

Original Article

Relationship of vasculogenic mimicry, SphK1 expression, and Cx43 expression to metastasis and prognosis in colorectal cancer

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Abstract: Objective: To determine the presence of vasculogenic mimicry (VM) and expression of Sphingosine kinase 1 (SphK1) and Connexin43 (Cx43) in colorectal cancer (CRC) tissues, and to identify their inter-relationships and associations with multiple pathologic parameters. Methods: Ninety-two CRC specimens and normal pericarcinoma tissues were analyzed for expression of SphK1 and Cx43 using immunohistochemistry, and for identification of VM using CD34-periodic acid-Schiff dual staining. Results: The positive rate of SphK1 expression was greater in CRC cells than pericarcinoma cells (85.87% vs. 33.70%, $P < 0.05$). In contrast, the positive rate of Cx43 expression was greater in pericarcinoma cells than in CRC cells (58.70% vs. 92.39%, $P < 0.05$). Analysis of CRC tissues indicated that expression of SphK1 was associated with poor differentiation, advanced tumor stage, lymph node metastasis, and the presence of VM ($P < 0.05$ for each comparison). Expression of Cx43 was associated with high differentiation and the presence of VM ($P < 0.05$ for each comparison). Patient sex, age, tumor size, depth of invasion, and distant metastasis were unrelated to the expression of either protein. There was a significant correlation between the expression of SphK1 and Cx43 ($P < 0.05$). Analysis of overall patient survival indicated that SphK1 positivity and the presence of VM were significantly associated with poor survival, but Cx43 positivity had no relationship with survival. Conclusion: SphK1 protein expression was significantly greater in CRC tissues than pericarcinoma tissues, suggesting this protein may be associated with the pathogenesis of CRC. In addition, the significant correlation between expression of SphK1 and Cx43 in CRC tissues suggests their interaction may impact the pathogenesis of CRC.

Keywords: SphK1, Cx43, colorectal cancer, vasculogenic mimicry, prognosis

Introduction

Colorectal cancer (CRC) is the third-leading cause of cancer-related deaths, and will cause an estimated 50,630 deaths in the United States during 2018 [1]. Thus, despite the implementation of aggressive screening procedures to improve early detection and our extensive knowledge of critical events in the pathogenesis of CRC, this cancer continues to be a major health concern in developed countries.

Sphingolipids, especially ceramide, sphingosine, ceramide-1-phosphate, and sphingosine-1-phosphate (S1P), play key roles in the pathogenesis of cancer [2]. In particular, S1P promotes cell proliferation and survival and regulates angiogenesis, whereas sphingosine and

ceramide inhibit cell proliferation and stimulate apoptosis. Sphingosine kinase (SphK) phosphorylates sphingosine to form S1P, and is a critical regulator of sphingolipid-mediated functions [3]. Growing evidence indicates that SphK1 plays a pivotal role in the pathogenesis of CRC [4-8], although the mechanisms are still unclear.

Previous studies suggested that connexins (gap junction proteins) are frequently down-regulated or not assembled into identifiable gap junctions during the early stages of many cancers, although more recent research indicated that connexins can be up-regulated during the later phases of metastasis [9]. In addition, accumulating evidence suggests that connexins may facilitate invasion, intravasation,

Table 1. Expression of SphK1 and Cx43 in CRC tissues and pericarcinoma (normal) tissues

Protein	Result	CRC tissues	Pericarcinoma tissues	χ^2	P^a
SphK1	Positive	79	31	52.081	0.000
	Negative	13	61		
Cx43	Positive	54	85	28.269	0.000
	Negative	38	7		

^aChi square tests indicated that SphK1 positivity was significantly higher in CRC tissues, and Cx43 positivity was significantly lower in CRC tissues.

extravasation, and metastasis in a variety of cancers [10-13]. Cx43 is the best known and studied of the 21 connexins. This connexin is highly expressed in epithelial cells, hematopoietic cells, neurons and astrocytes, cardiac neuronal crest cells, and fibroblasts [14]. Recent reports suggest that Cx43 plays multiple roles during the different stages of tumor progression [15, 16]. In particular, Cx43 downregulation is associated with cancer phenotype [17, 18]. However, the presence of aberrant Cx43 upregulation in advanced carcinomas suggests that cancer cells may communicate with normal host cells across hemichannels, thus suggesting that Cx43 has a role in the migration and growth of metastatic cells. However, it is unknown whether Cx43 level is associated with patient outcome or can be used as a marker of cancer prognosis.

Vasculogenic mimicry (VM) is a newly described process in which highly aggressive tumor cells form fluid-conducting channels that supply nutrition to tumor cells [19]. It is now believed that VM plays an essential role in the pathology of a wide variety of human cancers, including carcinomas, sarcomas, glioblastomas, astrocytomas, and melanomas [8, 19-21]. The development of VM strongly correlates with poor prognosis in a number of cancers, including osteosarcoma [7], gastric cancer, [21] and ovarian cancer [22]. However, little is known about the role of VM in CRC.

Protein internalization, lysosomal or proteasomal degradation, and other molecular mechanisms that cause post-translation modifications can regulate the expression of Cx43 [23]. In addition, various kinases can initiate several subsequent events, such as rapid gap-junctional intercellular communication (GJIC), down-regulation or internalization of Cx43, and Cx hexamer stabilization [24, 25]. A recent study re-

ported that S1P, a bioactive sphingolipid mainly produced by SphK1 metabolism, can regulate Cx43 protein expression and GJIC in skeletal muscle cells [26]. Thus, we hypothesized that SphK1 may also regulate Cx43 during tumor progression. Therefore, in the present study, we assessed the expression of SphK1 and Cx43 in CRC and pericarcinoma tissues, their associations with clinicopathological characteristics (especially VM formation), and their inter-relationships.

Materials and methods

Patients and tissue specimens

Patients with CRC who received surgical resection at the First Affiliated Hospital of Guangxi Medical University (Nanning) from March 2013 to November 2013 were prospectively recruited for this study. Each patient had a diagnosis of CRC based on clinicopathological characteristics, and none of them received radiotherapy or adjuvant chemotherapy before surgery. All patients were followed up until death or when last seen alive at a clinical visit during March 2018 (median follow-up time: 54 months). A total of 92 paraffin-embedded CRC tissues and 92 adjacent normal epithelial tissues were collected for immunohistochemistry (IHC) and CD34/periodic acid Schiff (PAS) double staining. The study protocols were approved by Ethics Committee of First Affiliated Hospital of Guangxi Medical University. Each patient signed a written informed consent agreement before surgery.

IHC procedures

The formalin-fixed paraffin-embedded tissue samples were cut into 4 μ m sections, and then baked at 60°C for 30 min. The slides were then dewaxed in xylene, rehydrated in ethanol, and then incubated in 3% hydrogen peroxide to inhibit endogenous peroxidase activity. Next, the slides were boiled in antigen-retrieval EDTA buffer for 10 min. After blocking with goat serum, the slides were incubated with rabbit polyclonal antibody against Cx43 (1:500, Santa Cruz) and rabbit monoclonal antibody against SphK1 (1:700, Sigma), and then incubated at 4°C overnight. After washing with phosphate-buffered saline (PBS), the sections were incubated with a second antibody for 30 min, then washed again with PBS. Finally, the sections

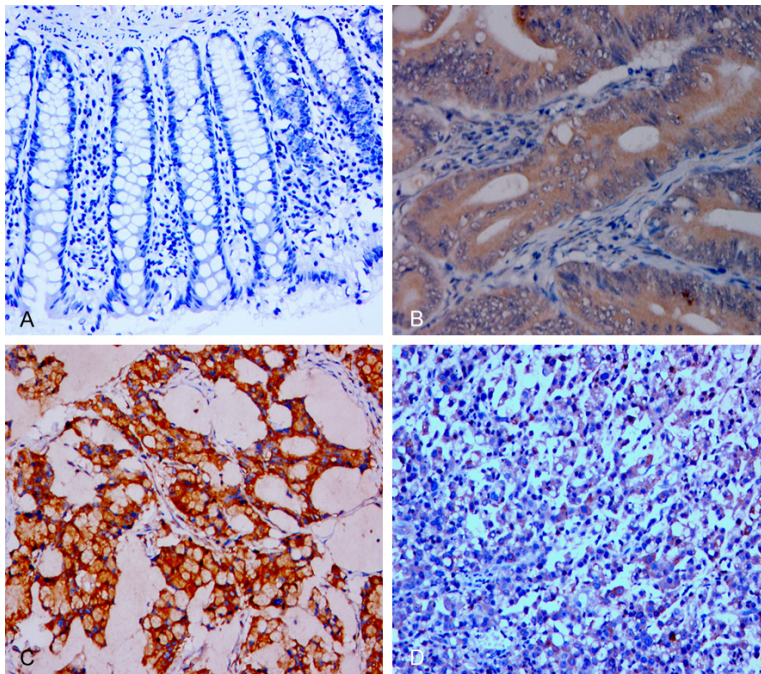


Figure 1. IHC analysis of SphK1 expression in representative pericarcinoma (normal) and CRC tissues ($\times 400$). A. No expression in pericarcinoma colorectal cells; B. Positive expression in the cytoplasm of colorectal tubular adenocarcinoma cells; C. Positive expression in colorectal mucinous adenocarcinoma cells; D. Positive expression in the cytoplasm of undifferentiated colorectal carcinoma cells.

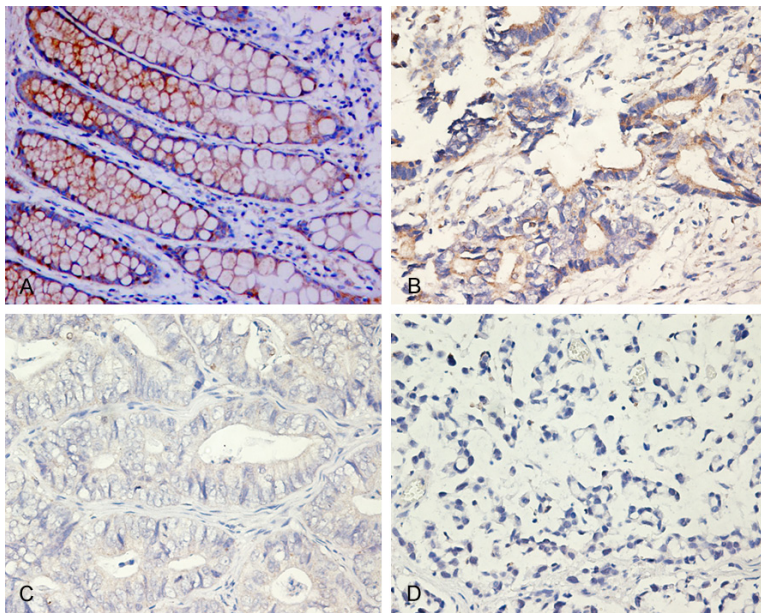


Figure 2. IHC analysis of Cx43 expression in representative pericarcinoma (normal) and CRC tissues ($\times 400$). A. Positive expression in the membrane and cytoplasm of pericarcinoma colorectal cells; B. Low expression in the membrane and cytoplasm of well-differentiated colorectal adenocarcinoma cells; C. Low expression in the membrane and cytoplasm of moderately differentiated colorectal adenocarcinoma cells; D. No expression in poorly differentiated colorectal adenocarcinoma cells.

were visualized by addition of a diaminobenzidine solution, and counterstained with hematoxylin. Negative controls were performed by incubation with rabbit IgG instead of the primary antibody.

Evaluation of IHC staining

Brown-yellow staining was considered as positive. For each section, 10 visual fields were randomly examined under a microscope (Olympus, Japan) at $400\times$, and 200 CRC cells were counted in each visual field. The percentage of positive cells in each field was used for scoring. Thus, the cut-off was 10% malignant positive cells for separation between positive and negative results. A four-grade scale was applied: “-” indicated less than 10% staining; “+” indicated 10 to 50% staining; “++” indicated 50 to 75% staining; and “+++” indicated 75% or more staining.

CD34/periodic acid Schiff (PAS) double staining.

CD34/PAS double staining was also performed to detect VM formation in the paraffin-embedded sections. First, IHC staining for CD34 using a mouse monoclonal antibody (1:50; cat. no. ZM-0046; Zhongshan Goldenbridge, Beijing, China), was performed to detect endothelial cells. Then, sections were washed in running water for 1 min and incubated with PAS for 30 min to detect the basement membrane of tubular structures. VM is indicated by the presence of tubular structures containing red blood cells, with PAS staining of the basement membrane, that are surrounded by tumor

Table 2. Correlation between the expression of SphK1 and Cx43 in CRC tissues

Expression	SphK1 expression					Total	r_s	P value
	+++	++	+	-				
Cx43	+++	10	0	0	1	92	0.595	0.000
	++	11	1	0	0			
	+	14	8	6	3			
	-	4	11	14	9			

r_s : Spearman's rank correlation coefficient.

cells with no CD34 staining. The presence of 1 or more red blood cells in the tubular structure was needed for identification of VM.

Statistical analysis

The significance of the relationships between SphK1 expression, Cx43 expression, and clinicopathological characteristics were determined using the Pearson chi-square test or Fisher's exact test. Survival curves were evaluated by the Kaplan-Meier method, and compared using the log-rank test. Spearman rank correlation analysis was used to assess the association between the expressions of different proteins. Statistical analysis was performed using SPSS software 17.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered significant.

Results

Clinicopathological features of patients

The median age of the 92 CRC patients was 56 years (range: 25 to 83 years), and there were 56 males and 36 females. A total of 78 patients had tubular or papillary adenocarcinoma, 13 had mucinous adenocarcinoma, and 1 had signet-ring cell carcinoma. Cancer cells were highly differentiated in 3 patients, moderately differentiated in 68 patients, and poorly differentiated in 21 patients. Thirty-nine patients had lymph node metastases, 17 patients had distant metastases, and 29 patients had evidence of VM. The median follow-up time was 54 months (range: 3 to 60 months).

SphK1 and Cx43 expression in normal and malignant colorectal tissue

We performed IHC to determine SphK1 and Cx43 expression in colorectal tissue samples and matched pericarcinoma (normal) tissue

samples. The expression of SphK1 was predominantly cytoplasmic in tumor cells and pericarcinoma epithelial cells (**Table 1; Figure 1**). In particular, 31 of 92 normal epithelial cells (34%) expressed SphK1, but 79 of 92 cancer cells (86%) expressed SphK1. Cx43 expression in normal epithelia was in the membranes and cytoplasmic regions (**Table 1; Figure 2**). The opposite trend occurred for Cx43, in that 85 of 92 normal epithelial cells (92%) expressed Cx43, but 54 of 92 cancer cells

(58.7%) expressed Cx43. Most examined cells only had cytoplasmic expression of this protein (**Figure 2A-D**), although mixed staining (cytoplasmic and membranous) occurred in a few cases (**Figure 2B, 2C**). Interestingly, Spearman rank correlation analysis indicated a significant positive correlation between expression of SphK1 and Cx43 in CRC cells ($r = 0.595$, $P < 0.01$) (**Table 2**).

Clinical and pathological implications of SphK1 and Cx43 expression in CRC

We also examined the correlations of multiple clinicopathologic characteristics with the expression of SphK1 and Cx43 in CRC cells (**Table 3; Figure 3**). SphK1 expression was significantly and positively associated with poorly differentiated cells ($P = 0.041$), more advanced T stage ($P = 0.009$), lymph node metastases ($P = 0.033$), and the presence of VM ($P = 0.020$). Notably, there was no significant association of SphK1 expression with distant metastasis ($P = 0.142$), but all 17 colon cancer tissues with distant metastasis tested positive for expression of SphK1. Cx43 expression was greater in more highly differentiated cells ($P < 0.001$) and was associated with the presence of VM ($P = 0.023$). There were no significant relationships of sex, age, tumor size, depth of invasion, and distant metastases with the expression of Cx43 or SphK1 (**Table 3; Figure 3**).

Association of SphK1 expression, Cx43 expression, and the presence of VM with clinical outcome

The Kaplan-Meier survival analysis indicated that overall survival was worse for patients with SphK1 positivity ($P = 0.0443$) and with the presence of VM in colorectal tumor infiltrates ($P = 0.0388$) (**Figure 4A, 4C**). However, Cx43 posi-

Vasculogenic mimicry, SphK1 and Cx43 in colorectal cancer

Table 3. Relationship of SphK1 and Cx43 expression with clinical and pathological characteristics of patients with CRC

Groups	Total	SphK1				Cx43			
		Positive (n)	Positive (%)	χ^2	P	Positive (n)	Positive (%)	χ^2	P
Sex									
Male	56	46	82.1	1.638	0.201	32	57.1	0.142	0.706
Female	36	33	91.7			22	61.1		
Age									
≤ 50	32	30	93.8	1.614	0.204	18	56.2	0.121	0.728
> 50	60	49	81.7			36	60.0		
Tumor diameter									
< 5 cm	41	36	87.8	0.228	0.633	28	68.3	2.810	0.094
≥ 5 cm	51	43	84.3			26	51.0		
Tumor Differentiation									
High	3	1	33.3	6.308	0.041	2	66.7	13.525	0.000
Moderate	68	58	85.3			47	69.1		
Low	21	20	95.2			5	23.8		
Tumor stage									
T1/T2	47	36	76.6	6.811	0.009	28	59.6	0.031	0.861
T3/T4	45	43	95.6			26	57.8		
Lymph node metastasis									
Yes	39	37	94.9	4.521	0.033	23	59.0	0.002	0.963
No	53	42	79.2			31	54.5		
Invasion depth									
Mucosal/superficial muscularis	5	4	80.0		0.542	3	60.0		1.000
Deep muscle or full layer	87	75	86.2			51	58.6		
Distant metastasis									
Yes	17	17	100.0	2.152	0.142	12	70.6	1.217	0.270
No	75	62	82.7			42	56.0		
Vasculogenic mimicry									
Yes	29	29	100.0	5.372	0.020	22	75.9	5.148	0.023
No	63	50	79.4			32	50.8		

tivity had no significant relationship with overall survival ($P = 0.7732$, **Figure 4B**).

Discussion

Previous studies demonstrated elevated expression of SphK1 in the colons of patients with CRC, suggesting its potential use as a biomarker for CRC, and that increased expression of SphK1 was associated with poor outcomes (mainly due to metastasis to the lymph nodes, liver, and other organs) [27]. In this study, we found that SphK1 expression was significantly correlated with tumor differentiation, lymph node metastasis, and T stage, and that the expression of SphK1 was higher in CRC tissues than adjacent non-cancer tissues. Although SphK1 expression was not significantly different in patients with distant metastasis, all 17 CRC tissues with distant metastasis had ex-

pression of SphK1. Furthermore, Kaplan-Meier survival analysis showed that SphK1-positive CRC patients had significantly shorter OS than SphK1-negative patients. These findings are consistent with previous studies that examined the role of this protein in CRC [28].

Previously, Cx43 was considered as a tumor suppressor [9, 29], but there is increasing recognition that it has multiple effects in the pathogenesis of cancer. Thus, recent research suggests that Cx43 can promote cancer progression [30], angiogenesis [31], invasion [32], intravasation, extravasation, metastasis [33], and drug resistance of cancer cells [34]. Kanczuga-Koda et al. [35] used IHC analysis to demonstrate that either upregulation of Cx43 occurred as tumors progressed to an invasive phenotype, or that a dominant cell type within the tumor which expressed cytoplasmic Cx43

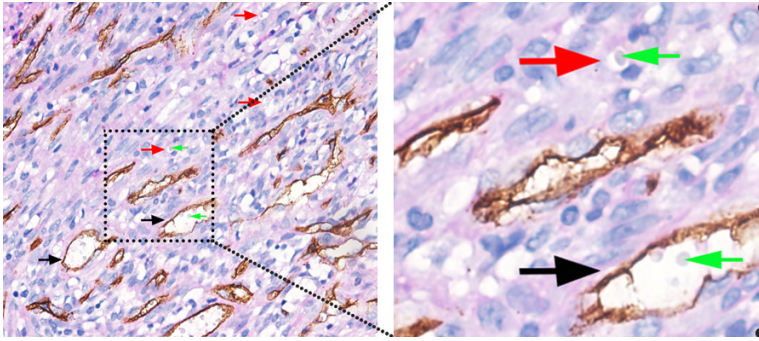


Figure 3. CD34/PAS double staining of colorectal cancer tissue samples for detection of VM. Black arrows indicate typical blood vessels, with brown CD34+ staining; green arrows indicate red blood cells; and red arrows indicate VM channels formed by tumor cells.

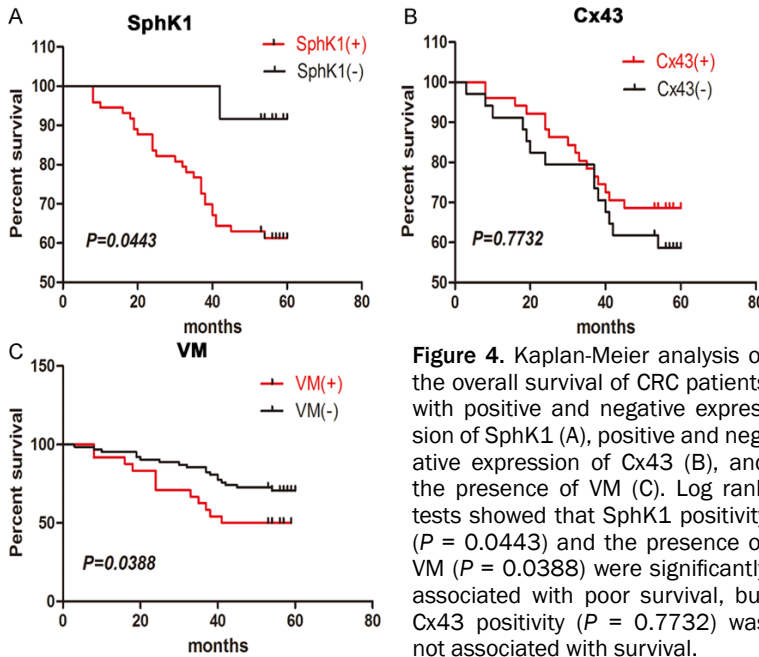


Figure 4. Kaplan-Meier analysis of the overall survival of CRC patients with positive and negative expression of SphK1 (A), positive and negative expression of Cx43 (B), and the presence of VM (C). Log rank tests showed that SphK1 positivity ($P = 0.0443$) and the presence of VM ($P = 0.0388$) were significantly associated with poor survival, but Cx43 positivity ($P = 0.7732$) was not associated with survival.

made up the bulk of the invasive tumors. Teleki et al. [36] used mRNA platforms to evaluate connexin gene expression and found that high expression of Cx43 was associated with poorer relapse-free survival (RFS) and overall survival (OS) in patients with ER-negative breast cancer. Other research reported that enhanced Cx43 expression was also associated with increased cell migration, tumor chemoresistance, and metastasis of different types of tumors [37, 38]. The role of Cx43 in cancer is tissue- and stage-specific [9, 39]. In particular, Cx43 has a role in the later stages of breast cancer progression, in that it mediates the interaction between the tumor and endothelial cells, and facilitates ad-

hesion and extravasation at secondary sites [40, 41]. Cx43 is also expressed at higher levels in lymph node metastases than in primary breast tumors [42]. Other studies reported aberrant Cx43 expression in advanced stages of prostate and colon adenocarcinomas [43, 44].

In this study, we found that Cx43 expression was significantly lower in CRC tissues than adjacent non-cancerous tissues, and that expression was inversely associated with tumor grade. Furthermore, our Kaplan-Meier analysis showed that Cx43-positive and Cx43-negative CRC patients had similar overall survival times. Our evidence thus suggests that the role of Cx43 depends on the cancer subtype, consistent with previous studies [45].

VM is the formation of a microcirculation pattern that differs from the traditional vascular system, and plays an important role in transferring blood and other nutrients to tumors. Researchers have identified VM in many solid tumors, such as melanoma, hepatocellular carcinoma, bladder cancer, and prostate cancer [46-49]. Furthermore, there is a strong correlation between VM and the presence of more malignant features of cancer, such as advanced stage or grade, poor cell differentiation, and short overall survival.

In our study, we identified VM in 29 of 92 specimens of CRC tissues (31.5%), similar to a previous study of CRC [50], and confirming the presence of VM in CRC. Moreover, our Kaplan-Meier analysis indicated that VM-positive CRC patients had significantly shorter OS than VM-negative patients ($P = 0.0388$). These findings suggest that VM could be useful as a biomarker for predicting progression and metastasis in CRC. Our results thus agree with a previous

immunohistochemical study which concluded that VM in CRC patients is associated with metastasis and poor prognosis [50].

Cx43 interacts with various proteins *via* its C-terminal region, including kinases (such as SphK1), cell-adhesion proteins, and Bax (a pro-apoptotic protein). These molecular interactions may affect the expression and function of these proteins, and thereby modulate cellular physiology. Recent studies reported that epithelial-mesenchymal transition (EMT) contributed to VM, and identified the upregulation of EMT-associated transcription factors in tumor cells that had VM [48, 51]. Mounting evidence also indicates that cancer stem cell (CSCs) can differentiate into tumor or endothelial lineages [52], as well as into vascular smooth muscle-like cells [53]. VM-engaging tumor cells have the phenotypes of endothelial and tumor tissues [54, 55], so VM may represent the incomplete progression of CSC differentiation into different endothelial lineages. Another study found that epithelial cancer cells may have the capacity of self-renewal of the stem cell phenotype *via* EMT [56]. VM allows tumor cells to express the endothelial phenotype and function as endothelial cells in forming blood vessel-like structures. In fact, both epithelial and mesenchymal markers are present in tumor cells undergoing VM [54, 57]. It is plausible that CSCs function in VM by induction of the EMT, because EMT plays a key role in tumorigenesis and in VM [48, 58, 59].

Furthermore, our previous studies showed that SphK1 modulates the expression of EMT-related markers and cell migration in CRC cells [60]. Thus, SphK1 may have a role in mediating EMT in CRC. Inoculation with CSCs overexpressing SphK1 increases the development of tumors in nude mice, relative to parent cells or CSCs that do not overexpress SphK1 [61]. Shipitsin et al. [62] studied breast tumor heterogeneity and found that Cx43 was significantly expressed in the subset of CD44+ stem-like cells, and considered it as a CD44+ specific gene. Another study found that Cx43, in addition functioning as a channel, also functioned as an adhesion site that enhanced cell aggregation, and that the adhesive effects did not require the formation of functional channels [63]. Thus, Cx43 and other connexins have multiple functions during the pathogenesis of cancer.

Overall, our findings suggest that complex associations among SphK1, VM, and Cx43 affect tumor progression and metastasis. Thus, we believe that examination of the interactions of these biomarkers could help to predict the biological behavior of CRC cells, and may allow identification of targets for novel therapeutic strategies.

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Disclosure of conflict of interest

None.

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Vasculogenic mimicry, SphK1 and Cx43 in colorectal cancer

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Vasculogenic mimicry, SphK1 and Cx43 in colorectal cancer

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