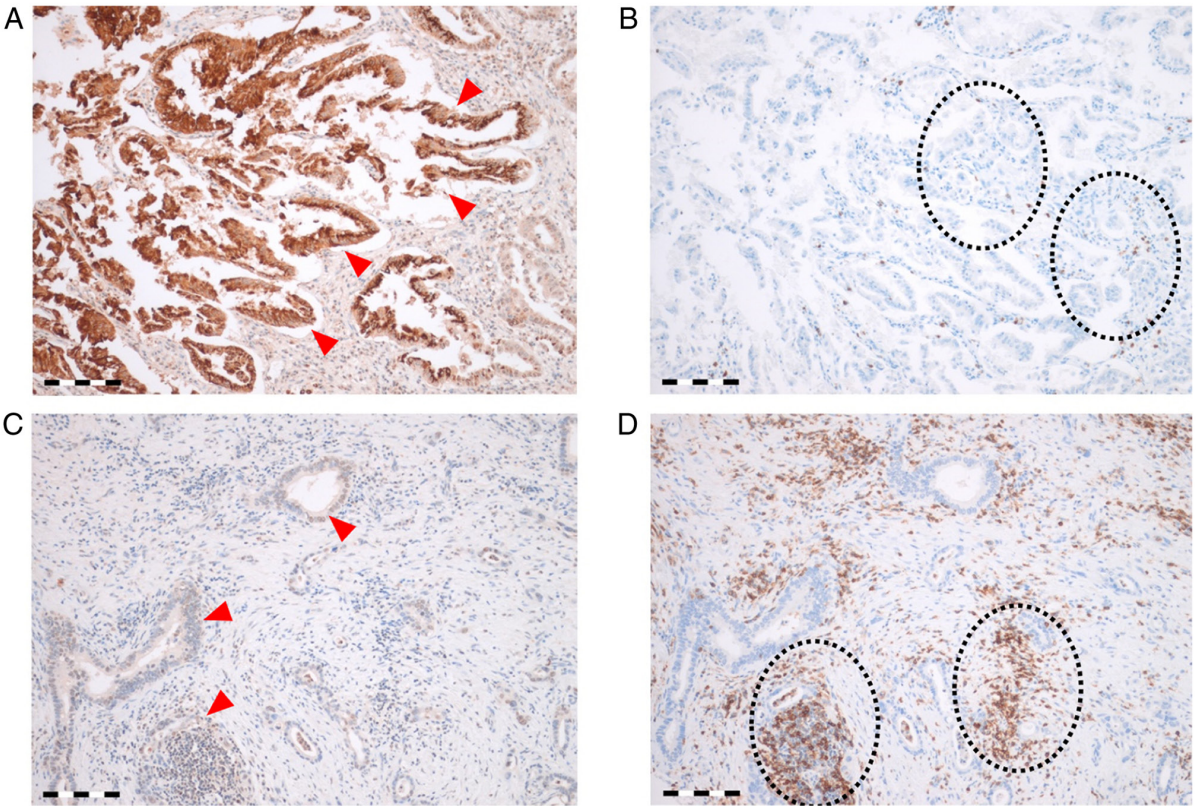

Figure 1. Immunohistochemical stains for IDO1 and CD8 expression. (A) A case with high IDO1 expression in the cytoplasm of tumor epithelial cells (indicated by arrowhead) with (B) rare CD8-positive TILs (indicated by dotted circle). (C) Another case with low IDO1 expression (indicated by arrowhead) with (D) frequent CD8-positive TILs (indicated by dotted circle). Scale bar, 200 μm; original magnification, x200). IDO1, indoleamine 2, 3-dioxygenase 1; TILs, tumor infiltrating lymphocytes.

Cox hazard ratio=2.168) in addition to tumor proximal location and perineural invasion was an independent prognostic factor for overall survival. Furthermore, IDO1^{high}/CD8^{low} expression (P=0.015, Cox hazard ratio=2.460) with tumor proximal location, lymph node metastasis, and perineural invasion was an independent prognosticator for disease-free survival.

Discussion

Since the immunosuppressive effects of IDO1 were discovered, IDO1 has been reported to be highly expressed and associated with clinical outcomes in a variety of solid tumors showing contradictory biologic behaviors. IDO1 expression is a predictor of poor clinical outcomes in many kinds of solid tumors, such as ovarian adenocarcinomas, colorectal adenocarcinomas, laryngeal squamous cell carcinomas, and endometrial and esophageal cancers (12-17). In contrast, patients with high IDO1 expression in some tumors, such as basal cell-like breast carcinoma, hepatocellular carcinoma, and renal cell carcinoma, have increased survival (13,18,19). In the present study of 76 EHBDC surgical specimens, we demonstrated that high IDO1 expression in the tumor epithelial cells was positively correlated with tumor recurrence and poor patient survival. As is well known, some stromal mononuclear immune cells are also positive for IDO1; however, this expression was not correlated with patient survival (data not shown).

The role of CD8$^+$ T lymphocytes in tumor progression has been examined in a variety of human malignancies (24-28). Most research has revealed a beneficial prognostic effect of high intratumoral/intraepithelial CD8$^+$ T lymphocyte infiltration. In accordance with our results, there are a few reports of a favorable prognostic effect of CD8$^+$ TILs in patients with EHBDC (24,29,30). IDO1 expression in tumor epithelial cells was inversely associated with number of CD8$^+$ TILs in the present study. Similar to our results, Ino *et al* reported that tumoral IDO1 expression was correlated with a reduced number of TILs and natural killer (NK) cells in endometrial cancer, possibly contributing to disease progression and poor clinical outcomes (14). Brandacher *et al* also reported that high IDO1 expression was associated with a significant reduction in CD3$^+$ TILs in colon cancer as compared to tumor samples with low IDO1 expression (31).

IDO1 is an immunomodulatory enzyme that catalyzes the degradation of tryptophan (Trp) to Kyn. The depletion of Trp and accumulation of Kyn have been reported to induce effector T-cell apoptosis/dysfunction and generate immunosuppressive regulatory T cells (32). Recently, functional inactivation of tumor-reactive T cells has been considered to be an essential mechanism of tumor immune evasion (33). The upregulation of IDO1 occurs in tumor cells in response to interferon-γ (IFN-γ) secreted by CD8$^+$ T cells (34) while the increased expression of IDO1 suppresses the CD8$^+$ T cell response, resulting in

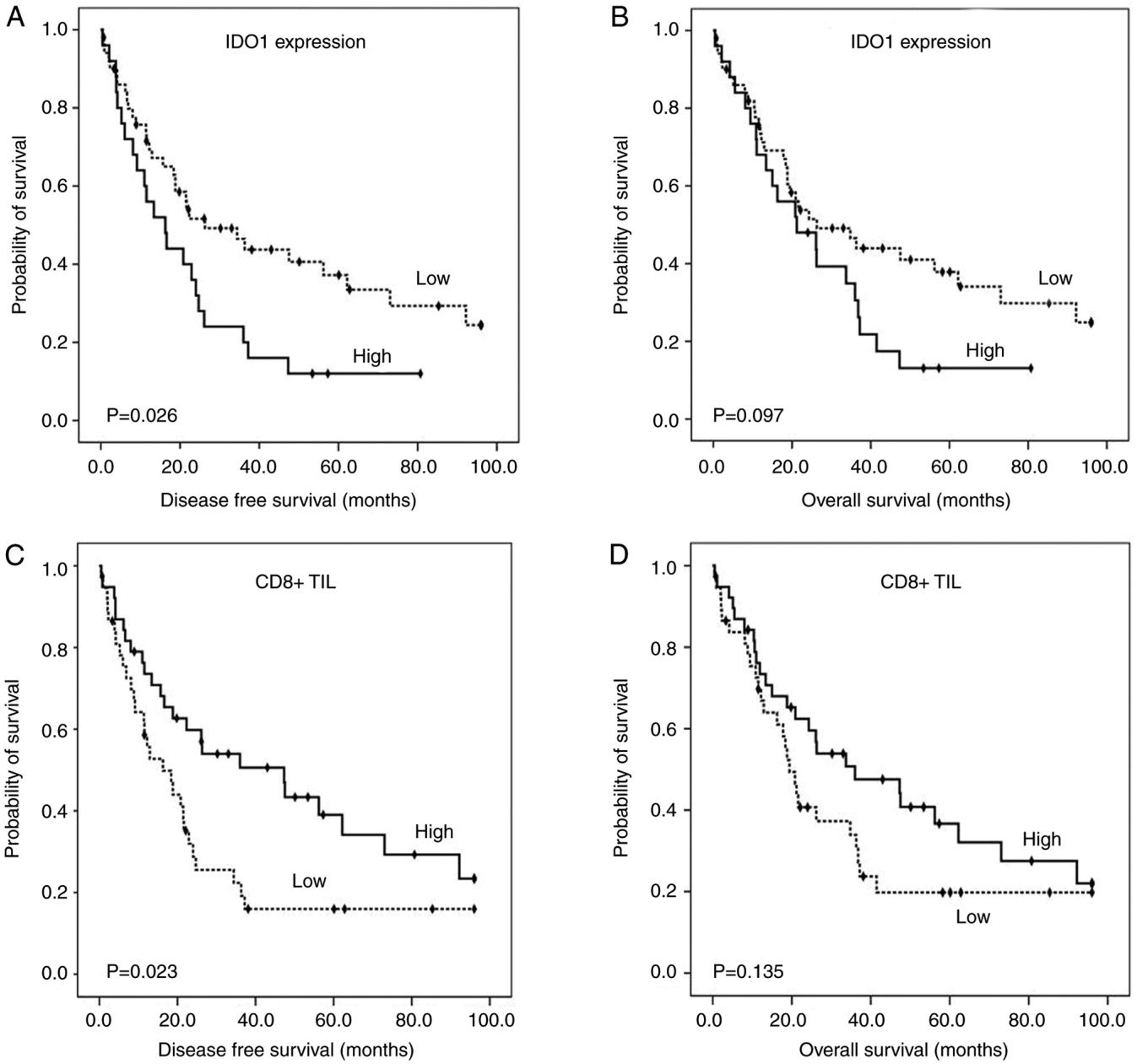

Figure 2. Kaplan-Meier curves according to IDO1 and $CD8^+$ TILs expression. Patients with high IDO1 expression show poor clinical outcomes for (A) disease-free survival and (B) overall survival compared with low IDO1 expression. Patients with high $CD8^+$ TIL expression tend to have a more favorable (C) disease-free survival and (D) overall survival compared with those with low $CD8^+$ TIL expression. IDO1, indoleamine 2, 3-dioxygenase 1; TILs, tumor infiltrating lymphocytes.

tumor immune evasion and tumor growth, suggesting a possible negative feedback loop to regulate T-cell activation (5). Since both PD-L1 and IDO1 are increased in tumor cells by IFN-γ induced by $CD8^+$ T cells (35), IDO1-and PD-L1-expressing tumors are expected to have similar clinical significance (36). However, IDO1 expression may have different clinicopathological implications from PD-L1 expression because there is the isolated mechanism to promote IDO1 secretion by activation pathway of RAS and PAMP (pathogen-associated molecular pattern) that is not shared with PD-L1 (37). In this regard, further research to elucidate the relationship between IDO1 and PD-L1 is needed in the future. These suggest that a more complex mechanism than previously evaluated acts between IDO1 expression and the immune microenvironment. Considering that single-agent treatments with IDO1 enzyme inhibitors have a negligible effect on decreasing the established cancer burden, a combination of select therapies with IDO1 blockade for a synergistic benefit against tumor growth is likely needed. Capitalizing on this background and the negative association between IDO1 expression and $CD8^+$ TILs as shown in our study, patients with $IDO1^{high}/CD8^{low}$ or $IDO1^{low}/CD8^{high}$ subgroups can represent a stronger interaction between IDO1-expressing tumor and $CD8^+$ TILs compared to other subgroups. Therefore, IDO1 blockers are expected to be more effective for the $IDO1^{high}/CD8^{low}$ subgroup with worst prognosis by inhibiting a patent link between IDO1-expressing tumor and $CD8^+$ TILs.

Table III. Univariate analyses (log-rank test) for overall and disease-free survival.

A, Overall survival

Parameters		Mean survival (months)	CI (95%) Lower	CI (95%) Upper	P-value
Age, years	<70	36.8	8.41	65.19	0.056
	>70	20.9	10.67	31.13	
Sex	Male	20.9	9.54	32.26	0.769
	Female	26.1	9.11	43.09	
Size, cm	<2.3	47.3	13.54	81.16	0.03
	>2.3	20.8	17.56	24.04	
Location	Distal	36.3	23.55	49.05	0.007
	Proximal	13.4	4.39	22.41	
Differentiation	Well	20.9	0	44.36	0.303
	Moderately	26.2	11.5	40.9	
	Poorly	8.1	4.14	12.06	
LVI	Absent	26.2	8.09	44.31	0.392
	Present	26.1	13.7	38.49	
PNI	Absent	56.2	7.56	104.84	0.026
	Present	20.9	14.84	26.96	
LN metastasis	Absent	36	21.29	50.71	0.1
	Present	20.8	12.76	28.84	
pT stage[a]	pT1	41.5	0	115.37	0.439
	pT2	24.3	17.87	30.73	
	pT3	18.4	10.95	25.85	
pN stage[a]	pN0	36	21.29	50.71	0.092
	pN1	20.9	17.61	24.19	
	pN2	10.9	3.75	18.09	
pTNM stage[a]	I	73	7.37	138.63	0.112
	II	26.1	11.68	40.52	
	III	10.9	3.72	18.09	
Postoperative chemotherapy	No	26.1	10.54	41.67	0.705
	Yes	24.3	15.16	33.44	
IDO1 expression	Low	26.3	7.94	44.67	0.097
	High	21.2	5.68	36.72	
$CD8^+$ TIL	Low	19.4	15.42	23.38	0.135
	High	36	8.2	63.8	
IDO1/$CD8^+$ TIL, 2 tiers	Other than High/Low	33.7	17.5	49.9	0.013
	High/Low	16.3	0	34.45	
IDO1/$CD8^+$ TIL, 4 tiers	Low/Low	19.4	14.5	24.3	0.097
	High/Low	16.3	0	34.45	
	High/High	33.7	18.36	49.04	
	Low/High	47.5	5.98	89.02	

B, Disease-free survival

Parameters		Mean survival (months)	CI (95%) Lower	CI (95%) Upper	P-value
Age, years	<70	24.7	4.43	44.97	0.082
	>70	18.8	12.13	25.47	
Sex	Male	20.8	11.26	30.34	0.827
	Female	21.6	15.91	27.09	

Table III. Continued.

B, Disease-free survival

Parameters		Mean survival (months)	CI (95%)		P-value
			Lower	Upper	
Size, cm	<2.3	47.3	8.15	86.45	0.011
	>2.3	18.4	8.45	28.35	
Location	Distal	26.1	9.43	42.77	0.007
	Proximal	12.9	10.31	15.49	
Differentiation	Well	20.8	12.35	29.26	0.184
	Moderately	26.1	15.1	37.1	
	Poorly	8.1	2.1	14.1	
LVI	Absent	24.7	7.98	41.42	0.151
	Present	15.7	9.76	21.64	
PNI	Absent	92.2	38.03	146.37	0.005
	Present	16.6	6.22	26.98	
LN metastasis	Absent	36	19.53	52.47	0.012
	Present	11.4	1.55	21.25	
pT stage[a]	pT1	20.8	0	95	0.573
	pT2	22.9	14.2	31.61	
	pT3	18.4	4.09	32.71	
pN stage[a]	pN0	36	19.53	52.47	0.017
	pN1	15.7	7.92	23.48	
	pN2	8.1	0.66	15.54	
pTNM stage[a]	I	73	0	150.25	0.063
	II	22.3	16.17	28.43	
	III	8.1	0.66	15.42	
Postoperative chemotherapy	No	21.5	13.6	29.4	0.728
	Yes	22.3	10.58	34.02	
IDO1 expression	Low	26.3	9.23	43.87	0.026
	High	16.3	7.98	24.62	
$CD8^+$ TIL	Low	16.3	7.03	25.57	0.023
	High	47.3	20.93	73.67	
IDO1/$CD8^+$ TIL, 2 tiers	Other than High/Low	26.3	10.19	42.42	<0.001
	High/Low	8.1	2.42	13.78	
IDO1/$CD8^+$ TIL, 4 tiers	Low/Low	18.8	5.75	31.85	0.005
	High/Low	8.1	2.42	13.78	
	High/High	26.1	0	56.16	
	Low/High	47.5	0	97.81	

[a]AJCC Stage grouping, 8th edition (20). CI, confidence interval; LVI, lymphovascular invasion; PNI, perineural invasion; LN, lymph node; TNM, tumor node metastasis; TIL, tumor-infiltrating lymphocyte.

This is the first study to demonstrate the detailed clinicopathological and prognostic impact of IDO1 expression associated with decreased numbers of $CD8^+$ TILs. In the future, our results can be utilized as a novel candidate biomarker in EHBDC for the development of immunotherapeutic drugs by augmenting our understanding of complex immune mechanisms.

The limitations of this study include the retrospective nature of the study design and the relatively small number of cases. In addition, the fundamental problem in TMA studies, such as tumor heterogeneity, remains unresolved.

In conclusion, IDO1 was highly expressed in the tumor epithelial cells in approximately one-third of cases of EHBDC. High expression of IDO1 was associated with decreased numbers of $CD8^+$ TILs, an increased pN category, an advanced overall stage, and frequent recurrence. When further stratified by combining IDO1 expression with $CD8^+$ TIL status, the $IDO1^{high}/CD8^{low}$ subgroup had the worst

Table IV. Multivariate analyses (Cox proportional hazards model).

Parameter	Comparison	Overall survival			Disease-free survival		
		HR	95% CI	P-value	HR	95% CI	P-value
Location	Proximal vs. Distal	1.349	1.004-1.812	0.047	1.449	1.073-1.957	0.016
PNI	Presence vs. Absence	2.159	0.984-4.737	0.055	2.486	1.060-5.830	0.036
LN metastasis	Presence vs. Absence	-	-	-	1.956	1.026-3.732	0.042
IDO1/CD8$^+$ TIL	IDO1^{high}/CD8^{low} vs. Others	2.168	1.1-4.272	0.025	2.460	1.195-5.065	0.015

CI, confidence interval; HR, hazard ratio; PNI, perineural invasion; LN, lymph node; TNM, tumor node metastasis; TIL, tumor-infiltrating lymphocyte.

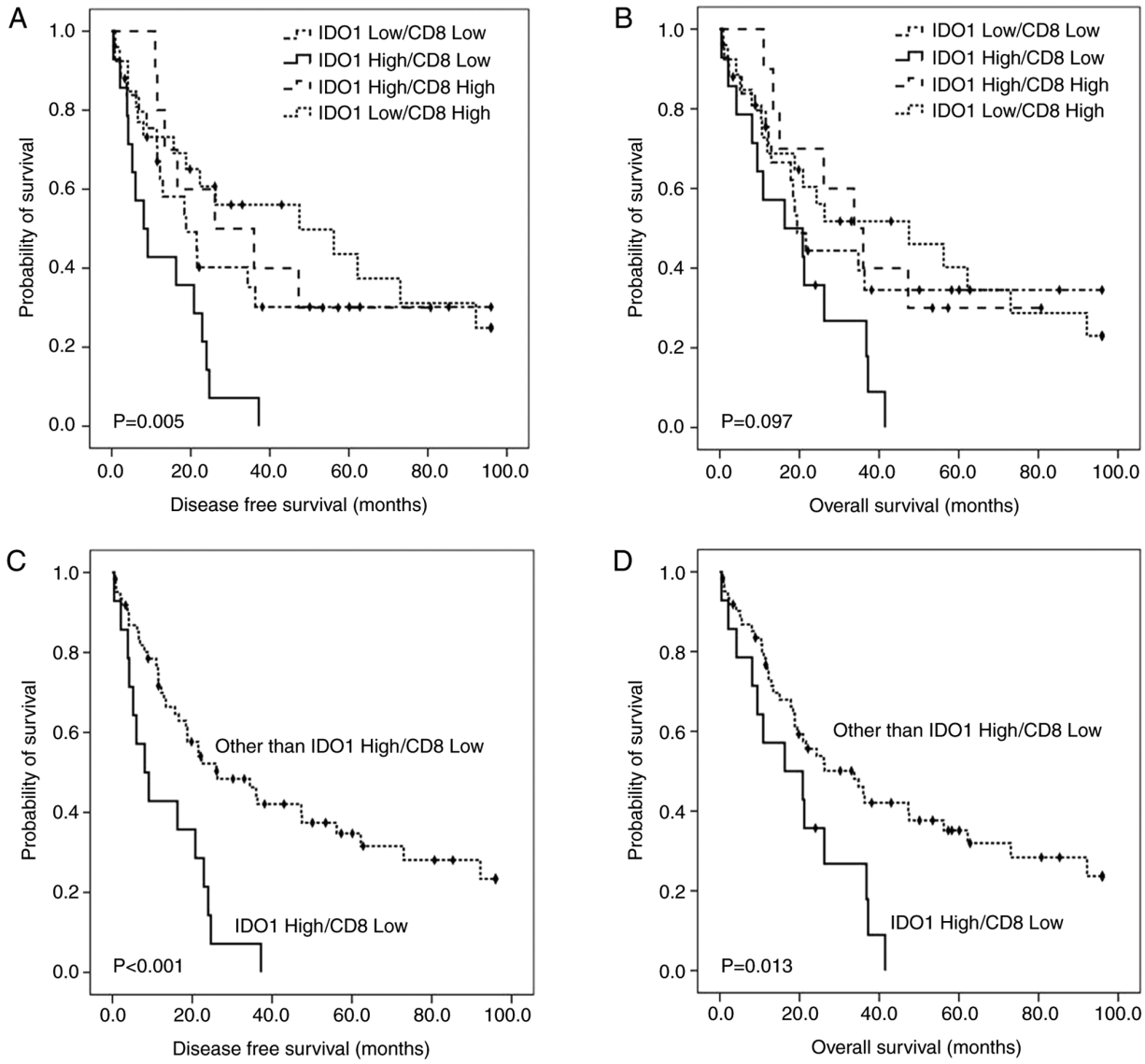

Figure 3. Kaplan-Meier curves according to immune subgroups using combined IDO1 and CD8$^+$ TILs expression. The IDO1^{high}/CD8^{low} subgroup demonstrated the worst prognosis, while the IDO1^{low}/CD8^{high} subgroup demonstrates the best clinical outcome for (A) disease-free survival and (B) overall survival compared with IDO1^{low}/CD8^{low} and IDO1^{high}/CD8^{high} subgroups. Patients with IDO1^{high}/CD8^{low} expression demonstrated a statistically favorable survival outcome for (C) disease-free survival and (D) overall survival compared with those in the other three groups. IDO1, indoleamine 2, 3-dioxygenase 1; TILs, tumor infiltrating lymphocytes.

prognosis for overall survival and disease-free survival. High IDO1 expression with decreased numbers of CD8$^+$ TILs is an independent prognostic indicator, and expected to be an IDO1 blocker-targetable candidate in patients with EHBDC.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BJN, GMC, HJJ, CHM, HSO, MK and DWE conceived and designed the research. BJN and DWE performed the experiments, analysed the data and wrote the manuscript. GMC, HJJ, CHM, HSO and MK reviewed the manuscript and approved the final version. All authors have read and approved the final manuscript. BJN and DWE confirm the authenticity of all the raw data.

Ethics approval and consent to participate

This study was approved by the Institutional Review Board of Gangneung Asan Hospital (approval no. GNAH 2020-06-018). All procedures were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions. Informed consent to be included in the study, or the equivalent, was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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