WALAILAK JOURNAL
http://wjst.wu.ac.th Health Sciences

Sphingosine 1-Phosphate Receptor 4 Expression in Colorectal Cancer Patients

Suchittra SAMUHASANEETO[1,*], Suthiporn PROMAGSORN[2] and Chuchard PUNSAWAD[1]

[1]School of Medicine, Walailak University, Nakhon Si Thammarat 80161, Thailand
[2]Department of Pathology, Maharat Nakhon Si Thammarat Hospital, Nakhon Si Thammarat 80000, Thailand

([*]Corresponding author's e-mail: ssuchitt@wu.ac.th)

Received: 21 February 2016, Revised: 20 May 2016, Accepted: 9 June 2016

Abstract

The aim of this study was to investigate the expression of S1PR4 in colorectal cancer and its correlation with clinicopathologic characteristics. Paraffin-embedded colorectal cancer samples and matched adjacent normal tissues were collected from 32 patients at Maharat Nakhon Si Thammarat Hospital. The expression of SphK1 and S1PR4 in colorectal cancer and matched normal tissues were determined by immunohistochemistry and compared with the clinicopathologic characteristics for each of the 32 colorectal cancer patients. There were 20 males (62.50 %) and 12 (37.50 %) females. The mean age was 59.66±12.66 years. The majority of tumors were moderately differentiated (62.50 %). A high level of SphK1 and S1PR4 protein expression was observed in tumor samples compared with adjacent normal mucosa (P = 0.005 and P = 0.02, respectively). The neoplastic cells showed positive cytoplasmic staining expression for both SphK1 and S1PR4 proteins. The distribution of these 2 proteins in colorectal cancer patients showed that neither were significantly correlated to clinicopathologic features such as sex, age, pathological stage, T stage, N stage, M stage, tumor differentiation, tumor location or tumor size. It was found that S1PR4 was overexpressed in colorectal cancer and may influence tumor progression, however, there was no correlation between S1PR4 expression and clinicopathologic characteristics.

Keywords: Sphingosine 1-phosphate receptor 4, Sphingosine-1-phosphate, sphingosine kinase 1, colorectal cancer

Introduction

Colorectal cancer is the third most common cancer in the world [1]. The highest incidence of colorectal cancer is in developed countries such as the USA, Canada, Australia and Europe. However, the incidence and mortality of colorectal cancer are rapidly rising in several countries in Asia [2-4]. The incidence of colorectal cancer in Thailand is the third most common cancer in males and fourth in females [5]. Survival of colorectal cancer patients is directly correlated with the tumor stage at the time of diagnosis. When colorectal cancer is detected at a localized stage, the 5-year survival rate is 90.3 % and drops to 12.5 % if the cancer has spread to distant organs [6]. The tumor stage, predictive and prognostic markers are important indicators in colorectal cancer patients. Additionally, studies of genes and protein changes in cancer have paved the way for the development of new drugs for cancer therapy.

Sphingosine-1-phosphate (S1P), a bioactive lipid, is catalyzed by sphingosine kinase. There are 2 isoforms of sphingosine kinase (SphK1 and SphK2) which differ in their subcellular localisations, regulation and function [7]. S1P exerts its action via binding to G protein-coupled S1P receptors (S1PR), termed S1PR1-5 or specific intracellular target protein. A role for S1P in cancer is evident from studies demonstrating increased SphK1 expression in many different tumor types such as breast cancer, lung

cancer, salivary gland carcinoma, esophageal carcinoma and colorectal cancer [8-12]. SphK1 regulates tumor cell proliferation, apoptosis and invasion in colon cancer [13]. S1PR4 is also involved in cancer progression. For instance, overexpression of S1PR4 in breast cancer patients is associated with shorter disease-free and disease-specific survival [8]. High S1PR4 expression in tumors of ER negative breast cancer patients is also correlated with node positive status, suggesting a role for S1PR4 in metastasis [8]. However, no study has specifically reported S1PR4 expression in colorectal cancer. In the present study, we investigate the expression of S1PR4 in colorectal cancer and its correlation with clinicopathologic characteristics.

Materials and methods

Study protocol

This study is a retrospective design. Patients with a histopathologically confirmed diagnosis of colorectal cancer during January to December 2013 from Maharat Nakhon Si Thammarat Hospital, in Southern Thailand were eligible. A total of 32 patients, consisting of 20 males (62.50 %) and 12 (37.50 %) females, with primary tumor resection who had been classified using tumor node metastasis (TNM) staging system [14] were enrolled in the study. The mean age was 59.66±12.66 years (youngest patient being 39 years and oldest being 82 years of age). The clinicalpathological characteristics of patients are listed in **Table 1**. Most of the patients (43.75 %) exhibited pathological stage IIIB. The majority of tumors were moderately differentiated (62.50 %). Four patients had metastases at the time of diagnosis, 2 had liver metastases and 2 had liver and lung metastases. In most cases, the tumor was localized in the sigmoid colon (48.87 %). The study protocol was reviewed and approved by the Ethics Committee of Walailak University (Protocol No.14/002).

Immunohistochemical staining

The presence of SphK1 and S1PR4 in paraffin-embedded tumor samples and adjacent normal mucosal tissues was investigated using immunohistochemical staining. Tissue sections (4 μm thick) were deparaffinized with xylene and rehydrated with graded alcohol. Then, antigen was retrieved in Tris-EDTA buffer (pH 9.0) using a microwave. The endogenous peroxidase was inactivated with 3 % hydrogen peroxide for 5 min at room temperature. After washing the sections in Tris-buffered saline (pH 7.6), the nonspecific binding site was blocked with protein block (EXPOSE Mouse and Rabbit Specific HRP/DAB Detection IHC kit ab80436; Abcam plc, UK). The sections were incubated with the primary antibody. Anti-SphK1 antibody (ab61148; Abcam plc, UK) was used at a dilution of 1/200 and incubated at 25 °C for 60 min and anti-S1PR4 antibody (ab150512; Abcam plc, UK) was used at a dilution of 1/200 and incubated at 4 °C overnight. Then, they were incubated with Goat anti-rabbit HRP conjugate (EXPOSE Mouse and Rabbit Specific HRP/DAB Detection IHC kit ab80436; Abcam plc, UK) for 15 min at room temperature. Subsequently, diaminobenzidine (DAB) chromogen solution was applied for 5 min. After counterstaining with Hematoxylin, the sections were dehydrated and mounted with a coverslip.

Evaluation of SphK1 and S1PR4 immunostaining

Each immunostained section was randomly counted in 5 microscopic fields under a high-power field (×400) microscope. The percentage of labeled cells was graded as follows: grade 0, no positive cells; grade 1, 1 - 25 % positive cells; grade 2, 25 - 50 % positive cells; grade 3, > 50 % positive cells. The staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong) [15]. Finally, the total score was calculated by multiplying the grade by the intensity, with a maximum score of 9. The immunohistochemistry was evaluated by 2 independent observers.

Statistical analysis

All statistical analyses were performed using the SPSS 17.0 statistical software. Descriptive values of study data were provided as number and percentage. Differences in expression between normal mucosa and tumor were tested using the Wilcoxon signed-rank test. Correlation between the expression of SphK1, S1PR4 and clinical data were performed using a Chi-square test. Statistical significance was determined at P-value < 0.05.

Results and discussion

Expression of SphK1 and S1PR4 proteins were detected in tumors and adjacent normal mucosa in all 32 patients. Both SphK1 and S1PR4 showed weak cytoplasmic staining in the normal sample mucosa (**Figures 1A** and **1C**). The neoplastic cells showed diffusely positive cytoplasmic staining for SphK1 (**Figure 1B**) and faint, focally positive cytoplasmic staining of S1PR4 (**Figure 1D**). This revealed a high level of SphK1 and S1PR4 expression in the tumor samples compared with adjacent normal mucosa (P = 0.005 and P = 0.02, respectively; **Table 2**).

The distribution of expression in colorectal cancer patients showed that SphK1 and S1PR4 were not significantly correlated to any clinicopathologic features including sex, age, pathological stage, T stage, N stage, M stage, tumor differentiation, tumor location or tumor size.

Several previous studies have reported a role of SphK1 in carcinogenesis. SphK1 is upregulated in breast cancer, esophageal carcinoma, cholangiocarcinoma, uterine cervical cancer, adrenocortical carcinoma and colorectal cancer [8,11-13,16-19]. Elevation of SphK1 in cancer suggests a potential prognostic application. SphK1 expression correlates with poor prognosis in various types of cancers [16-18,20].

SphK1 expression is functionally associated to cell proliferation and invasion in several types of cancer. For instance, SphK1 expression was overexpressed at transcription and protein level in cholangiocarcinoma, uterine cervical cancer and adrenocortical carcinoma [16-18]. Further analysis of human colon cancer demonstrates that SphK1 promotes tumor cell proliferation, apoptosis and invasion by regulating the focal adhesion kinase pathway, adhesion molecules, MAPK pathways and nuclear factor-κB p65 [13,21,22]. Our study found overexpression of SphK1 in colorectal cancer patients. This is consistent with previous data [12,13,22], suggesting that SphK1 plays an important role in tumor progression. Recent studies have demonstrated that knocked down SphK1 and SphK1 inhibitors inhibited cancer cell proliferation [16,17,23,24]. Thus, SphK1 gene or SphK1 inhibitors could be a useful target for cancer treatment.

Liu *et al*. found high SphK1 expression in 66 colon cancer patients correlated with tumor differentiation, lymph node metastasis and distance [13]. Another study of 303 colon cancer cases showed that the presence of SphK1 expression correlated with the pathological stage [24]. In our study, SphK1 expression was evaluated in 32 colon cancer patients. However, we did not find statistically significant associations of SphK1 expression with clinicopathologic characteristics, this may have been due to the small sample size.

S1PR4 has been shown to have an important role to induce cell migration in breast cancer [8,26]. Long *et al*. reported that S1PR4 induced cell migration in MDA-MB-453 breast cancer cells [26]. High tumor expression of S1PR4 and SphK1 are associated with shorter disease-specific survival and disease recurrence times in patients with estrogen receptor-negative breast cancer [8]. In addition, high S1PR4 expression in tumors is also correlated with node positive status, suggesting a role for S1PR4 in metastasis [8]. Our study is the first to investigate the significance of S1PR4 expression in colorectal cancer patients and the finding that S1PR4 is overexpressed suggests it may influence tumor progression. In the present study, we did not observe a significant correlation of S1PR4 expression and clinicopathologic characteristics, but the power for detection was low considering that the sample size was limited. A larger group of patients is needed to definitely address any association between S1PR4 expression and cancer progression.

Table 1 Clinicalpathological characteristics of 32 colorectal cancer patients.

Characteristics	Number of patients (%)
Sex	
Male	20 (62.50)
Female	12 (37.50)
Age (years)	
< 50	9 (28.12)
≥ 50	23 (71.88)
Pathological stage (TNM)	
I	2 (6.25)
IIA	8 (25.00)
IIIB	14 (43.75)
IIIC	4 (12.50)
IVA	2 (6.25)
IVB	2 (6.25)
T stage	
T2	2 (6.25)
T3	28 (87.50)
T4b	2 (6.25)
N stage	
N0	10 (31.25)
N1a+N1b+N2a+N2b	22 (68.75)
M stage	
M0	28 (87.50)
M1a+M1b	4 (12.50)
Tumor differentiation	
Well	10 (31.25)
Moderately	20 (62.50)
Poorly	2 (6.25)
Tumor location	
Ascending colon	1 (3.12)
Transverse colon	1 (3.12)
Descending colon	2 (6.25)
Sigmoid colon	15 (48.87)
Rectum	11 (34.37)
Other	2 (6.25)
Tumor size	
< 5 cm	7 (21.88)
≥ 5 cm	25 (78.12)

Note: TNM is Tumor Node Metastasis

Table 2 Score of SphK1 and S1PR4 expression in 32 colorectal cancer patients

Score	SphK1			S1PR4		
	Tumor (%)	Normal mucosa (%)	P-value	Tumor (%)	Normal mucosa (%)	P-value
3	14 (43.75)	26 (81.25)	0.005	21 (65.63)	28 (87.50)	0.02
6	18 (56.25)	6 (18.75)		11(37.37)	4 (12.50)	

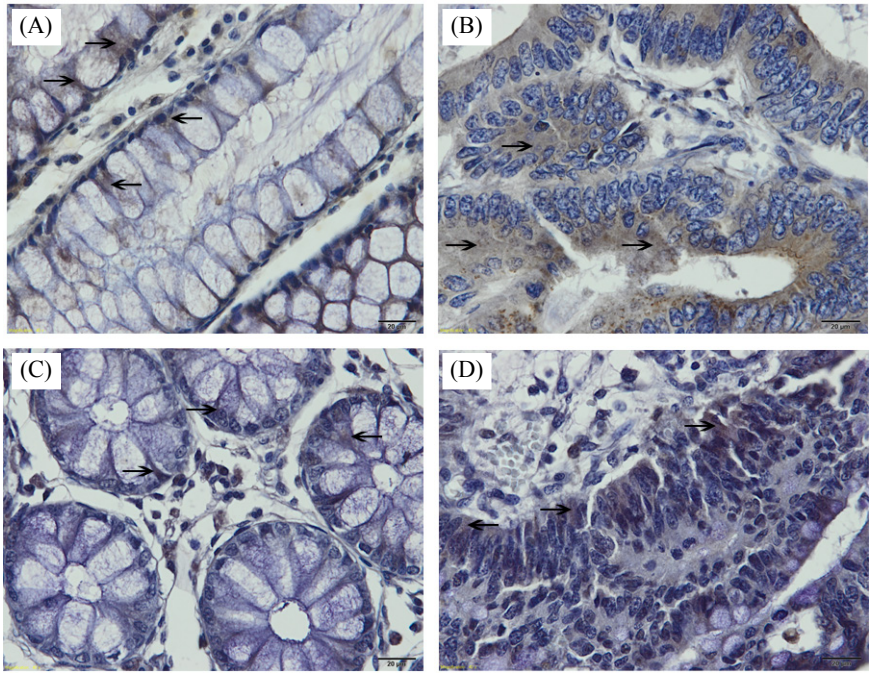

Figure 1 Immunohistochemistry staining of SphK1 and S1PR4 (×400 magnification). (A) SphK1 and (C) S1PR4 showed weak cytoplasmic staining (arrows) in a normal colonic mucosa patient. (B) SphK1 and (D) S1PR4 showed diffusely positive cytoplasmic staining (arrows) in a colorectal cancer patient.
Note: Pathological stage of patient in **Figure 1** was IIA.

Conclusions

In summary, overexpression of S1PR4 was found in colorectal cancer more often than would be expected by chance suggesting it may influence tumor progression. However, there was no correlation between S1PR4 expression and clinicopathologic characteristics detected in this set of samples. Further studies with a larger number of patients might provide a clearer understanding of this issue.

Acknowledgements

This research was financially supported by the Institute of Research and Development, Walailak University.

References

[1] J Ferlay, I Soerjomataram, M Ervik, R Dikshit, S Eser, C Mathers, D Forman and F Bray. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int. J. Cancer.* 2015; **136**, E359-E386.

[2] CJ Chen, SL You, LH Lin, WL Hsu and YW Yang. Cancer epidemiology and cancer control in Taiwan: a brief review. *Jpn. J. Clin. Oncol.* 2002; **32**, S66-S81.

[3] JJ Sung, JY Lau, KL Goh and WK Leung. Increasing incidence of colorectal cancer in Asia: Implications for screening. *Lancet. Oncol*. 2005; **6**, 871-6.

[4] J Ferlay, HR Shin, F Bray, D Forman, C Mathers and DM Parkin. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int. J. Cancer* 2010; **127**, 2893-917.

[5] W Imsamram, A Chaiwerawattana, S Wiangnon, D Pongnikorn, K Suwanrungrung, S Sangrajrang and R Buasom. *Cancer in Thailand*. Vol VIII. New Thammada Press, Bangkok, 2015, p. 50.

[6] CE DeSantis, CC Lin, AB Mariotto, RL Siegel, KD Stein, JL Kramer, R Alteri, AS Robbins and A Jemal. Cancer treatment and survivorship statistics, 2014. *CA. Cancer J. Clin.* 2014; **64**, 252-71.

[7] S Pyne, SC Lee, J Long and NJ Pyne. Role of sphingosine kinases and lipid phosphate phosphatases in regulating spatial sphingosine 1-phosphate in health and disease. *Cell Signal* 2009; **21**, 14-21.

[8] J Ohotski, JS Long, C Orange, B Elsberger, E Mallon, J Doughty, S Pyne, NJ Pyne and J Edwards. Expression of sphingosine 1-phosphate receptor 4 and sphingosine kinase 1 is associates with outcome in oestrogen receptor-negative breast cancer. *Br. J. Cancer* 2012; **106**, 1453-9.

[9] KR Johnson, KY Johnson, HG Crellin, B Ogretmen, AM Boylan, RA Harley and LM Obeid. Immunohistochemical distribution of sphingosine kinase 1 in normal and tumor lung tissue. *J. Histochem. Cytochem.* 2005; **53**, 1159-66.

[10] G Liu, H Zheng, Z Zhang, Z Wu, H Xiong, J Li and L Song. Overexpression of sphingosine kinase 1 is associated with salivary gland carcinoma progression and might be a novel predictive marker for adjuvant therapy. *BMC Cancer* 2010; **10**, 495-506.

[11] J Pan, YF Tao, Z Zhou, BR Cao, SY Wu, YL Zhang, J Wang, GL Lou, Z Li, X Feng and J Ni. A novel role of sphingosine kinase-1 (SPHK1) in the invasion and metastasis of esophageal carcinoma. *J. Trans. Med.* 2011; **9**, 157-71.

[12] T Kawamori, T Kaneshiro, M Okumura, S Maalouf, A Uflacker, J Bielawski, YA Hannun and LM Obeid. Role for sphingosine kinase 1 in colon carcinogenesis. *FASEB J.* 2009; **23**, 405-14.

[13] SQ Liu, YJ Su, MB Qin, YB Mao, JA Huang and GD Tang. Sphingosine kinase 1 promotes tumor progression and confers malignancy phenotypes of colon cancer by regulating the focal adhesion kinase pathway and adhesion molecules. *Int. J. Oncol.* 2013; **42**, 617-26.

[14] SB Edge, DR Byrd, CC Compton, AG Fritz, FL Greene and A Trotti. *AJCC Cancer Staging Handbook.* 7th ed. Lippinocott Raven Publishers, Philadelphia, 2010, p. 173-4.

[15] JP Spano, C Lagorce, D Atlan, G Milano, J Domont, R Benamouzig, A Attar, J Benichou, A Martin, JF Morere, M Raphael, F Penault-Llorca, JL Breau , R Fagard, D Khayat and P Wind. Impact of EGFR expression on colorectal cancer patient prognosis and survival. *Ann. Oncol.* 2005; **16**, 102-8.

[16] MH Chen, CC Yen, CT Cheng, RC Wu, SC Huang, CS Yu, YH Chung, CY Liu, PMH Chang, Y Chao, MH Chen, YF Chen, KC Chiang, TS Yeh, TC Chen, CYF Huang and CN Yeh. Identification of SPHK1 as a therapeutic target and marker of poor prognosis in cholangiocarcinoma. *Oncotarget* 2015; **6**, 23594-608.

[17] HS Kim, G Yoon, JY Ryu, YJ Cho, JJ Choi, YY Lee, TJ Kim, CH Choi, SY Song, BG Kim, DS Bae and JW Lee. Sphingosine kinase 1 is a reliable prognostic factor and a novel therapeutic target for uterine cervical cancer. *Oncotarget* 2015; **6**, 26746-56.

[18] S Pyne, J Edwards, J Ohotski and NJ Pyne. Sphingosine 1-phosphate receptors and sphingosine kinase 1: Novel biomarkers for clinical prognosis in breast, prostate, and hematological cancers. *Front. Oncol.* 2012; **2**, 1-5.

[19] Y Xu, B Dong, J Huang, W Kong, W Xue, Y Zhu, J Zhang and Y Huang. Sphingosine kinase 1 is overexpressed and promotes adrenocortical carcinoma progression. *Oncotarget* 2016; **7**, 3233-44.

[20] W Li, CP Yu, JT Xia, L Zhang, GX Weng, HQ Zheng, QL Kong, LJ Hu, MS Zeng, YX Zeng, M Li, J Li and LB Song. Sphingosine kinase 1 is associated with gastric cancer progression and poor survival of patients. *Clin. Cancer Res.* 2009; **15**, 1393-9.
[21] SQ Liu, JA Huang, MB Qin, YJ Su, MY Lai, HX Jiang and GD Tang. Sphingosine kinase 1 enhances colon cancer cell proliferation and invasion by upregulation the production of MMP-2/9 and uPA via MAPK pathways. *Int. J. Colorectal Dis*. 2012; **27**, 1569-78.
[22] YJ Su, JA Huang, SQ Liu, JX Huang, YY Zhong, GD Tang and HX Jiang. The expression and clinical significance of SphK1 and nuclear factor-kB p65 in human colon carninoma. *Zhonghua. Nei. Ke. Za. Zhi.* 2012; **51**, 220-4.
[23] J Long, Y Xie, J Yin, W Lu and S Fang. SphK1 promotes tumor cell migration and invasion in colorectal cancer. *Tumor. Biol.* 2016; **37**, 6831-6.
[24] T Ju, D Gao and ZY Fang. Targeting colorectal cancer cells by a novel sphingosine kinase 1 inhibitor PF-543. *Biochem. Biophys. Res. Commun.* 2016; **470**, 729-34.
[25] SSL Tan, LW Khin, L Wong, B Yan, CW Ong, A Datta, M Salto-Tellez, Y Lam Y and CT Yap. Sphingosine kinase1 promotes malignant progression in colon cancer and independently predicts survival of patients with colon cancer by competing risk approach in south Asian population. *Clin. Trans. Gastroenterol.* 2014; **5**, e51.
[26] JS Long, Y Fujiwara, J Edwards, CL Tannahill, G Tigyi, S Pyne and NJ Pyne. Sphingosine 1-phosphate receptor 4 use HER2(ERBB2) to regulate extracellular signal regulated kinase-1/2 in MDA-MB-453 breast cancer cells. *J. Biol. Chem.* 2010; **285**, 35957-66.