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Clinical Significance of Sphingosine 1-phosphate Receptor 2 and Takeda G Protein-coupled Receptor 5 in Extrahepatic Cholangiocarcinoma Patients

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ABSTRACT

Background & Aims: In biliary epithelial cells, two bile acid receptors, sphingosine 1-phosphate receptor 2 (S1PR2) and Takeda G protein-coupled receptor 5 (TGR5) have been reported to trigger cell proliferation, as well as neoplastic cell invasiveness. In this study, we aimed to investigate the clinical significance of S1PR2/TGR5 expression in extrahepatic cholangiocarcinoma (CCA) patients.

Methods: Patients who underwent surgical resection of extrahepatic CCA at Korea University Guro Hospital between 2002 and 2018 were included. Data on immunohistochemical staining and H-score of S1PR2 and TGR5 were evaluated using digital image analysis.

Results: A total of 115 cases of invasive CCA were analyzed. The H-score of S1PR2 showed a decrease in invasive CCA (p=0.052) but that of TGR5 showed a significant increase (p=0.02). Overall survival and disease-free survival were significantly lower in the low S1PR2 expression group (p<0.05) than in the control group; however, TGR5 expression was not significant (p=0.096). In multivariate analysis, low S1PR2 was only significant for poor prognosis.

Conclusion: Low S1PR2 level was the only independent poor prognostic factor in patients with resected extrahepatic CCA.

Key words: extrahepatic – cholangiocarcinoma – S1PR2 – TGR5 – S1P.

Abbreviations: BA: bile acids; CCA: cholangiocarcinoma; DBD: distal bile duct; DFS: disease-free survival; IHC: Immunohistochemistry; PBD: perihilar bile duct; S1P: Sphingosine-1-phosphate; S1PR2: S1P receptor 2; TGR5: Takeda G protein-coupled receptor 5.

INTRODUCTION

Bile acids (BA) not only play a fundamental role in bile formation and secretion but is also important in protective and injurious processes involving the biliary tract. However, in the last decade, awareness regarding the physiological and chemical heterogeneity of this category of compounds and their possible beneficial or injurious effects on the biliary tree has increased [1].

Since the discovery of BA receptors in bile acid signaling mechanisms, there has been increased interest in liver diseases, and it has been found that dysregulation is involved in cytotoxicity, inflammation, and fibrosis [2].

Recently, it has been identified that specific BA receptors, Takeda G protein-coupled receptor 5 (TGR5) and sphingosine-1-phosphate receptor 2 (S1PR2), are involved in the proliferation of and secretion from cholangiocyte, which seems to protect cholangiocyte from the toxicity of BA [3]. Decreased TGR5 expression may also contribute to the development or progression of cholangiopathies, such as primary biliary cholangitis and primary sclerosing cholangitis, as reduced TGR5-dependent cell-protective mechanisms, such as bicarbonate secretion, render cholangiocytes more vulnerable towards bile salt toxicity [4].

Sphingosine-1-phosphate (S1P), formed by the phosphorylation of sphingosine, is released from cells via specific transporters in the plasma membrane and then binds to a variety of receptors (S1PR 1–5) in different organ systems [5, 6]. As a bioactive lipid, it regulates several biological processes, including cell growth, survival, differentiation, migration, lymphocyte circulation, and immune cell

regulation. Furthermore, S1P and S1PR2 are associated with liver fibrosis. Although there is substantial evidence that S1P plays a significant role in cancer, the role of S1PR2 in cancer and extrahepatic cholangiocarcinoma (CCA) is controversial.

Bile acids receptors have been suggested to be involved in CCA proliferation and cell spreading; however, little is known about the clinical significance of S1PR2 and TGR5 in extrahepatic CCA in humans.

Thus, we aimed to investigate the clinical significance of S1PR2/TGR5 expression in extrahepatic CCA patients.

METHODS

Patient Selection and Clinicopathologic Data Collection

Patients who underwent surgical resection of extrahepatic CCA at Korea University Guro Hospital (KUGH) between 2002 and 2018 were initially screened. Patients who underwent preoperative systemic chemotherapy, those who died within 7 days of surgery, those with in situ carcinoma lesions only, and those without available archival tissue blocks were excluded. Finally, 115 patients were included in the study.

Clinical and demographic data, including gender, age, tumor, nodes, and metastases (TNM) stage, and the day of disease recurrence or last hospital visit, were retrieved by reviewing electronic medical records. Pathological characteristics of the tumors, including tumor location, histologic grade, histologic type, lymph node metastasis, surgical margin status, and lymphatic/venous/perineural invasion of the tumor, were obtained by reviewing pathology reports and archival slides by a pathologist (H.K.). The histological types were determined based on the 5th edition of the World Health Organization classification of digestive system tumors [7]. Tumor staging was performed according to the 8th edition of the staging manual of the American Joint Committee on Cancer [8].

This study was approved by the Institutional Review Board of KUGH (accession no. 2020GR0394 and 2021GR0400). We confirm that informed consent was obtained from all subjects or their legal guardian. All methods were performed in accordance with the relevant guidelines and regulations.

Tissue Microarray Construction

Tissue microarray (TMA) blocks were constructed using tissue cores from the tumor-adjacent normal epithelium and representative lesion areas of dysplasia and invasive carcinoma. Collected tissue cores of 2-mm diameter, one–four per lesion, were transferred onto TMA recipient blocks.

Immunohistochemical Staining and Interpretation by Digital Image Analysis

Immunohistochemical (IHC) staining was performed on 4 μm thick tissue sections cut from TMA blocks according to the routine streptavidin-biotin-peroxidase method. The cut sections were deparaffinized and rehydrated, and endogenous peroxidase blocking was performed by treating with 3% hydrogen peroxide for 20 min. Antigen retrieval was performed using 10mM citrate buffer (pH 6.0) for 20 min. The BOND-MAX automated staining system (Leica) was used for staining. The antibodies used for IHC staining were anti-S1PR2 antibody (1:200, rabbit polyclonal, PA5-72868, Invitrogen, Thermo Fisher) and anti-TGR5 antibody (1:500, rabbit polyclonal, PA5-27076, Invitrogen, Thermo Fisher).

The staining results of IHC slides were interpreted using the open-source digital image analysis software, QuPath ver 0.3 [9]. A cytoplasmic staining pattern was observed for both antibodies. To quantitatively evaluate IHC staining, the H-score was calculated for each lesion [10]. Each tumor cell detected using QuPath was assigned one of the following four categories and intensity scores: no staining (score 0), weak staining (score 1), moderate staining (score 2), and strong staining (score 3). The final H-score was the sum of the multiplied value of the intensity score and percentage of cells showing staining intensity.

The low-and high-expression groups for each antibody were divided according to the third quartile (Q3) values of the H-score as cutoffs. The median and Q3 values of the H-score of S1PR2 expression were 136.40 and 228.17, respectively (range, 2.46–282.62). The median and Q3 values of the H-score for TGR5 expression were 105.70 and 157.61, respectively (range, 6.65–275.22).

Thirteen patients were diagnosed with extrahepatic CCA, and the control group included five patients with benign biliary conditions. We analyzed the bile proteome using liquid chromatography-mass spectrometry. We compared the relative abundances of various proteins in the CCA and control groups.

Statistical Analysis

To assess correlations between clinicopathologic parameters and S1PR2/TGR5 expression, the chi-square or Fisher's exact test was applied. The Kaplan–Meier method and log-rank test were used to compare survival in different expression groups. The disease-free survival (DFS) was defined as the duration from the day of surgery to the day of disease recurrence, death, or last follow-up. Multivariate Cox proportional hazards regression analysis was performed using factors that showed p-values < 0.1 in univariate analyses. A p-value < 0.05 was considered statistically significant. All statistical analyses were performed using R software, version 4.1.0.

RESULTS

Patient Characteristics

The enrolled patients were predominantly male (85 men and 30 women) (Table I). Forty-four (38.3%) patients were aged < 65 years. There were 70 (60.9%) patients with distal bile duct (DBD) cancers and 45 (39.1%) with perihilar bile duct (PBD) cancers. Lymph node metastasis was observed in 34 (29.6%) patients. Distant metastasis was discovered in one (0.9%) patient during surgery. The median follow-up period was 857 days (range: 9–5809 days).

Clinicopathologic Characteristics and S1PR2/TGR5 Expression

The clinicopathologic characteristics of 115 extrahepatic CCA patients and their relationships with S1PR2/TGR5 expression status are summarized in Table I. The low S1PR2 group was more frequent in PBD patients than in DBD patients (p=0.001). Patients with low TGR5 expression had more venous invasion than in high expression (p=0.006). Other than these

Table I Correlations between clinicopathologic characteristics and S1PR2/TGR5 expression

	Number	Low S1PR2	High S1PR2	p	Low TGR5	High TGR5	p
Age				0.355			0.966
<65	44	35	9		33	11	
≥65	71	51	20		53	18	
Gender				0.832			0.093
Male	85	64	21		67	18	
Female	30	22	8		19	11	
Histologic type				0.710			0.710
Adenocarcinoma	113	84	29		84	29	
Adenosquamous carcinoma	1	1	0		1	0	
Undifferentiated carcinoma	1	1	0		1	0	
Histologic grade				0.739			0.146
Well-differentiated	26	21	5		16	10	
Moderately differentiated	63	45	18		47	16	
Poorly differentiated	25	19	6		22	3	
Undifferentiated	1	1	0		1	0	
Tumor location				0.001			0.774
Distal bile duct	70	45	25		53	17	
Perihilar bile duct	45	41	4		33	12	
TNM stage				0.052			0.705
I	31	19	12		23	8	
II	63	47	16		45	18	
III	17	16	1		14	3	
IV	4	4	0		4	0	
Surgical margin status				0.497			0.230
Free from carcinoma	90	66	24		65	25	
Involved by carcinoma	25	20	5		21	4	
Lymph node metastasis				0.459			0.093
Absent	81	59	22		57	24	
Present	34	27	7		29	5	
Distant metastasis				0.311			0.319
Absent	114	56	58		57	57	
Present	1	1	0		1	0	
Lymphatic invasion				0.082			0.405
Absent	76	53	23		55	21	
Present	39	33	6		31	8	
Venous invasion				0.232			0.006
Absent	98	71	27		69	29	
Present	17	15	2		17	0	
Perineural invasion				0.398			0.765
Absent	22	18	4		17	5	
Present	93	68	25		69	24	

S1PR2: S1P receptor 2; TGR5: Takeda G protein-coupled receptor 5.

findings, there were no significant associations between S1PR2 or TGR5 expression and the clinicopathologic parameters.

S1PR2 and TGR5 in Normal Epithelium, Dysplasia, and Invasive Carcinoma Tissues

The number of tissue cores evaluated for each lesion was as follows: 16 for tumor-adjacent normal epithelium, 37 for dysplasia, and 118 for invasive carcinoma. Following disease progression, S1PR2 showed a trend towards lower expression (p=0.059; when normal epithelium and invasive carcinoma were compared).

The expression of TGR5 significantly increased as the disease progressed (p=0.003 and 0.001; when normal epithelium and dysplasia were compared with invasive carcinoma, respectively) (Fig. 1).

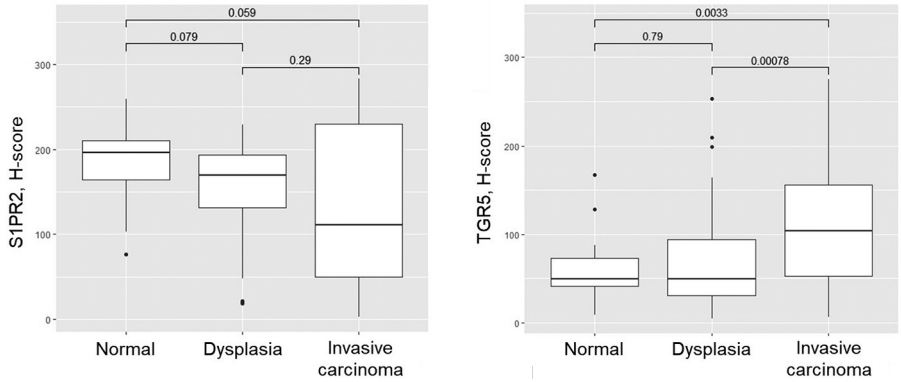

Fig. 1. Expression of S1PR2 and TGR5 in tumor-adjacent normal epithelium, dysplasia, and invasive lesion in extrahepatic cholangiocarcinoma.

Prognostic Significance of S1PR2 and TGR5 Expression in Extrahepatic CCA

Using Kaplan–Meier survival analysis with log-rank tests, we observed that low S1PR2 expression was significantly associated with worse 10-year DFS (p=0.006). For TGR5, no significant correlation was observed with survival. However, there was a trend towards an association between low TGR5 levels and shorter survival (p=0.096) (Fig. 2).

We performed univariate Cox analyses for major clinicopathologic variables and S1PR2/TGR5 expression levels. Statistically significant risk factors in the univariate analyses were surgical margin status, lymphatic invasion, TNM stage, and S1PR2 expression. Factors that showed a p-value < 0.1 in univariate analyses were included in the multivariate analyses. In the multivariate analyses, low S1PR2 expression was an independent prognostic factor for 10-year DFS (p=0.040) (Table II).

DISCUSSION

Recently, there have been advances in the study of BA and its receptors in the biliary epithelium. Bile acids and BA receptors play a protective role in cholangiocytes, as well as showing anti-apoptotic effects [1]. TGR5 overexpression has been reported to promote cholangiocyte proliferation, leading to cyst growth in polycystic liver disease and even progression of CCA [4]. Also, it was demonstrated that TGR5 is over expressed in intrahepatic CCA tissue [11]. Another recent study TGR5 expression in human extrahepatic CCA tissues, positively correlates with well-differentiated pathological grade but not with other clinical characteristics, including age, gender, lymphatic metastasis, and TNM stage [12]. In this study, TGR5 expression was measured using a relative quantitative evaluation of IHC staining with digital image analysis of extrahepatic CCA specimens and adjacent tissues. The expression of TGR5 significantly increased as the disease progressed from tumor-adjacent normal epithelium to dysplasia and invasive carcinoma; however, it did not show a significant correlation with survival, except for a trend in the association of low TGR5 and shorter survival.

S1PR2 is the predominant S1PR expressed in cholangiocytes. It has been suggested that conjugated BAs promote cell growth through S1PR2 in CCA [13], and the accumulation of conjugated BAs attributed to the bile duct obstruction results in the activation of S1PR2, which further activates the extracellular signal-regulated protein kinase 1/2 signaling pathway [14]. However, the role of S1PR2 in cancer is controversial, demonstrating that this receptor can not only promote tumorigenesis but also inhibit the motility of cancer cells and tumor angiogenesis [15-17]. To date, there have been few studies on S1PR2 in extrahepatic CCA, which have been carried out on human CCA cell lines or mice [14, 18, 19]. Our

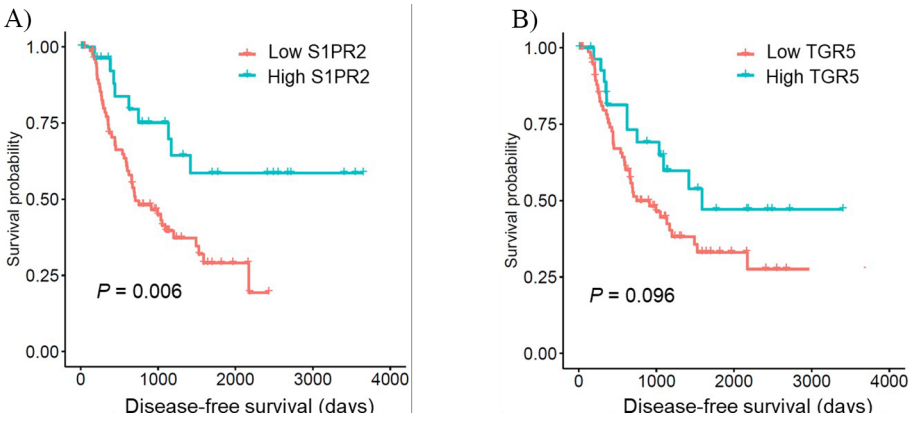

Fig. 2. Kaplan-Meier analysis of disease-free survival according to S1PR2 and TGR5 expression status in extrahepatic cholangiocarcinoma.

Table II. Univariate and multivariate Cox analysis of 10-year disease free survival in 115 extrahepatic cholangiocarcinoma patients

	Univariate		Multivariate	
	HR (95% CI)	p	HR (95% CI)	p
Age (<65 vs. ≥65 years)	0.8509 (0.506 - 1.432)	0.543		
Gender (Male vs. female)	0.9207 (0.510 - 1.662)	0.784		
Tumor grade (WD, MD vs. PD, UD)	1.151 (0.629 - 2.106)	0.648		
Tumor location (DBD vs. PBD)	1.666 (0.981 - 2.83)	0.059	0.967 (5.001 - 1.870)	0.921
Surgical margin status	2.571 (1.438 - 4.595)	0.001	1.793 (0.923 - 3.484)	0.085
TNM stage				
I	Reference	-	Reference	-
II	2.154 (1.060 - 4.378)	0.034	1.873 (0.871 - 4.027)	0.108
III	2.294 (0.952 - 5.532)	0.064	1.205 (0.424 - 3.426)	0.726
IV	9.954 (3.043 - 32.565)	0.000	3.028 (0.664 - 13.813)	0.153
Lymph node metastasis	1.512 (0.876 - 2.612)	0.138		
Distant metastasis	5.702 (0.756 - 43.01)	0.091	3.323 (0.274 - 40.265)	0.345
Lymphatic invasion	1.846 (1.090 - 3.124)	0.023	1.599 (0.839 - 3.048)	0.154
Venous invasion	1.377 (0.672 - 2.822)	0.382		
Perineural invasion	2.002 (0.906 - 4.42)	0.086	1.688 (0.743 - 3.836)	0.211
S1PR2 (low vs. high)	0.3804 (0.185 - 0.782)	0.009	0.457 (0.211 - 0.992)	**0.048**
TGR5 (low vs. high)	0.5852 (0.309 - 1.108)	0.100		

CI: confidence interval; DBD: distal bile duct, HR: hazard ratio; MD: moderately differentiated; PBD: perihilar bile duct; PD: poorly differentiated; UD: undifferentiated; WD: well-differentiated.

study found S1PR2 expression in extrahepatic CCA tissues. In this study, we observed that low S1PR2 expression was an independent poor prognostic factor for extrahepatic CCA and showed a trend towards lower expression as the disease progressed to invasive cancer, contrary to the expression of TGR5. These results suggest that TGR5 and S1PR2, as the dual BA receptors of large bile duct cholangiocytes, are conversely expressed as the CCA progress, although both receptors are expressed in extrahepatic CCA. It might be considered that invasive cell growth through S1PR2 overexpression is activated in the marginal area of tumors rather than obstructed central lesions because S1PR2 is known to be activated by conjugated BA [20]. In addition, S1PR2 showed locational differences and significantly lower expression in PBD tumors than in DBD tumors. However, TGR5 did not show any difference, although tumor location was not a prognostic factor in univariate and multivariate analyses. Taken together, lower S1PR2 expression in tumors and poor survival could be suggested, probably due to longstanding and massive bile duct obstruction. In addition, adjuvant management is considered depending on S1PR2 expression, given its association with poor prognosis.

CONCLUSIONS

We identified two BA receptors in extrahepatic CCA tissue and observed that low S1PR2 expression was correlated with a poor prognosis.

Conflicts of interest: None to declare.

Authors' contribution: H.K. and H.J.K. conceived and designed the study. H.J.K., Y.T.P. and J.S.K. collected subjects, samples, and data. H.K. and H.J.K. performed the IHC analysis and reviewed statistic analyzed the data. H.K. and H.J.K. wrote the initial draft of the manuscript. All the authors critically revised the paper and approved the final version of the manuscript.

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