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Increased Sphingosine Kinase 1 Expression Predicts Distant Metastasis and Poor Outcome in Patients With Colorectal Cancer

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Abstract. *Background/Aim: Sphingosine kinase 1 (SPHK1) is up-regulated in many different cancers and plays a crucial role in tumor development and progression. However, the prognostic value of SPHK1 in colorectal cancer (CRC) remains unclear. Materials and Methods: The expression of SPHK1 in CRC cell lines and 328 CRC tissue samples was examined. It was also investigated whether SPHK1 expression is associated with clinicopathological characteristics and outcomes in patients with CRC. Results: HCT 116 and HT-29 cells expressed significantly higher SPHK1 levels than did CCD 841 CoTr. On immunohistochemistry, SPHK1 expression was significantly higher in CRC tissue than in normal colonic mucosal tissue, with 34.1% of CRC patients exhibiting high SPHK1 expression. High SPHK1 expression in CRC was significantly associated with higher histological grade, deeper invasion depth, lymphatic invasion, vascular invasion, and development of distant metastasis, and was shown to be an independent predictor of distant metastasis. Furthermore, patients with high SPHK1 expression had significantly lower overall survival rates than those with low expression. Conclusion: High SPHK1 expression was significantly associated with aggressive CRC behavior and worse overall survival, and was an independent predictor of distant metastasis. SPHK1 may thus be a potential prognostic biomarker and therapeutic target in CRC patients.*

Colorectal cancer (CRC) is the third most common cancer and the fourth leading cause of cancer-related death worldwide (1, 2). Also, it is the second and third most common cancer in men and women, respectively, in the Republic of Korea (3). Although advances in the molecular characterization of CRC have provided remarkable progress in treatment methods, approximately 56% of patients with CRC die from their cancer (4). The majority of CRC patient deaths occur after the cancer metastasizes; moreover, approximately 20% of patients already have metastases at diagnosis (5). Metastasis is a multistep process associated with complex genetic alterations that enable cancer cell survival, proliferation, and migration away from the primary site through the vascular or lymphatic system (6), whereupon they disseminate to establish secondary lesions in distant organs (7).

Sphingolipids have emerged as key components in cancer development owing to their role in cancer stem cell-driven angiogenesis and lymphangiogenesis (8, 9). Many studies have documented the roles of various sphingolipid enzymes, sphingolipid-binding proteins, and transmembrane transporters in human cancers (10). Among these, sphingosine kinase (SPHK) family members are key enzymes in cancer biology, as their catalytic activity is critical for the regulation of sphingolipid metabolism (9). SPHK exists as two functional isoenzymes in humans, SPHK1 and SPHK2 (11). SPHK1 is an oncogenic enzyme that phosphorylates sphingosine to form sphingosine-1-phosphate, a sphingolipid metabolite that plays a crucial role in various aspects of cellular processes including

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apoptosis, survival, growth, proliferation, and angiogenesis (12, 13). Previous studies have shown that SPHK1 is upregulated in many human cancers, including those of the breast, ovaries, cervix, stomach, lung, head and neck, and brain (9, 12-17). Although evidence of SPHK1 involvement in cancer development and metastasis, as well as in tumor microenvironment neovascularization, has been reported, studies of SPHK1 in CRC are scarce (18).

This study analyzed the expression levels of SPHK1 in CRC cell lines as well as the expression patterns of SPHK1 in CRC tissue samples using tissue microarrays and immunohistochemical staining. Furthermore, the association between SPHK1 expression status and the clinicopathological characteristics and outcomes of CRC patients were investigated.

Materials and Methods

Cell lines. The human normal colonic epithelial cells CCD 841 CoTr as well as the CRC cell lines HCT 116 and HT-29 were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle's Medium or Roswell Park Memorial Institute 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) (Gibco, Life Technologies, Grand Island, NY, USA). All cell lines were cultured in a humidified atmosphere of 5% carbon dioxide at 37˚C.

Western blotting. Protein extracts were prepared using radio-immunoprecipitation assay buffer (Thermo Fisher Scientific, Waltham, MA, USA) containing freshly added protease and phosphatase inhibitor cocktails (Thermo Fisher Scientific). The concentrations of the total cell lysates were measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Twenty micrograms of total protein were mixed with 5× sample buffer and heated at 95˚C for 5 min. The samples were loaded on 12% sodium dodecyl sulfate-polyacrylamide gels and transferred to a polyvinylidene fluoride membrane (EMD Millipore, Burlington, MA, USA) via a Transblot apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% non-fat skim milk in Tris-buffered saline solution (10 mmol/l Tris-hydrogen chloride [pH 7.4] and 0.5 mol/l sodium chloride) with 0.1% v/v of Tween-20. The blots were probed with anti-SPHK1 antibody (1:200, polyclonal; Abcam, Cambridge, MA, USA) and anti-glyceraldehyde 3-phosphate dehydrogenase (1:1,000; Abcam). They were then incubated in horseradish peroxidase-conjugated secondary antibodies (1:2,000, Thermo Fisher Scientific). Proteins were detected using an enhanced chemiluminescence reagent (Santa Cruz Biotechnology Inc., Dallas, TX, USA). The experiment was repeated three times and the data shown are representative.

Tissue samples. Tissue samples were obtained from 328 consecutive patients who underwent surgery for primary adenocarcinoma of the colon and rectum. Two independent board-certified pathologists reviewed all hematoxylin and eosin-stained slides and selected the most representative for each case. Clinicopathological data including age, sex, histological grade, tumor size, pathological tumor stage, pathological lymph node stage, distant metastasis, local recurrence, lymphovascular invasion, perineural invasion, and postoperative follow-up were collected. All tumors were assessed for histological grade according to the World Health Organization classification and were postoperatively staged according to the American Joint Committee on Cancer staging system (19). None of the patients underwent preoperative neoadjuvant chemotherapy or neoadjuvant concurrent chemoradiation therapy. Informed consent was obtained from all subjects. This study (2018-04-028) was reviewed and approved by the institutional review board of Kangbuk Samsung Hospital (Seoul, Republic of Korea).

Tissue microarray technique. SPHK1 protein expression in tissue microarray blocks from 328 CRC and 22 normal colonic mucosal tissue samples was examined using immunohistochemical staining. The tissue microarray blocks were produced as previously described (20). Briefly, all available hematoxylin and eosin-stained slides were reviewed thoroughly, and the two most representative tumor areas were marked on the corresponding formalin-fixed, paraffin-embedded tissue blocks. Two tissue cores that were 2 mm in diameter were acquired from each donor block and manually arranged into recipient microarray blocks. The array was held in an X-Y position guide with a 1 mm increment between the individual tissue cores, and the instrument was used to create holes in a recipient block with defined array cores. An appropriate-gauge needle was used to transfer the tissue cores into the recipient block, and a pair of tissue microarray blocks was created for each patient sample. The proportion of tumor volume in each core was greater than 70%.

Immunohistochemical staining. The 4 μm-thick consecutive sections were obtained from each formalin-fixed, paraffin-embedded tissue microarray block and placed onto charged slides. Sections were deparaffinized, dehydrated with xylene, and then rehydrated in a graded series of alcohol solutions. Immunohistochemical staining was conducted using an automatic immunostainer using the compact polymer method (Bond Intense Detection Kit, Leica Biosystems, Newcastle upon Tyne, UK) according to the manufacturer's recommendations (9, 20-25). The primary antibody used was SPHK1 (1:100, polyclonal, Abgent, Inc., San Diego, CA, USA). After chromogenic visualization using peroxidase/3,3'-diaminobenzidine (EnVision+ Detection Systems, Dako), slides were counterstained with hematoxylin, after which coverslips were mounted.

Interpretation of immunohistochemical staining. The degree of immunohistochemical expression of SPHK1 was determined based on the assessment of the entire core area by combining the scores of the proportion of positively stained cancer cells and the staining intensity, as previously described (9, 17, 26). Briefly, the total scores were computed as the product of the proportion score (0, no staining; 1, 1-9%; 2, 10-49%; and 3, 50% or more cancer cells) and the intensity score (0, absent; 1, weak; 2, moderate; and 3, strong), resulting in scores of 0, 1, 2, 3, 4, 6, and 9. The optimal cutoff value for high and low SPHK1 expression level was chosen based on the distribution of the staining results as well as the extent of heterogeneity as determined using the log-rank test with respect to overall survival (OS). A final score of 4 or more signified tumors with high SPHK1 expression, while a final score of less than 4 was indicative of low SPHK1 expression. All slides were examined and scored by two board-certified pathologists who were blinded to the clinicopathological data and patient identities. Disagreements between the two pathologists were resolved by consensus.

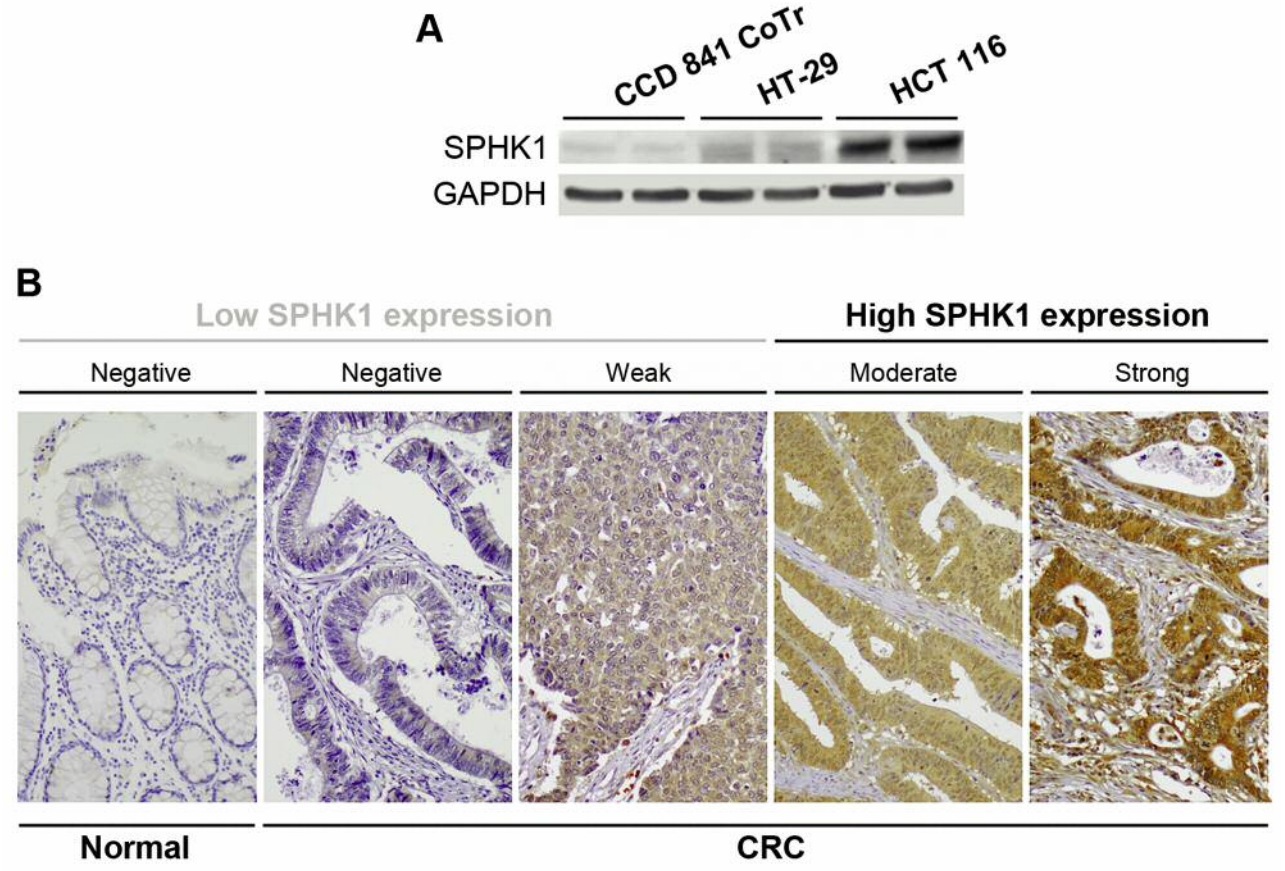

Figure 1. *Sphingosine kinase 1 (SPHK1) expression in human normal colonic (CCD 841 CoTr) and colorectal cancer (CRC; HT-29 and HCT 116) cell lines and tissue samples. (A) Western blot for SPHK1 expression in cell lines. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. (B) Immunohistochemical staining for SPHK1 in tissue samples using the polymer method. Original magnification 100×.*

Statistical analysis. To determine the association between SPHK1 expression status and clinicopathological characteristics, the chi-square test, Fisher's exact test, or linear-by-linear association test was performed. Multivariate logistic regression analysis with a backward stepwise elimination method was used to identify independent predictors of distant metastasis. Univariate and multivariate survival analyses were performed to examine the prognostic significance of SPHK1 expression. Kaplan–Meier plots for OS were constructed, and differences were analyzed by applying the log-rank test for univariate analysis. Multivariate survival analysis was performed for parameters that achieved statistical significance on univariate survival analysis using the Cox proportional hazards model [95% confidence interval (CI)] with a backward stepwise elimination method. Statistical analyses were performed using SPSS for Windows, Version 16.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was defined as a p-value less than 0.05.

Results

SPHK1 expression in colorectal cancer cell lines. Western blotting revealed that SPHK1 protein expression was significantly higher in the CRC cell lines HCT 116 and HT-29 than in the normal colonic epithelial cell line CCD 841 CoTr (Figure 1A).

SPHK1 expression in colorectal cancer tissue samples. Representative photomicrographs of SPHK1 immunostaining are shown in Figure 1B. No staining was observed in 72.7% (16 of 22) of normal colonic mucosal tissue samples examined. In contrast, CRC tissues expressed SPHK1 in varying intensities and proportions. Among the 328 CRC patient samples, 112 (34.1%) showed high SPHK1 expression and 216 (65.9%) showed low expression. SPHK1 expression was primarily observed in the cytoplasm of cancer cells; cancer cells with strong cytoplasmic SPHK1 expression also had weak-to-moderate nuclear SPHK1 immunoreactivity. The frequency of high SPHK1 expression in CRC was significantly higher than that in normal colonic mucosa (p<0.001).

Association between SPHK1 expression status and the clinicopathological characteristics of colorectal cancer. The relationships between SPHK1 expression status and the

Table I. *Relationship between sphingosine kinase 1 expression and clinicopathological characteristics of colorectal cancer.*

Characteristic	Total	Sphingosine kinase 1 expression		*p*-Value
		High (%)	Low (%)	
Age (years)				
≥59	176	56 (31.8)	120 (68.2)	0.339
<59	152	56 (36.8)	96 (63.2)	
Gender				
Man	205	77 (37.6)	128 (62.4)	0.092
Woman	123	35 (28.5)	88 (71.5)	
Histological grade				
1	16	1 (6.3)	15 (93.8)	0.029
2	298	105 (35.2)	193 (64.8)	
3	14	6 (42.9)	8 (57.1)	
Tumor size (cm)				
≥5.0	179	63 (35.2)	116 (64.8)	0.661
<5.0	149	49 (32.9)	100 (67.1)	
Pathological tumor stage (pT)				
pT1	15	1 (6.7)	14 (93.3)	<0.001
pT2	44	4 (9.1)	40 (90.9)	
pT3	220	81 (36.8)	139 (63.2)	
pT4	49	26 (53.1)	23 (46.9)	
Pathological lymph node stage (pN)				
pN0	158	50 (31.6)	108 (68.4)	0.088
pN1	91	27 (29.7)	64 (70.3)	
pN2	79	35 (44.3)	44 (55.7)	
Distant metastasis				
Present	95	48 (50.5)	47 (49.5)	<0.001
Absent	233	64 (27.5)	169 (72.5)	
Local recurrence				
Present	11	6 (54.5)	5 (45.5)	0.147
Absent	317	106 (33.4)	211 (66.6)	
Lymphatic invasion				
Present	161	72 (44.7)	89 (55.3)	<0.001
Absent	167	40 (24.0)	127 (76.0)	
Vascular invasion				
Present	49	27 (55.1)	22 (44.9)	0.001
Absent	279	85 (30.5)	194 (69.5)	
Perineural invasion				
Present	82	28 (34.1)	54 (65.9)	1.000
Absent	246	84 (34.1)	162 (65.9)	

clinicopathologic characteristics of patients with CRC are summarized in Table I. High SPHK1 expression was significantly correlated with higher histological grade (p=0.029), development of distant metastasis (p<0.001), higher pathological tumor (pT) stage (p<0.001), presence of lymphatic invasion (p<0.001), and presence of vascular invasion. (p=0.001). However, there were no significant correlations between SPHK1 expression status and patient age, sex, tumor size, pathological lymph node stage (pN), presence of local recurrence, and perineural invasion.

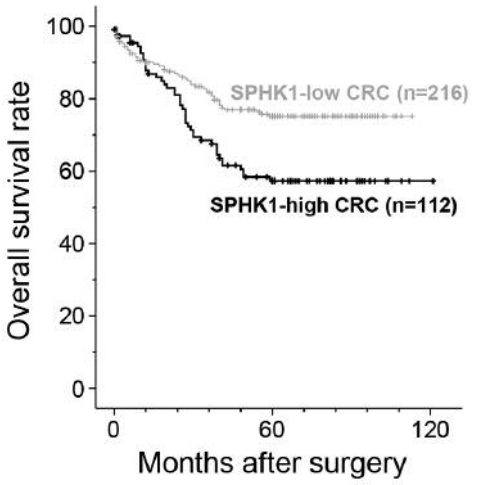

Figure 2. *Kaplan–Meier plot showing survival rates in 328 colorectal cancer (CRC) patients with high versus low sphingosine kinase 1 (SPHK1) expression.*

Factors independently predicting distant metastasis in patients with colorectal cancer. The relationships between distant metastasis, clinicopathological characteristics, and SPHK1 expression are summarized in Table II. Tumor size (p=0.013), higher pT (p<0.001), higher pN (p<0.001), local recurrence (p<0.001), lymphatic invasion (p<0.001), vascular invasion (p<0.001), perineural invasion (p<0.001), and high SPHK1 expression were found to be significantly associated with distant metastasis development in patients with CRC. To identify the factors that independently predict the development of distant metastasis, these covariates were subjected to a multivariate logistic regression analysis. Higher pN (p=0.003, relative risk=2.800; 95% CI=1.412-5.551), local recurrence (p=0.043, relative risk=6.806; 95% CI=1.001-46.262), perineural invasion (p=0.006, relative risk=2.651; 95% CI=1.325-5.304), and high SPHK1 expression (p=0.025, relative risk=2.124; 95% CI=1.100-4.101) were independent predictive factors for distant metastasis. On the other hand, tumor size, pT, lymphatic invasion, and vascular invasion did not independently predict the development of distant metastasis.

Prognostic significance of SPHK1 expression in patients with colorectal cancer. Univariate survival analysis for OS revealed that larger tumor size (p<0.001), higher pT (p<0.001), higher pN (p<0.001), distant metastasis (p<0.001), local recurrence (p<0.001), lymphatic invasion (p<0.001), vascular invasion (p<0.001), perineural invasion (p<0.001), and high SPHK1 expression

Table II. *Factors predicting distant metastasis in patients with colorectal cancer.*

Characteristic	Univariate			Multivariate	
	Distant metastasis		*p*-Value	*p*-Value	Relative risk (95% confidence interval)
	Present (%)	Absent (%)			
Age (years)					
≥59	49 (27.8)	127 (72.2)	0.630	Not applicable	Not applicable
<59	46 (30.3)	106 (69.7)			
Gender					
Male	63 (30.7)	142 (69.3)	0.362	Not applicable	Not applicable
Female	32 (26.0)	91 (74.0)			
Histological grade					
1	2 (12.5)	14 (87.5)	0.150	Not applicable	Not applicable
2	88 (29.5)	210 (70.5)			
3	5 (35.7)	9 (64.3)			
Tumor size (cm)					
≥5.0	62 (34.6)	117 (65.4)	0.013	0.792	1.101 (0.537-2.251)
<5.0	33 (22.1)	116 (77.9)			
Pathological tumor stage					
pT1	0 (0.0)	15 (100.0)	<0.001	0.083	3.235 (0.856-12.221)
pT2	3 (6.8)	41 (93.2)			
pT3	56 (25.5)	164 (74.5)			
pT4	36 (73.5)	13 (26.5)			
Pathological lymph node stage					
pN0	23 (14.6)	135 (85.4)	<0.001	0.003	2.800 (1.412-5.551)
pN1	22 (24.2)	69 (75.8)			
pN2	50 (63.3)	29 (36.7)			
Local recurrence					
Present	9 (81.8)	2 (18.2)	<0.001	0.043	6.806 (1.001-46.262)
Absent	86 (27.1)	231 (72.9)			
Lymphatic invasion					
Present	67 (41.6)	94 (58.4)	<0.001	0.777	1.105 (0.555-2.201)
Absent	28 (16.8)	139 (83.2)			
Vascular invasion					
Present	30 (61.2)	19 (38.8)	<0.001	0.205	1.779 (0.730-4.333)
Absent	65 (23.3)	214 (76.7)			
Perineural invasion					
Present	44 (53.7)	38 (46.3)	<0.001	0.006	2.651 (1.325-5.304)
Absent	51 (20.7)	195 (79.3)			
Sphingosine kinase 1 expression					
High	48 (42.9)	64 (57.1)	<0.001	0.025	2.124 (1.100-4.101)
Low	47 (21.8)	169 (78.2)			

(p=0.003) were significant predictors of worse OS (Table III). The 5-year OS rates were 57.3% for patients with SPHK1-high CRC and 75.1% for those with SPHK1-low CRC (Figure 2). Multivariate survival analysis of OS was performed using tumor size, pT, pN, distant metastasis, local recurrence, lymphovascular invasion, vascular invasion, perineural invasion, and SPHK1 expression status as covariates. Three factors including tumor size (p=0.018), distant metastasis (p<0.001), and lymphatic invasion (p=0.002) independently predicted OS (Table III). By contrast, SPHK1 expression *per se* did not predict OS (p=0.461).

Discussion

Sphingolipid networks and their associated enzymes and receptors are closely associated with carcinogenesis. As an oncogenic enzyme, SPHK1 has been reported to be involved in the survival, proliferation, and transformation of cancer cells. Its activation is mediated by biological processes driven by a variety of growth factors, cytokines, and mitogenic factors (16, 27-31). The association between SPHK1 expression and CRC progression and aggressiveness remains to be clarified. This study revealed a significantly higher SPHK1 protein expression in CRC cell lines and

Table III. *Factors predicting worse overall survival of patients with colorectal cancer.*

Characteristic	Overall survival		
	Univariate	Multivariate	
	p-Value	*p*-Value	Hazard ratio (95% confidence interval)
Age (years): ≥59 vs.<59	0.265	Not applicable	Not applicable
Gender: Male *vs*. female	0.166	Not applicable	Not applicable
Histological grade: 2-3 *vs*. 1	0.436	Not applicable	Not applicable
Tumor size (cm): ≥5.0 *vs*. <5.0	<0.001	0.018	1.698 (1.096-2.631)
Pathological tumor stage: pT3-4 *vs*. pT1-2	<0.001	0.381	1.542 (0.585-4.062)
Pathological lymph node stage: pN1-2 *vs*. pN0	<0.001	0.997	1.001 (0.605-1.657)
Distant metastasis: Present *vs*. absent	<0.001	<0.001	6.044 (3.762-9.711)
Local recurrence: Present *vs*. absent	0.012	0.594	1.242 (0.560-2.757)
Lymphatic invasion: Present *vs*. absent	<0.001	0.002	2.143 (1.323-3.472)
Vascular invasion: Present *vs*. absent	<0.001	0.071	1.527 (0.964-2.419)
Perineural invasion: Present *vs*. absent	<0.001	0.473	1.178 (0.753-1.842)
Sphingosine kinase 1 expression: High *vs*. low	0.003	0.461	1.190 (0.749-1.891)

tissues than in normal colonic epithelial cells and colonic mucosal tissue. In our study, 34.1% of CRC patient samples showed high SPHK1 expression; moreover, the frequency of high SPHK1 expression in CRC was significantly higher than in normal colonic tissues. These findings indicate that SPHK1 expression is upregulated in CRC, and point to this enzyme as a potential diagnostic biomarker for CRC.

A significant relationship between high SPHK1 expression and aggressive oncogenic characteristics such as higher histological grade, distant metastasis, higher pT, and lymphatic and vascular invasion was also found. These findings suggest that SPHK1 plays an important role in CRC progression, which is consistent with recent data showing that increased SPHK1 expression is associated with colorectal carcinogenesis and tumor progression (32, 33). Our results are also in agreement with those of previous studies demonstrating an association between increased SPHK1 expression and aggressive oncogenic behaviors, such as larger tumor size, deeper invasion depth, advanced stage, worse histological differentiation, higher invasive capacity, and/or chemotherapeutic resistance in a variety of different cancer types (9, 26, 34-38).

Furthermore, a high SPHK1 expression was observed to be an independent predictor of distant metastasis development. This finding is consistent with that of our previous study in which we found elevated SPHK1 expression to be predictive of distant metastasis development in patients with invasive breast carcinoma (35). Primary tumor invasion depth, lymph node metastasis, and vascular invasion are the most important prognostic factors in CRC, and are the main determinants of an aggressive tumor behavior and distant metastasis. Notably, our data revealed that the relative risk of distant metastasis associated with high SPHK1 expression (2.124) was comparable to the risk associated with pN (2.800) and perineural invasion (2.651), and was higher than that associated with vascular invasion (1.779). To the best of our knowledge, the use of SPHK1 expression status to predict the development of distant metastasis has not been previously reported in patients with CRC. Our data suggest that immunostaining for SPHK1 provides clinically useful information for patients with CRC, and that SPHK1 expression status is a robust identifier of patients at high risk of developing distant metastasis.

We further observed that CRC patients whose tumors showed high SPHK1 expression had shorter OS than patients with low SPHK1-expressing tumors. Our results are consistent with those of a recent study that showed that increased SPHK1 expression is associated with shorter OS rates in mice with human colon cancer xenografts (32). The prognostic significance of SPHK1 has been documented in several types of human malignancies, including breast cancer, esophageal cancer, gastric cancer, glioblastoma, and head and neck cancer (12, 13, 16, 35, 36); all these studies suggested that patients with high SPHK1-expressing tumors had shorter survival periods than patients with low SPHK1 expression. Although the conventional staging system successfully grades patients with respect to their prognoses according to clinicopathological characteristics, it does not provide critical information that may influence treatment strategy. As such, many potential biomarkers have been investigated to overcome the limitations of the conventional system, and have been found to be predictive of prognoses. However, reliable biomarkers that can stratify patients with CRC are relatively scarce. As such, SPHK1 overexpression can be used as a novel predictor of poor outcome in patients with CRC.

In conclusion, SPHK1 expression was significantly elevated in CRC compared to normal colorectal tissue, and an up-regulated SPHK1 expression was significantly associated with aggressive CRC tumor behavior. High SPHK1 expression was also found to be an independent predictor of distant metastasis. Elevated SPHK1 expression potentially promotes tumor development, progression, and metastasis of CRC; as such, SPHK1 expression status may serve as a predictor of distant metastasis and patient outcomes.

Author's Contributions

All Authors were responsible for: substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data, drafting the manuscript, revising the manuscript critically for important intellectual content, and final approval of the version to be published.

Conflicts of Interest

None of the Authors have any conflicts of interest to declare regarding this study.

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