


RESEARCH PAPER



Chloride intracellular channel protein 2 in cancer and non-cancer human tissues: relationship with tight junctions

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ABSTRACT

Chloride intracellular channel protein 2 (CLIC2) belongs to the CLIC family of conserved metazoan proteins. Although CLICs have been identified as chloride channels, they are currently considered multifunctional proteins. CLIC2 is the least studied family member. We investigated CLIC2 expression and localization in human hepatocellular carcinoma, metastatic colorectal cancer in the liver, and colorectal cancer. Significant expression of mRNAs encoding CLIC1, 2, 4, and 5 were found in the human tissues, but only CLIC2 was predominantly expressed in non-cancer tissues surrounding cancer masses. Fibrotic or dysfunctional (aspartate aminotransferase ≥ 40) non-cancer liver tissues and advanced stage HCC tissues expressed low levels of CLIC2. Endothelial cells lining blood vessels but not lymphatic vessels in non-cancer tissues expressed CLIC2 as well as high levels of the tight junction proteins claudins 1 and 5, occludin, and ZO-1. Most endothelial cells in blood vessels in cancer tissues had very low expressions of CLIC2 and tight junction proteins. CD31⁺/CD45⁻ endothelial cells isolated from non-cancer tissues expressed mRNAs encoding CLIC2, claudin 1, occludin and ZO-1, while similar cell fractions from cancer tissues had very low expressions of these molecules. Knockdown of CLIC2 expression in human umbilical vein endothelial cells (HUVECs) allowed human cancer cells to transmigrate through a HUVEC monolayer. These results suggest that CLIC2 may be involved in the formation and/or maintenance of tight junctions and that cancer tissue vasculature lacks CLIC2 and tight junctions, which allows the intravasation of cancer cells necessary for hematogenous metastasis.

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Introduction

Chloride intracellular channel proteins (CLICs) were recently identified, and currently six related human genes encoding CLIC1–6 have been reported^{1,2} Because CLIC proteins are highly conserved throughout metazoa, they are thought to play critical roles in maintaining life^{3,4} Although they are considered multifunctional proteins, the common functions of CLICs have not been well defined⁵ When inserted into an artificial lipid bilayer, they formed chloride ion channels^{6,7} However, most cellular CLICs are not present as membrane-bound proteins but as soluble proteins or localized in cell nuclei. The intimate interactions of CLICs with the actin

cytoskeleton have been frequently reported, suggesting pivotal roles in the regulation of cytoskeletal organization^{6,8,9} Nuclear CLIC4 may be involved in transforming growth factor β (TGF β)-mediated signaling. Studies of CLIC4-null mice indicated CLIC4 might be involved in renal tubulogenesis¹⁰ CLIC1-null mice had impaired phagosome acidification, which caused the dysfunction of macrophages, which expressed CLIC1 at a high level in normal animals¹¹ Jitterbug mice have a natural mutation in the *clic5* gene and have impaired hearing and balance¹² Many other functions of CLICs have been reported and therefore it is difficult to determine the specific nature and roles of CLICs. Identifying which proteins or factors interact with

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CLICs might help elucidate their roles; however, traditional methods such as immunoprecipitation or cross-linking techniques have not produced significant results¹

CLIC2 is the least investigated CLIC family member, and its nature and functions are yet to be elucidated. This is partly because the CLIC2 gene is absent in the murine genome; therefore, knockout mice cannot be used to gain insights into its function. Human CLIC2 gene is present in the telomeric region of chromosome Xq28, and the duplication or lack of this gene were reported to cause mental retardation, developmental disability or epilepsy mainly in male children^{13–15} CLIC2 gene mutations also caused cardiomegaly, in part because of its stimulating action on the ryanodine receptor while amplifying intracellular Ca²⁺ signals¹⁶

Recently, our pathologic investigation of surgically dissected tissue samples from cancer patients demonstrated that CLIC2 was expressed predominantly in non-cancer tissues rather than in cancer tissues. In this study, we investigated the localization of CLIC2 in cancer and surrounding non-cancer tissues from cases bearing hepatocellular carcinoma (HCC), metastatic colon cancer in the liver (Meta) and colorectal cancer (Colon) to gain insights into the functions of CLIC2 in normal and cancer cells and tissues. CLIC2 was mainly expressed in blood vessels in non-cancer tissues and co-expression with tight junction proteins was found. Therefore, CLIC2 might be involved in the construction and/or maintenance of tight junctions between endothelial cells in normal tissues, which might contribute to the prevention of hematogenous cancer cell spread.

Results

Cancer tissues and surrounding non-cancer tissues were obtained from 32 hepatocellular carcinoma (HCC), 14 metastatic colon cancer in the liver (Meta) and 6 colorectal cancer (Colon) cases as shown in Table 1. The tissues were investigated for their expression of CLICs using qPCR, western blotting and immunohistochemical analyses. Some non-cancer and cancer liver tissues from HCC cases were used for cell sorting and subsequent qPCR.

Figure 1 shows CLIC mRNA expression in cancer and surrounding non-cancer tissues. CLIC1 mRNA was expressed at the highest level among CLIC1–5

both in cancer and non-cancer tissues from HCC cases (Figure 1A). CLIC3 mRNA expression was weak (data not shown). Among the CLICs, only CLIC2 mRNA expression was significantly higher in non-cancer tissues than in the cancer tissues. We investigated whether liver dysfunction affected CLIC mRNA expression (Figure 1B,C). Liver dysfunction was evaluated by the value of plasma aspartate aminotransferase (AST) activity. Differences in AST values did not affect CLIC1, 4, and 5 mRNA expression, but CLIC2 mRNA expression was decreased in cancer (Figure 1B) and non-cancer tissues (Figure 1C) in dysfunctional cases (AST values ≥ 40). Furthermore, liver fibrosis correlated with decreased CLIC2 mRNA expression in non-cancer tissues (Figure 1D). When the HCC cases were divided by disease stages, CLIC2 mRNA expression in cancer tissues at the initial stage (stage IA) was significantly higher than that in HCC at advanced stages (Figure 1E). The higher expression of CLIC2 mRNA in non-cancer tissues compared with cancer tissues was also observed in Meta (Figure 1F) and Colon (Figure 1G) cancer cases. The predominant CLIC2 protein expression in non-cancer tissues was confirmed by western blotting (Figure 2). Although the CLIC2 gene is located in the X chromosome, there were no significant differences in its expression between male and female cases with any of the three types of cancers.

Immunohistochemical localization of CLIC2 in cancer and non-cancer tissues was investigated (Figure 3). In accordance with the qPCR and western blotting data, CLIC2 immunoreactivity was found mainly in non-cancer tissues (Figure 3Aa and Ba) in Kupffer cells (yellow arrowheads) and endothelial cells forming the liver sinusoids (pink arrowheads). Hepatocytes did not express CLIC2. In Colon cancer cases, cells in the interstitial tissues but not columnar epithelial cells expressed CLIC2 (Figure 3Ca). Only faint CLIC2 expression was found in cancer tissues of the HCC and Meta cases (Figure 3Ab and Bb). Although CLIC2 immunoreactivity was strongly observed in Colon cases, it was not found in cancer cells but in the interstitial tissues. Predominant CLIC2 expression in non-cancer tissues was clearly observed in the border zone between cancer and non-cancer tissues of Meta and Colon cases (Figure 3D,E). In contrast to CLIC2, CLIC1, 4, and 5 appeared to be evenly distributed in cancer and non-cancer tissues

Table 1. Background characteristics of patients.

Hepatocellular carcinoma (HCC)					
No	Stage (UICC8th)	Age	Sex	AST (U/L)	Fibrosis*
1	IA	62	Male	25	F1
2	IA	68	Female	51	F4
3	IA	69	Male	11	F3
4	IA	73	Female	33	F4
5	IA	74	Male	25	F3
6	IA	76	Female	37	F4
7	IB	58	Female	24	F1
8	IB	71	Male	17	F0
9	IB	72	Male	33	F4
10	IB	75	Male	17	F2
11	IB	75	Male	56	F2
12#	IB	75	Male	44	F3
13	IB	81	Female	26	F2
14	IB	83	Male	39	F2
15#	II	49	Male	56	F4
16	II	57	Male	44	F4
17	II	63	Female	52	F4
18	II	64	Male	28	F3
19	II	69	Male	29	F2
20	II	71	Male	16	F1
21	II	71	Male	33	F3
22	II	72	Male	43	F1
23	II	73	Male	17	F0
24	II	75	Male	59	F2
25	II	77	Male	64	F4
26	II	81	Female	50	F3
27	II	82	Female	30	F0
28	II	83	Male	30	F0
29	IIIA	73	Female	96	F4
30	IIIB	62	Male	216	F4
31	IIIB	65	Male	28	F4
32	IVA	63	Male	80	F2
Metastatic colon cancer in the liver (Meta)					
No	Stage (UICC8th)	Age	Sex	AST (U/L)	
1	IIA	61	Male	18	
2	IIA	78	Male	36	
3#	IIIB	80	Male	42	
4	IIIB	63	Male	24	
5	IIIB	68	Male	29	
6	IIIB	71	Male	14	
7	IIIB	79	Female	22	
8	IIIB	87	Female	42	
9	IVA	47	Male	21	
10	IVA	59	Female	20	
11	IVA	65	Male	21	
12§	IVA	70	Male	31	
13	IVA	85	Female	18	
14	No data	68	Male	34	
Colorectal cancer (Colon)					
No	Stage (UICC8th)	Age	Sex	AST (U/L)	
1	I	87	Female	27	
2	IIA	65	Female	14	
3	IIA	76	Female	21	
4	IIA	70	Male	18	
5	IIIB	61	Male	12	
6§	IVA	70	Male	31	

* based on New Inuyama classification

not included in the qPCR experiments

§ the same patient

(supplementary Figure 1). Strong CLIC1 immunoreactivity was diffusely distributed within the cytoplasm of cancer and non-cancer cells. CLIC5 was characteristically localized in the nuclei of cancer and non-cancer cells.

To identify the cell types expressing CLIC2, double or triple immunofluorescence staining was performed using cryo-sections of the colon and the liver. Figure 4 shows the localization of CLIC2 and CD11b, a myeloid cell marker. Strong CLIC2 fluorescence was found in vascular structures (Figure 4A), but weak fluorescence was also present in cells expressing CD11b (Figure 4B, yellow arrowheads) and stromal fibroblasts (pink arrowheads). CD31⁺ endothelial cells strongly expressed CLIC2 in the submucosal tissues (Figure 5A). Similarly, CD31⁺ endothelial cells in non-cancer tissues of the liver with HCC or Meta expressed CLIC2 strongly (supplementary Figure 2). Closer observation of CLIC2 expression in interstitial non-cancer tissues showed that it was localized mainly in CD31⁺ endothelial cells and that some fibroblast-like cells expressed low levels of CLIC2 (Figure 5B; yellow arrowheads). Although highly magnified images suggest the membranous (Bb' yellow arrowheads) localization of CLIC2, it had a narrower localization than the transmembrane protein CD31 in endothelial cells (Figure 5C), suggesting CLIC2 is not a transmembrane protein but a submembranous protein. CD31⁺ endothelial cells form both blood and lymphatic vessels. To determine whether CLIC2 was selectively expressed by blood and/or lymphatic vessels, triple-immunostaining was performed using antibodies to CLIC2, CD31 and podoplanin, a specific marker for lymphatic endothelia¹⁷ CLIC2 was localized to blood vessels (Figure 6A, yellow arrowheads) but not podoplanin-expressing lymphatic vessels (Figure 6A, blue arrowheads). However, most blood vessels in cancer tissues did not express CLIC2 or podoplanin (Figure 6B, blue arrowheads), although some endothelial cells in blood vessels expressed CLIC2 (Figure 6B, yellow arrowheads).

Because CLIC4 was reported to colocalize with tight junction protein ZO-1,⁹ the colocalization of CLIC2 with tight junction protein claudin 1 was investigated (Figure 7). Strong claudin 1 immunofluorescence was localized throughout CLIC2-

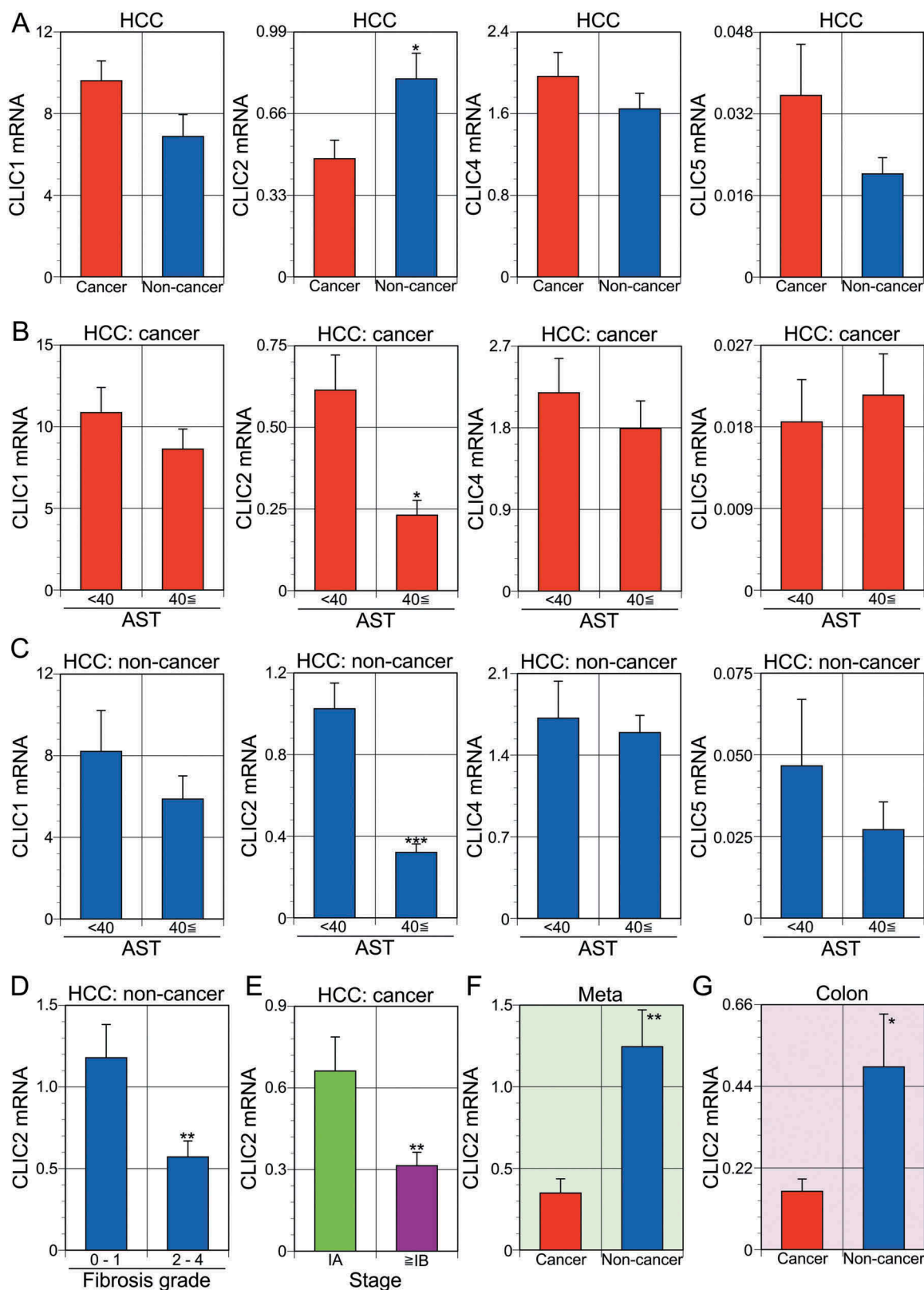


Figure 1. Expression of mRNAs for CLIC1, 2, 4, and 5 in cancer (red bars) and non-cancer (blue bars) tissues of HCC (A, B, C, D), Meta (F; green background), and Colon (G; pink background) cancer. (A) Only CLIC2 mRNA expression was increased in non-cancer liver tissues of HCC cases among the examined CLICs. High AST values were significantly correlated with lower CLIC2 mRNA expression in HCC (B) and non-cancer liver tissues of HCC cases (C). (D) CLIC2 mRNA expression in the non-cancer liver tissues of HCC cases was decreased when the fibrosis was advanced. (E) CLIC2 mRNA expression was higher in HCC cases at stage IA relative to those at stage IB or higher. (F and G) Higher CLIC2 mRNA expression was observed in non-cancer tissues compared with cancer tissues in Meta (F) and Colon (G). Data are expressed as the means \pm SEM and as a percentage of GAPDH mRNA levels. $n = 16$ for CLIC1, 4, and 5 mRNA in HCC; $n = 30$ for CLIC2 in HCC; $n = 13$ for Meta; $n = 6$ for colon. *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

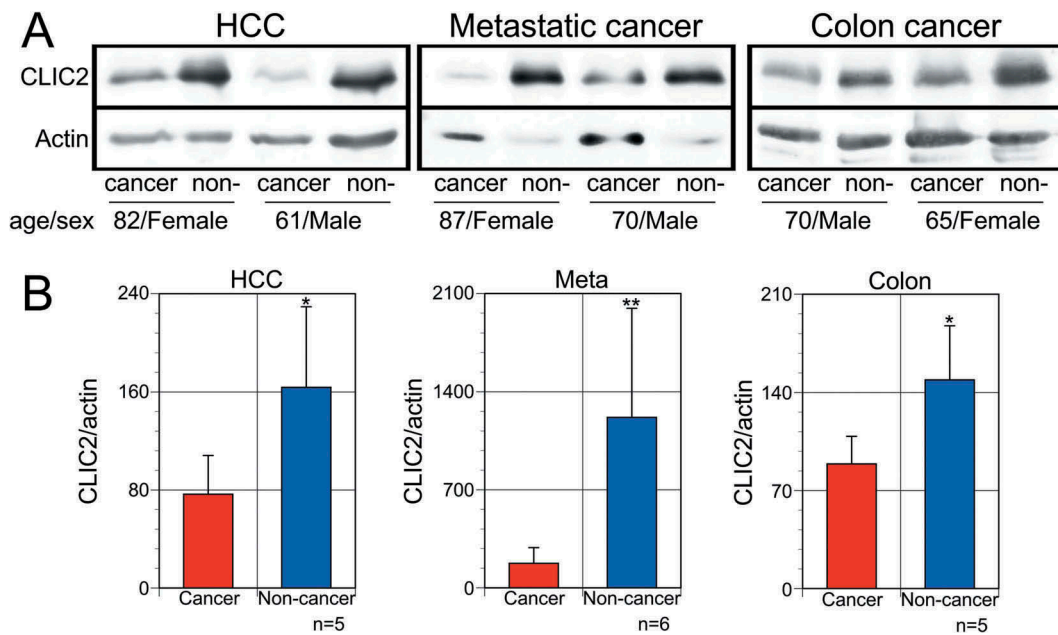


Figure 2. CLIC2 protein expression in cancer and non-cancer tissues of HCC, Meta and Colon cancer cases was analyzed by immunoblotting. (i) Representative immunoblots of CLIC2 and actin protein expressions showing the more prominent expression of CLIC2 protein in non-cancer (non-) tissues compared with cancer tissues. (ii) Densitometric evaluation of immunoreactive CLIC2 bands. Data [n = 5 (HCC) or n = 6 (Meta and Colon cancer)] are shown as the means \pm SD.

expressing endothelial cells in interstitial tissues in the non-cancer regions of a Colon cancer case (Figure 7A). Even small vessel endothelial cells expressed CLIC2 and claudin 1 (Figure 7A, yellow arrowheads). Notably, the strong fluorescence of CLIC2 was found in cell-cell contact regions (Figure 7B, yellow arrowheads), while colocalizing with claudin 1-fluorescence. In contrast to endothelial cells in non-cancer tissues, endothelial cells in interstitial tissues in the core of cancer tissues generally did not express claudin 1 or CLIC2 (Figure 7C, yellow arrowheads). Weak claudin 1 fluorescence was found at the cell-cell borders in cancer tissues (Figure 7C, blue arrows). Other tight junction proteins claudin 5, occludin and ZO-1 were immunolocalized in the colon tissues (Figure 8). Blood vessel endothelial cells in non-cancer tissues, but not cancer tissues and lymphatic endothelial cells, expressed these three proteins.

To confirm the expression of CLIC2 and tight junction proteins by endothelial cells, cancer, and non-cancer tissues from an HCC case were dissociated into individual cells and sorted with flow cytometry using antibodies to CD31 and CD45 (Figure 9). Total RNA was prepared from the sorted CD31⁺/CD45⁻ (endothelial cells), CD31⁺/CD45⁺ and CD31⁻/CD45⁺ cells, and then analyzed

by qPCR. The endothelial cells from non-cancer tissues expressed CLIC2 at the highest levels among the 6 types of cells. CD31⁺/CD45⁺, populations of invading leukocytes or resident macrophages, expressed CLIC2 at lower levels than CD31⁺/CD45⁻ cells. Very high expression of mRNA encoding the tight junction proteins claudin 1, ZO-1, and occludin were found only in the endothelial cell fractions from non-cancer tissues.

Finally, to gain insights into the roles of CLIC2 in endothelial cells, the effect of CLIC2 knockdown on the penetration of cancer cells was investigated by transendothelial migration assay using human umbilical vein endothelial cells (HUVECs) and human tongue squamous cell carcinoma cell line SAS cells (Figure 10). HUVECs expressed CLIC2, and this expression was knocked down with siRNA (Figure 10A). Transmigration assay results are shown in Figure 10B. Treatment with siRNA did not cause apparent changes in the morphology and growth of HUVECs (Figure 10C). SAS cells easily migrated through empty filters to the lower chambers, which were filled with FBS-containing DMEM (Figure 10D). However, confluent control HUVECs prevented transmigration (Figure 10Ea). HUVECs treated with siRNA with an irrelevant (irr) sequence

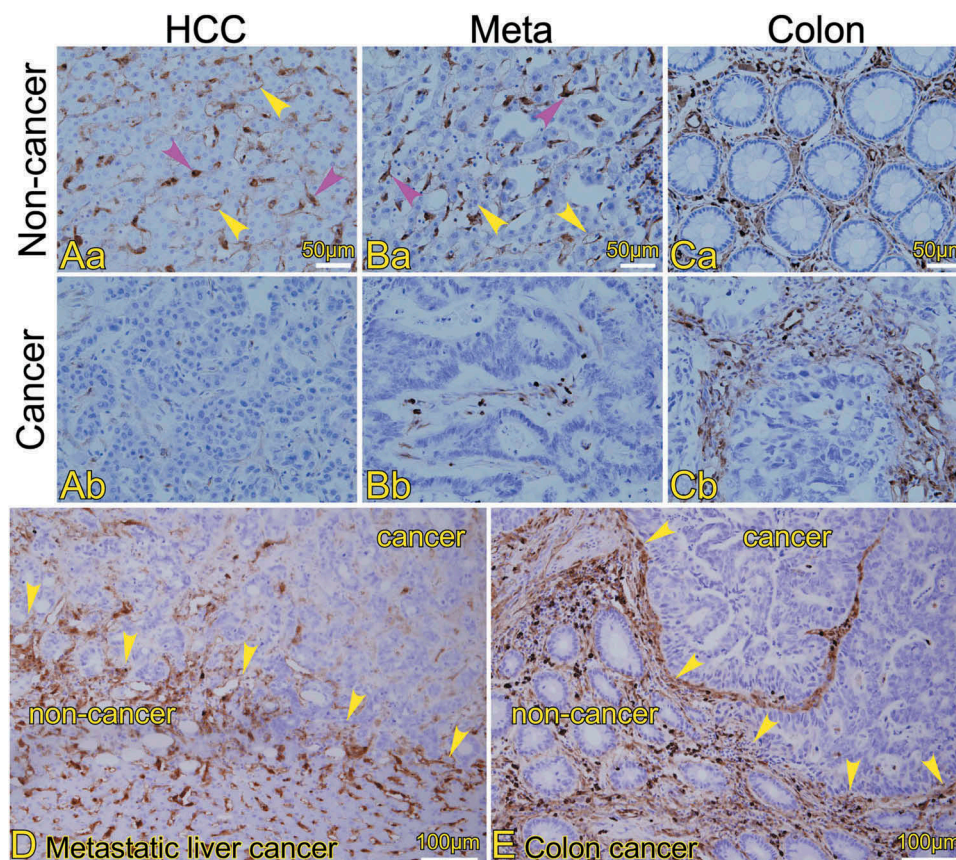


Figure 3. Localization of CLIC2-immunoreactivity in HCC (A: male 75-yo, AST44, InuyamaF3, stage IB), Meta (B: male 47-yo, stage IVA) (d: male, 80-yo, stage IIB) and Colon (C: female, 65-yo, stage IIA) (E: male 61-yo, stage IIIB) cancer cases was investigated by immunohistochemical staining. (D and E) show the border zone (denoted with yellow arrowheads) between cancer and non-cancer tissues in Meta and Colon cases, respectively. In non-cancer tissues, CLIC2 was expressed by cells in the interstitial tissues. Hepatocytes, colon epithelial cells, and cancer cells did not appear to express CLIC2.

suppressed the transmigration (Figure 10Eb). However, HUVECs with knocked down CLIC2 expression allowed many SAS cells to transmigrate (Figure 10Ec and 10F).

Discussion

Predominant expression of CLIC2 in non-cancer tissues and its intracellular localization

The present study revealed the predominant expression of CLIC2 in human non-cancer tissues, whereas CLIC1, 4, and 5 were similarly expressed in cancer and non-cancer tissues. Immunohistochemical investigation revealed that CLIC2 was expressed in endothelial cells, CD11b⁺ myeloid cells, and fibroblast-like cells in interstitial tissues. Among the CLIC2-expressing cells, the strongest expression was localized to blood vessel endothelial cells in non-cancer tissues as confirmed by

cell sorting experiments. This endothelial cell expression may be the cause of the predominant expression of CLIC2 in non-cancer tissues detected by qPCR and western blotting.

CLIC2 is the least investigated subtype among the CLIC family members. Its known physiological function may be the modulation of ryanodine receptor activities to regulate Ca²⁺-mediated intracellular signaling in skeletal and cardiac muscles^{18,19} CLIC2 may form chloride ion channels *in vitro* when it is inserted into an artificial lipid bilayer⁷ Compared with the localization of CD31 or PECAM, a transmembrane protein belonging to the immunoglobulin superfamily,²⁰ CLIC2 was distributed in the cytoplasm and not as a transmembrane protein as described previously,²¹ suggesting that CLIC2 does not function as a chloride channel in the plasma membrane.

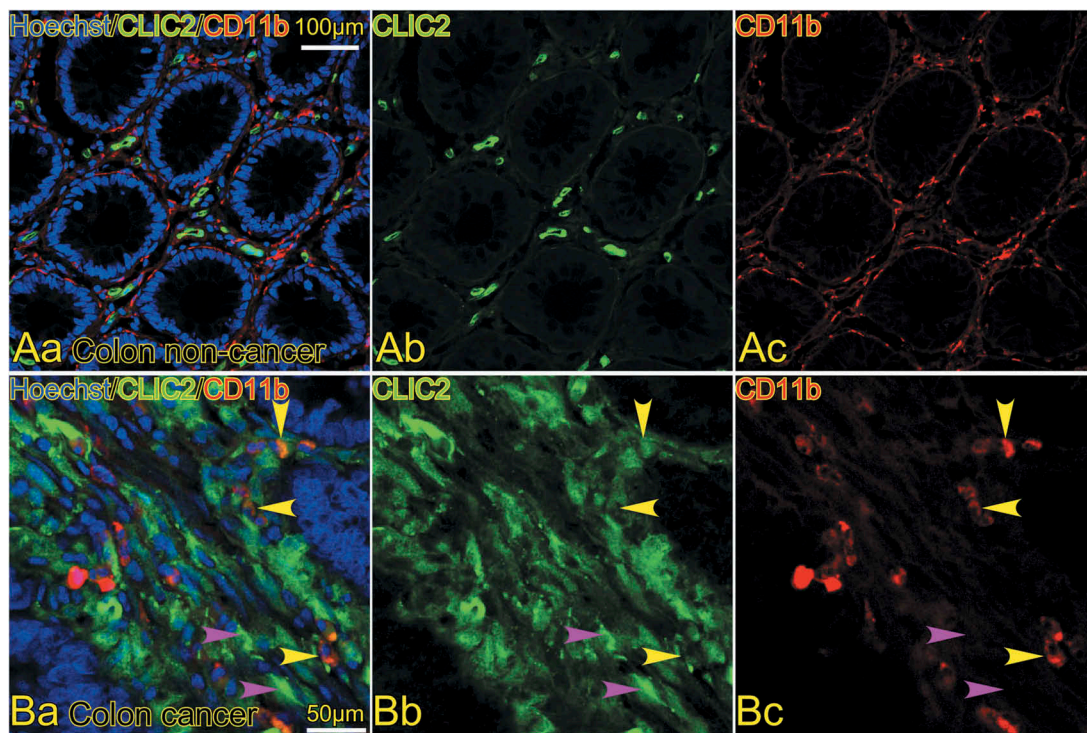


Figure 4. Localization of CLIC2 and the myeloid cell marker CD11b in normal colon tissues surrounding a colon cancer tumor (male 61-yo, stage IIIB). (A) Strong CLIC2 immunofluorescence was not localized in CD11b⁺ cells. (B) Close observation revealed that some CD11b⁺ cells had weak CLIC2 immunofluorescence (yellow arrowheads) in addition to stromal cells (pink arrowheads).

Tight junctions and CLIC2; relationship with the hematogenous spread of cancer

Endothelia in the blood vessels of non-cancer tissues express tight junction proteins claudins 1 and 5, ZO-1 and occludin in addition to CLIC2 as revealed by immunohistochemical staining. By contrast, most blood vessel endothelia in cancer tissues did not or only faintly expressed these proteins. Furthermore, CD31⁺/CD45⁻ endothelial cells isolated from non-cancer tissues expressed CLIC2 as well as claudin 1, ZO-1, and occludin at high levels. However, the same cell fraction from cancer tissues expressed these proteins at much lower levels. These results suggest that CLIC2 may be involved in the formation and/or maintenance of tight junctions between endothelial cells of blood vessels in non-cancer tissues. Furthermore, the intimate colocalization of CLIC2 and CD31 suggests the involvement of CLIC2 in CD31-mediated cell-cell contact between endothelial cells. Lymphatic endothelial cells normally form different types of cell-cell junctions from tight junctions²² The simultaneous lack of CLIC2 and tight junction proteins in lymphatic vessels suggests the specific involvement of CLIC2 in tight junction

formation in blood vessel endothelia. However, the molecular mechanisms involved in the relationship between CLIC2 and tight junction proteins are unclear. Whether CLIC2 has direct binding to proteins that form tight junctions including the actin cytoskeleton, or whether CLIC2 regulates the expression of these proteins is unclear.

Tight junctions are responsible for the regulation of endothelial permeability^{22,23} Endothelial tight junctions are necessary to prevent cancer cells freely migrating into the circulation during metastasis^{24,25} Cancer blood vessels are more permeable structures than those in normal tissue vessels, and they assist cancer metastasis^{24,26} The decrease or loss of claudins 1, 5, occludin and ZO-1 in many cancers has been reported^{24,27,28} As described above, CLIC2 may be involved in the formation and/or maintenance of tight junctions in normal blood vessel endothelia, which may lead to the inhibition of the hematogenous metastasis of cancer cells. Indeed, this study demonstrated that the knockdown of CLIC2 in HUVECs increased the FBS-induced transmigration of human cancer cells through a HUVEC monolayer.

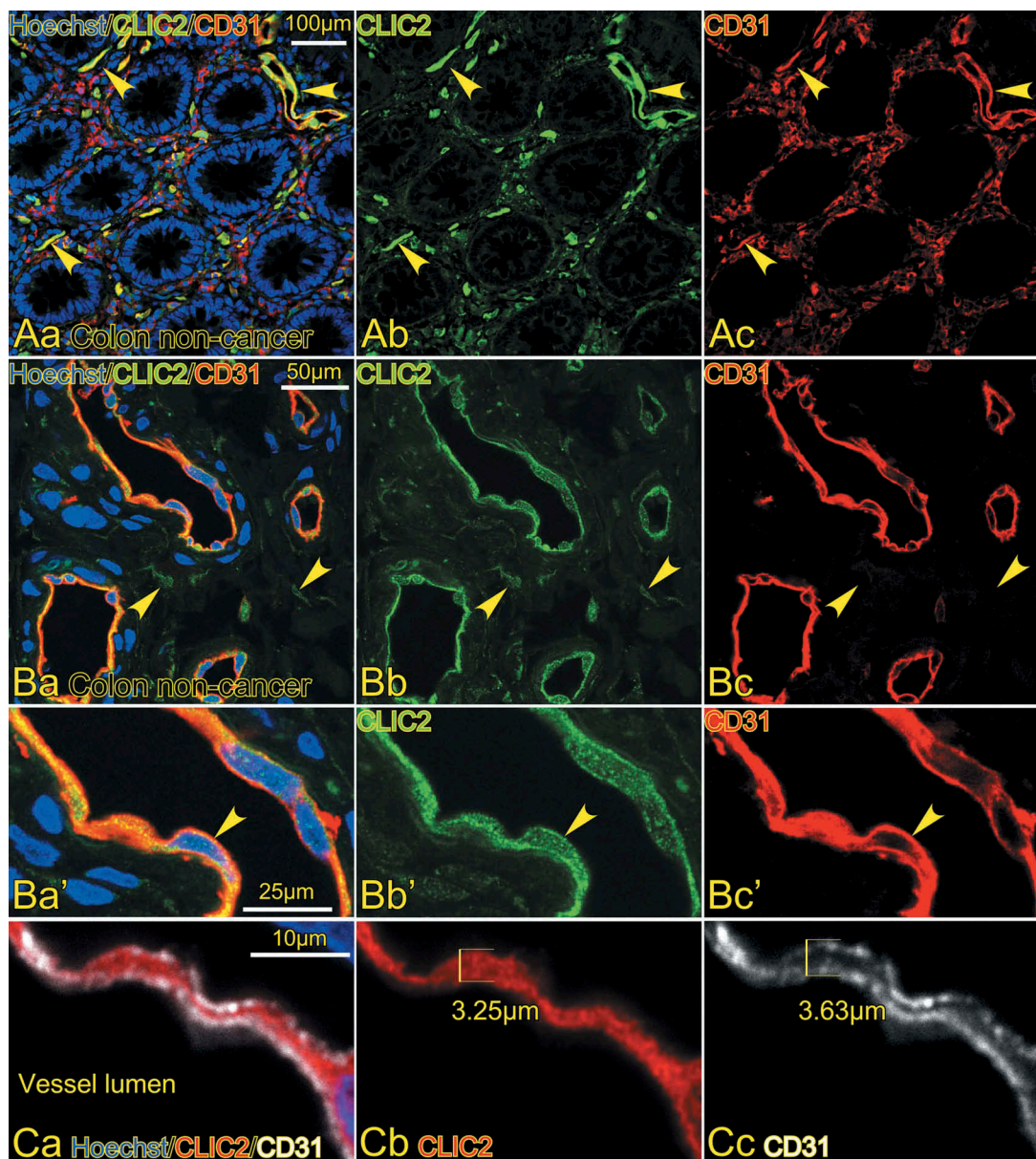


Figure 5. Colocalization of CLIC2 with CD31⁺ vasculature in colon non-cancer tissues from a female case (65-yo, stage IIA). (A) Strong CLIC2 fluorescence was localized in the CD31⁺ vasculature (yellow arrowheads). (B) Localization of CLIC2 in interstitial tissue from the normal colon. The majority of CLIC2 was localized in CD31⁺ endothelial cells. Fibroblast-like cells (yellow arrowheads) displayed weak CLIC2 immunofluorescence. (b') shows a magnified view of part of the optic field shown in (B). CLIC2 was localized mainly in the vicinity of the plasma membrane (yellow arrowheads). (C) Comparison of the localization of CLIC2 and CD31 in the vicinity of the plasma membrane. The numbers in Cb and Cc indicate the diameter based on two-protein fluorescence. Compared with CD31 fluorescence, CLIC2 fluorescence was localized in a smaller area, suggesting it was not present as a transmembrane protein.

This suggests that CLIC2 plays a role in preventing the hematogenous spread of cancer cells.

Potential involvement of HGF and VEGF in the decreased expression of CLIC2

Which factors cause the loss of CLIC2 or tight junction proteins in tumor endothelial cells (TECs)? Cancer cell-derived humoral factor(s) may enhance

the blood vessel permeability²⁹ Among these humoral factors, hepatocyte growth factor (HGF) may increase the permeability of blood vessels in liver cancers^{24,25} HGF was shown to suppress the expression of tight junction proteins including ZO-1, claudin 1 and occludin. Serum HGF levels are increased in various liver diseases such as acute and chronic hepatitis, liver cirrhosis and HCC, and AST values were positively correlated to the serum HGF

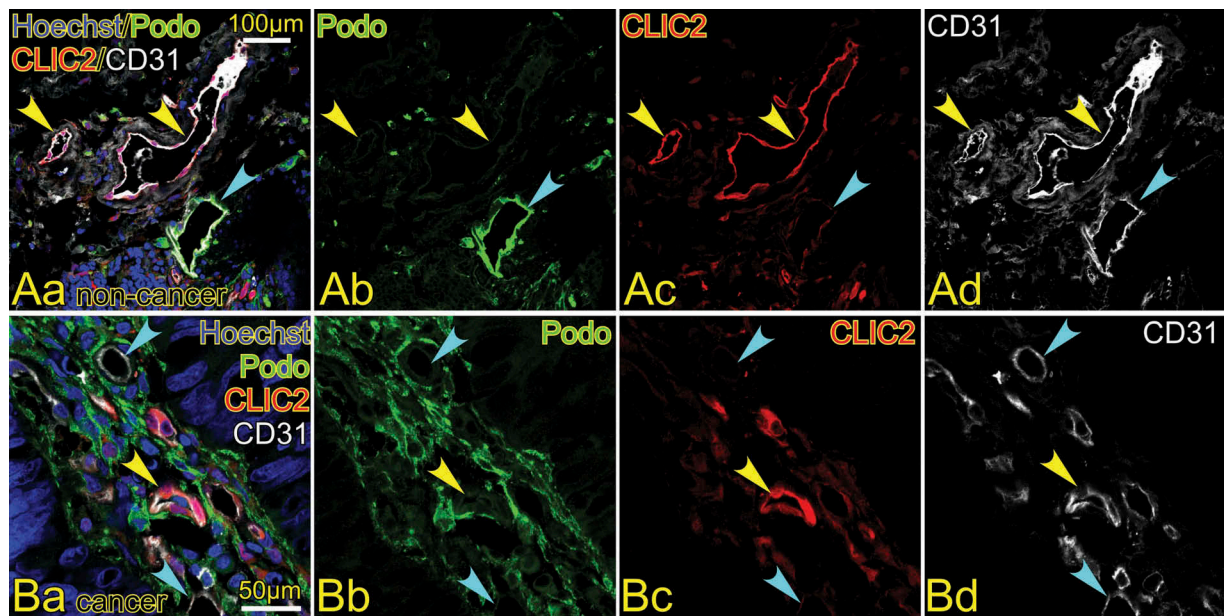


Figure 6. CLIC2 was exclusively expressed by endothelial cells of blood vessels but not of lymphatic vessels in colon tissues from the same case as that in Figure 5. (A) CLIC2 was only expressed by CD31⁺ endothelial cells that did not express podoplanin in the non-cancer interstitial tissue (yellow arrowheads). Podoplanin (Podo)-expressing lymphatic endothelial cells did not significantly express CLIC2 (blue arrowheads). (B) In colon cancer tissues, CLIC2 expression was absent in most podoplanin⁻/CD31⁺ endothelial cells (blue arrowheads). Yellow arrowheads denote CLIC2-expressing blood vessels.

level³⁰ The present findings showed that CLIC2 expression was reduced in non-cancer liver tissues with higher AST values or fibrosis. Collectively, increased HGF levels may reduce the expression of CLIC2 as well as tight junction proteins that likely allow cancer cells to penetrate blood vessel endothelia for hematogenous spread (Figure 11). Vascular endothelial growth factor (VEGF) is also a key regulator of blood vessel permeability²³ and was shown to reduce the expression of CLIC4 at the post-translational level³¹ VEGF receptor 2 (VEGFR2, or KDR in human) may mediate the increase in blood vessel permeability at least in part by reducing the expression or rearrangement of tight junction proteins^{32,33} Anti-VEGF antibody facilitated the formation of cell-cell adhesion partly through increasing the expression of tight junction proteins,³⁴ which might contribute to the anti-tumor effects of the antibody. Whether HGF and/or VEGF decrease the expression of CLIC2 in endothelial cells *in vivo* and *in vitro* should be investigated.

Conclusion

The identification of molecules such as CD90³⁵ exclusively expressed by the vasculature in cancer tissues has been one of the goals of cancer research

because it might provide a novel therapeutic mechanism to inhibit the supply of nutrients or oxygen to the tumor. In this study, CLIC2 was expressed exclusively in normal endothelial cells. By contrast, TECs in the cancer core were devoid of CLIC2 expression, which might correlate with the lack of tight junctions that allow the hematogenous metastasis of cancer cells. Therefore, cancer blood vessels might be in a state of loss-of-function regarding CLIC2. The elucidation of CLIC2 functions might help in unraveling the nature of cancer blood vessels as well as the functions of the normal vasculature. If CLIC2 expression is pharmacologically induced in blood vessels in cancer tissues, hematogenous spread might be prevented. Alternatively, the administration of agents that mimic the function of CLIC2 in and around cancer tissues might suppress distant metastases. It is necessary to elucidate the mechanisms underlying CLIC2 functions in endothelial cells.

Patients, materials and methods

Patients

From January 2015 to March 2018, 51 cancer patients who underwent surgery at Ehime University Hospital were included in this study. Surgically resected cancer

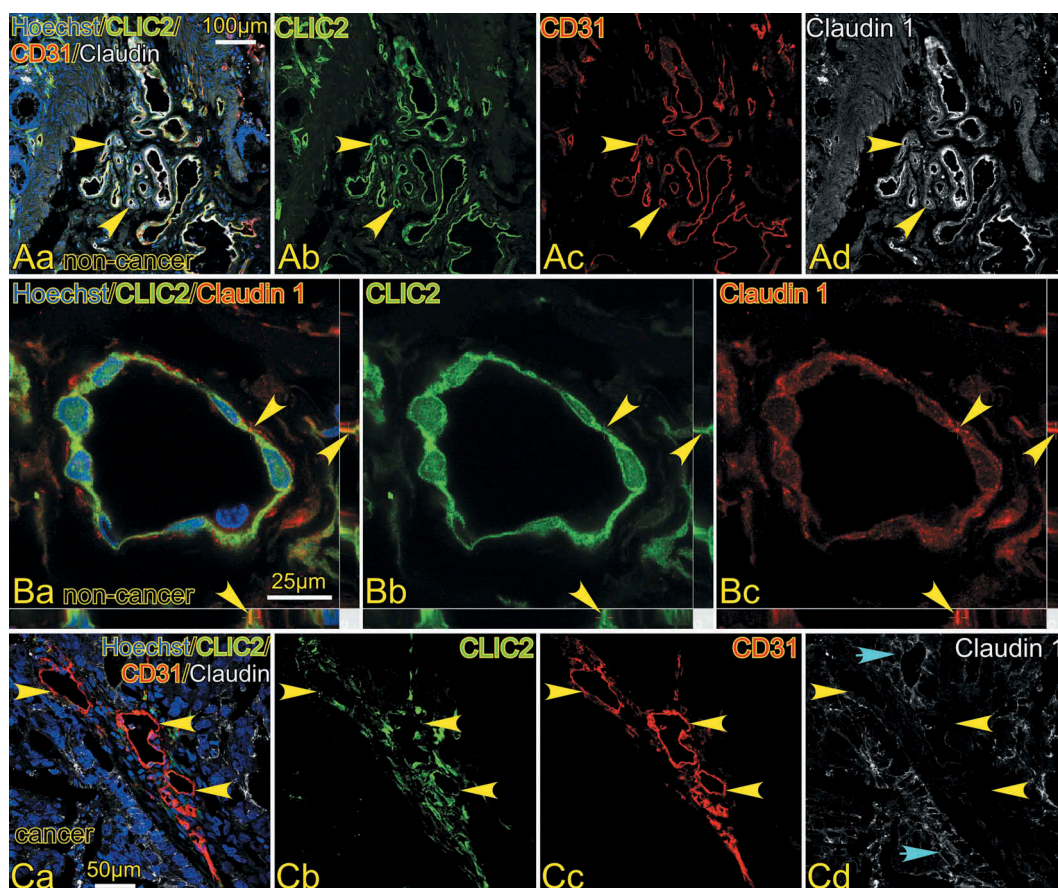


Figure 7. CLIC2 was often colocalized with claudin 1 in the colon tissue from the same case as that in Figure 5. A) Abundant vasculature in the non-cancer colon interstitial tissues expressed CLIC2 and claudin 1. Yellow arrowheads denote small vessels expressing CLIC2 and claudin 1. B) Close 3D observation of double-positive endothelial cells. CLIC2 was colocalized with claudin 1 at cell-cell contact regions (yellow arrowheads). (C) Blood vessels in the core of colon cancer tissues lacked CLIC2 and claudin 1 expression. Weak claudin 1 expression was present at the junctional regions of colon cancer cells (blue arrows).

tissues and surrounding the non-cancer tissues [32 cases with HCC, 14 cases with Meta, and 6 cases Colon] were obtained with written informed consent from patients under approval by the institutional review board at Ehime University Hospital. The background of patients is summarized in Table 1. From one patient (Meta no.12/Colon no.6), specimens of Meta and Colon were simultaneously obtained for our study. Histological diagnosis was performed using paraffin-embedded sections according to the Union for International Cancer Control (UICC) TNM classification of malignant tumors, 8th edition.

Quantitative RT-PCR (qPCR)

Surgically dissected HCC, Meta and Colon tissues were divided into cancer tissues and surrounding non-cancer tissues. Total RNA was extracted from the tissue homogenates using an RNeasy Lipid

Tissue Mini Kit (Qiagen) as described previously³⁶ cDNA was prepared using a ReverTra Ace qPCR RT Master Mix with a gDNA remover kit (Toyobo). qPCR analysis was performed using Fast Start Universal SYBR Green Master (Roche Diagnostic Japan) with an MJ mini instrument (BioRad). All gene-specific mRNA expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels and expressed as a % of GAPDH mRNA calculated as $100 \times 1/2^{\Delta Ct}$ ($\Delta Ct = \text{mean Ct of duplicate experiments of target genes} - \text{GAPDH mean Ct}$). All PCR primer sequences are listed in supplementary Table 1.

Immunohistochemical staining

The surgically dissected tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for

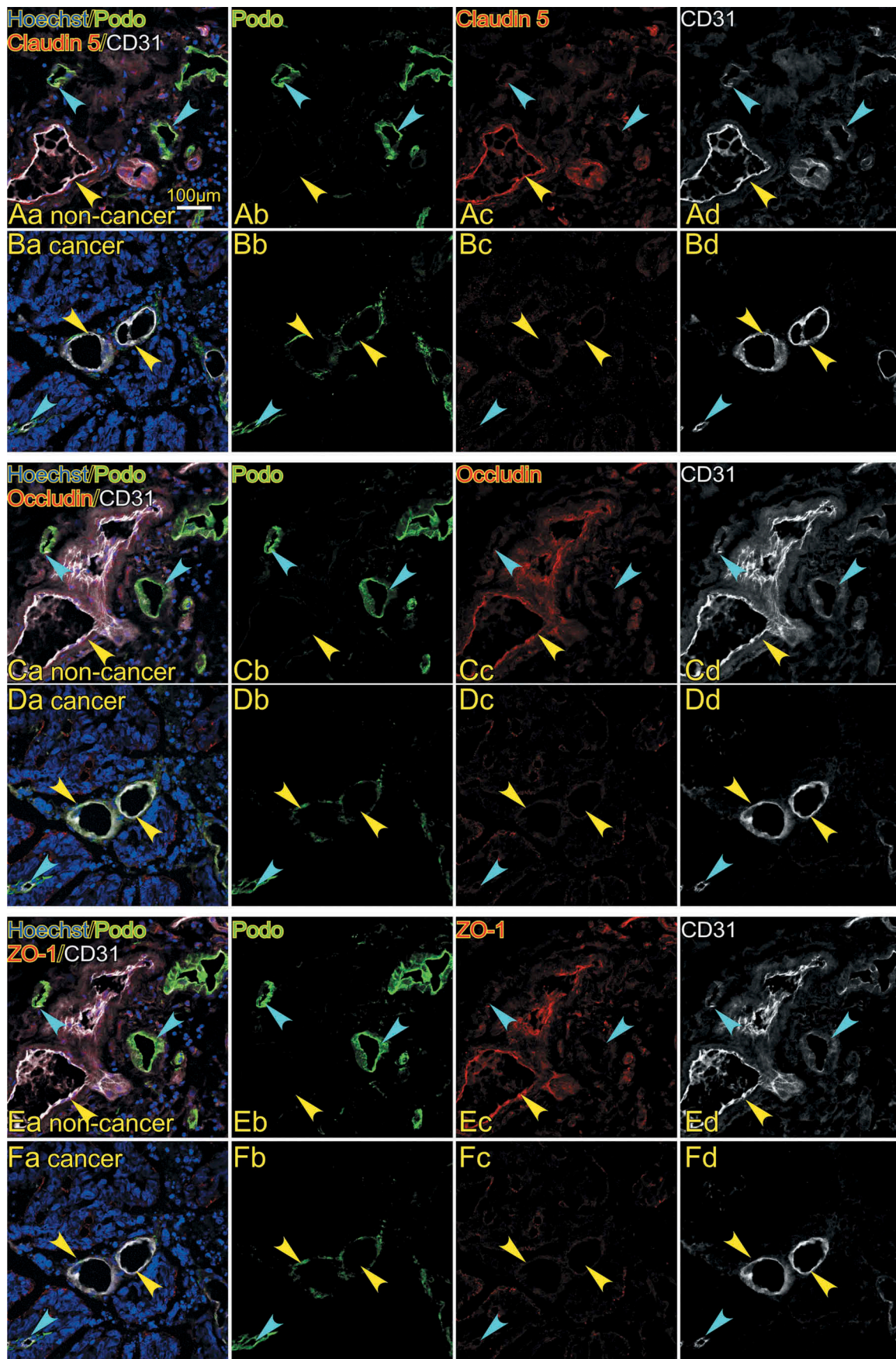


Figure 8. Expression of tight junction proteins, claudin 5 (A and B), occludin (C and D) and ZO-1 (E and F) in blood vessel endothelial cells in non-cancer and cancer tissues of the colon from the same case as that in [Figure 5](#). (A, C, and E) show non-cancer tissues, and (B, D and F) show cancer tissues. Podoplanin, tight junction proteins and CD31 immunofluorescence are shown in (b, c, and d), respectively. Yellow arrowheads denote blood vessel endothelial cells, and blue arrowheads denote podoplanin-expressing endothelial cells of lymphatic vessels. Blood vessel endothelial cells in non-cancer tissues, but not in cancer tissues, expressed tight junction proteins.

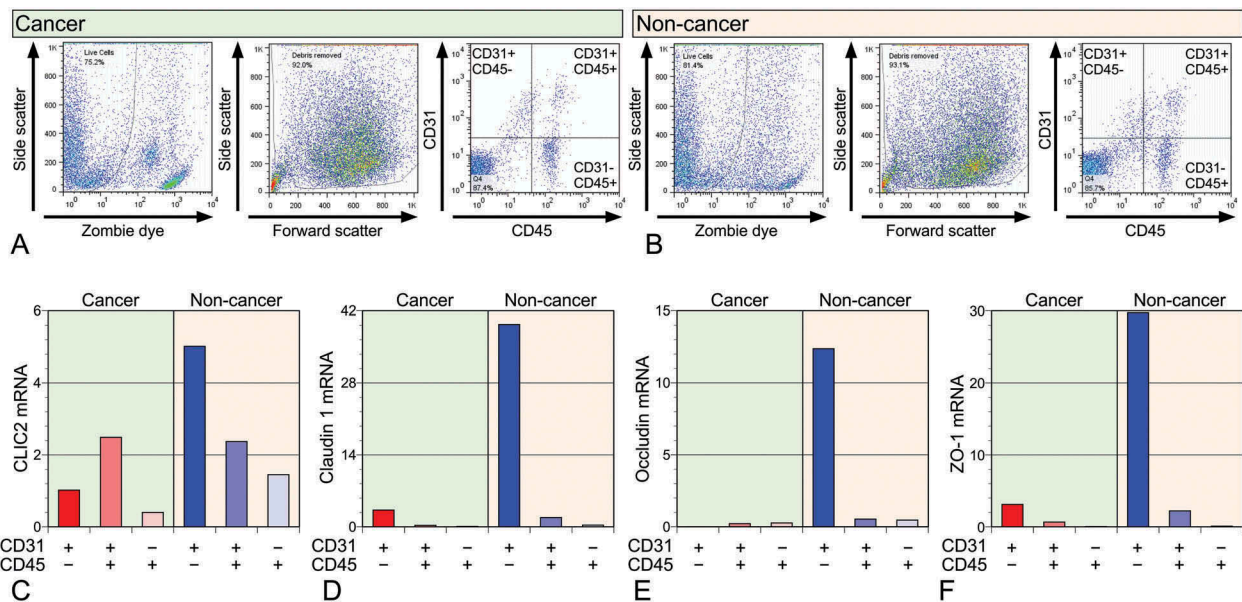


Figure 9. Expression of CLIC2 and tight junction proteins in sorted endothelial cells from liver tissue with HCC. (a and b) Representative dot plots of three similar flow cytometry analyses of cancer (A) and non-cancer (B) tissue cells from the HCC case. Gating strategy is shown for the analyses of CD31-expressing cells from cancer and non-cancer tissues by flow cytometry. CD31⁺/CD45⁻ endothelial cells as well as CD31⁺/CD45⁺ and CD31⁻/CD45⁺ cells were sorted and subjected to qPCR analyses. (C–F) mRNA expression encoding CLIC2 and tight junction proteins by the sorted cells. CD31⁺/CD45⁻ endothelial cells derived from non-cancer tissues expressed mRNAs encoding CLIC2 (C), claudin 1 (D), occludin (E) and ZO-1 (F) at the highest levels among the 6 types of sorted cells.

2–3 h at room temperature with slow shaking. After incubation in Tris-buffered saline containing 15% sucrose for 24 h, the fixed tissues were rapidly frozen in Tissue-Tek OCT compound (Sakura Finetek) and cryosectioned at a thickness of 10 μ m. Immunohistochemical staining was performed using the following two methods: 1) enzyme and 2) immunofluorescence immunohistochemistry.

Enzyme immunohistochemistry

After blocking endogenous peroxidase and non-specific binding, tissue sections were stained with the primary antibodies listed in supplementary Table 2. For enzyme immunohistochemistry, we diluted the primary antibodies with R.T.U. Animal-Free Block and Diluent (Vector Laboratories, Burlingame, USA, SP5035). After incubation with Envision+System-HRP Labelled Polymer anti-rabbit (Dako, K4001) and Envision+System-HRP

Labelled Polymer anti-mouse (Dako, K4003) for 30 min, tissue sections were stained with Histofine DAB Substrate Kit (Nichirei Biosciences, 425011) under a light microscope. Then, all slides were counterstained with hematoxylin, dehydrated with a series of graded ethanol solutions, and stabilized with mounting medium.

Immunofluorescence histochemistry

After incubation with tris-buffered saline containing 0.1% Tween-20 and 1 mg/ml bovine serum albumin, cryosections of non-cancer and cancer tissues were incubated with primary antibodies overnight. The sections were incubated with DyLight 488, DyLight 549, and/or DyLight 649-labeled secondary antibodies (Jackson ImmunoResearch Laboratories). Hoechst 33342 (Sigma-Aldrich) was used for nuclear staining³⁷ For the immunofluorescence staining of HCC and Meta cases, the tissue sections were incubated with 0.1% Sudan Black B diluted in

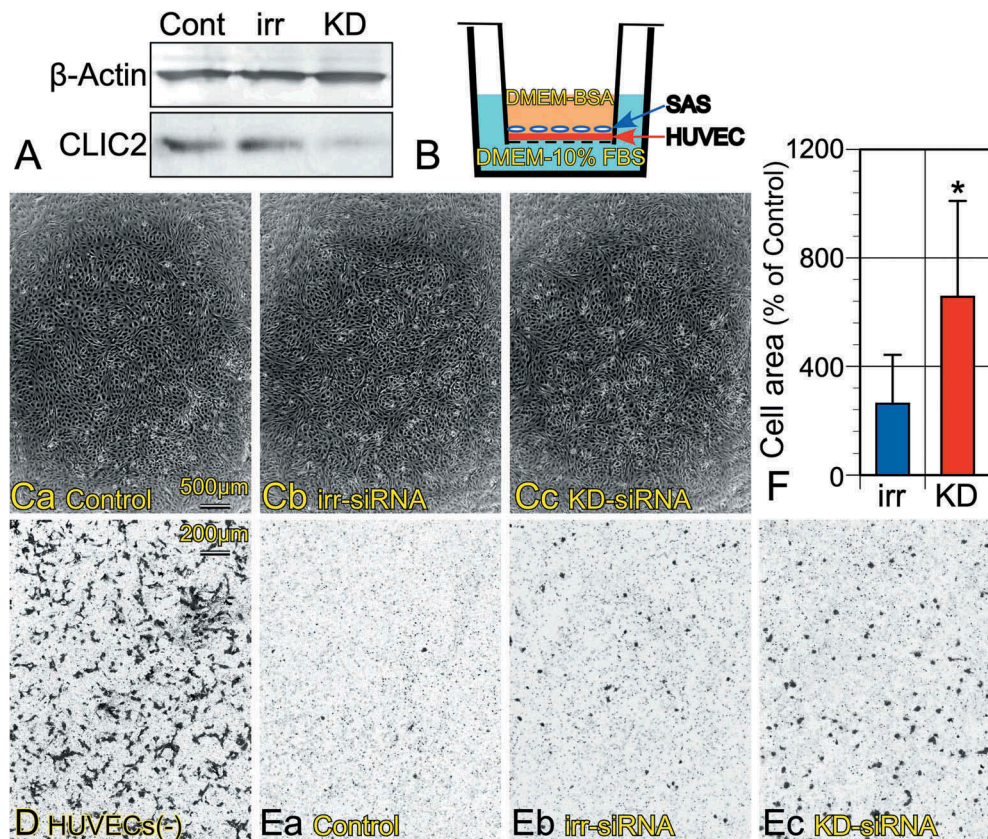


Figure 10. Effects of CLIC2 knockdown in HUVECs on the transmigration of SAS cells through HUVEC monolayers. (A) Knockdown of CLIC2 expression by HUVECs was confirmed by immunoblotting. Cont; non-treated control HUVECs, irr; HUVECs treated with siRNA with an irrelevant sequence, KD; HUVECs treated with siRNA for CLIC2 knockdown. Representative immunoblots are shown for six separate experiments. (B) Diagram showing the experimental set-up of the transmigration assay. HUVECs (50,000 cells) were seeded in the upper chamber and then SAS cells (30,000 cells) were placed on the HUVEC monolayer. The upper and lower chambers were filled with DMEM containing 0.5% BSA and that containing 10% FBS, respectively. (C) Phase contrast observation shows confluent control HUVECs (Ca), cells treated with irr-siRNA (Cb) or KD-siRNA (Cc). There were no apparent morphological differences in the confluent culture. (D) SAS cells freely migrated through the filter pore (8- μ m diameter) to the bottom surface in the absence of HUVECs. (E) SAS cells transmigrated through the monolayer of control (Ea), irr- (Eb) and KD- (Ec) siRNA treated HUVECs. Representative micrographs of six separate experiments are shown. (F) Statistical evaluation of the transmigration of SAS cells through the HUVEC monolayer. Transmigration was evaluated by measuring the total area occupied with SAS cells. Data show the mean \pm SD. *indicates $P < 0.05$.

70% ethanol for 5 min at room temperature followed by thorough washing to reduce autofluorescence before mounting as previously described³⁸. The stained specimens were observed with a Nikon A1 confocal laser scan microscope.

Western blotting

Tissue samples obtained from HCC, Meta and Colon cases were homogenized with Laemmli's sample solution containing 3% sodium dodecyl sulfate and subjected to electrophoresis and transfer as described previously³⁹. The lysates were immunoblotted with antibodies to β -actin and CLIC2. The blots were visualized with alkaline phosphatase-labeled

secondary antibodies (Promega) and analyzed by densitometry using ImageJ 1.43u (Wayne Rasband). The densities of immunoreactive bands were normalized against the immunoreactivity of β -actin.

Flow cytometry and cell sorting

Cancer and non-cancer tissues were dissociated into single cells in a gentleMACS dissociator using Tumor Dissociation Kit, human (Miltenyi Biotec) as reported previously⁴⁰. Remaining tissue aggregates, debris and contaminating erythrocytes were removed using MACS SmartStrainers with 100- μ m pores (Miltenyi Biotec), and debris and red blood cell removal

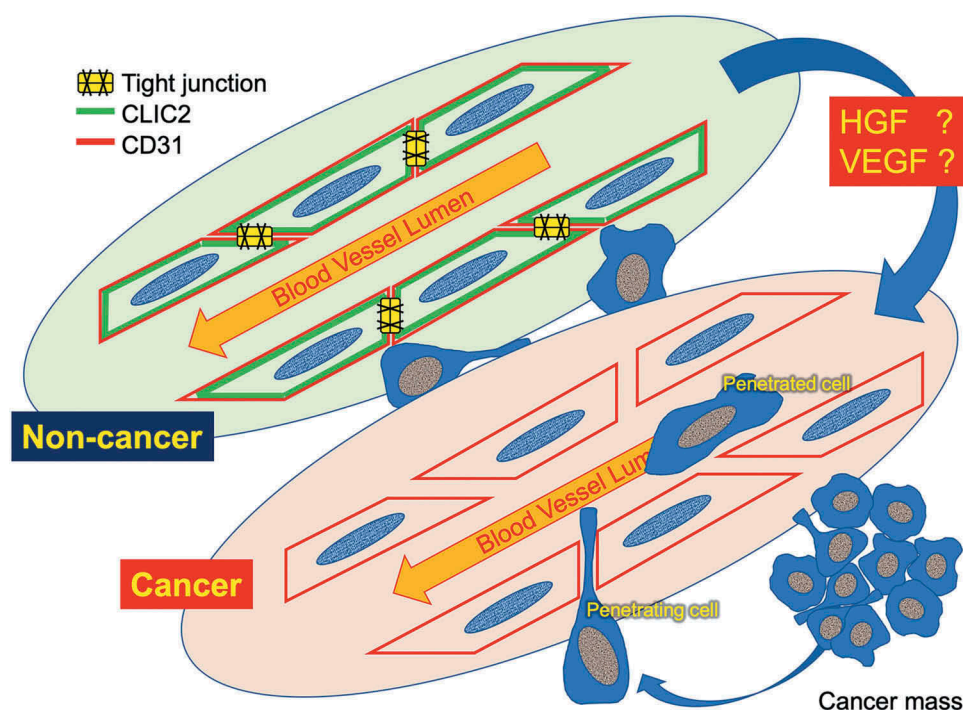


Figure 11. Diagram showing the mechanism of the inhibition of hematogenous cancer metastasis based on the current study findings. Normal blood vessel endothelia form tight junctions that prevent the intravasation of cancer cells. CLIC2 may be involved in the formation and/or maintenance of tight junctions. Blood vessels in cancer tissues lack CLIC2 and tight junction proteins, thereby allowing the intravasation of cancer cells leading to distant metastasis. HGF or VEGF may be candidates for the suppressed expression of CLIC2 and tight junction proteins in cancer tissues.

solutions (Miltenyi Biotec), respectively. The resultant cell suspensions were diluted and incubated with an anti-CD32 antibody to block Fc receptors. After incubation with fluorescence-labeled antibodies (see supplementary Table 3), the cells were analyzed on a Gallios flow cytometer (Beckman Coulter, Tokyo, Japan). Cell viability was investigated with Zombie NIR dye (BioLegend). Flow cytometry data were analyzed using FlowJo Software (version.7.6.5, TreeStar). For analyses of the mRNA expression in sorted cells, cells were incubated with Cell Cover (AL Anacyte Laboratories) to stabilize mRNA³⁶ before flow cytometry sorting. Total RNA was prepared from cells using a RNeasy micro kit (Qiagen) and then reverse-transcribed to obtain cDNA. qPCR was performed as described above.

Culture of HUVECs and siRNA-mediated knockdown

Human umbilical vein endothelial cells (HUVECs; Takara Bio) were maintained in

endothelial cell growth medium 2 (Takara Bio)³⁴ Only 2nd and 3rd passaged HUVECs were used for the following experiments. For the knock-down of CLIC2 expression, HUVECs were seeded at 50,000 cells/cm² and allowed to grow to 90%–100% confluency. Knockdown was performed using RNA interference as described previously⁴¹ The siRNA CLIC2 gene-targeting duplexes used were as follows: 5'-GCUAU AUUUGUGAUCAGAU TT-3' and 5'-AUCUGA UCACAAAUAUAGCTT-3' (Sigma-Aldrich). HUVECs were transfected with 20 nM of the labeled siRNA duplexes, SilenceMag siRNA delivery reagents (OZ Bioscience) on top of a magnetic plate (OZ Bioscience). The cells were incubated for 24 h with siRNA, then the culture medium was exchanged and the cells were maintained in fresh culture medium for 48 h. Western blotting was employed to confirm decreased CLIC2 protein expression. As a control, a siRNA duplex with an irrelevant sequence (irr-siRNA; 5'-GCGCGCUUUGUAGGAUUCGT

T-3' and 5'-CGAAUCCUACAAAGCGCGCTT-3', Dharmacon Research, Pittsburgh, PA) was used.

Transendothelial migration assays

Filter-based transendothelial migration assays were performed as described previously⁴² After HUVECs were incubated with irr- or CLIC2-knockdown siRNA, 50,000 cells were seeded on cups with polycarbonate membrane filter (8- μ m pore) bottoms set in 24-well culture plates (Corning). DMEM containing 10% fetal bovine serum (FBS) was added to the bottom of the plates and 0.5% bovine serum albumin (BSA; Sigma-Aldrich) was poured into the cups (Figure 10). Then, human tongue squamous cell carcinoma-derived cell line SAS cells were seeded into the cups (30,000 cells/cup) and cultured for 8 h. Cells that migrated to the bottom of the cups were stained with crystal violet and the cell areas were measured using ImageJ (Wayne Rasband, NIH)⁴³

Statistical analysis

Data expressed as means \pm SEM or SD were statistically analyzed using InStat3 software (GraphPad Software). Data were subjected to two-tailed Student's *t*-test (unpaired) or ANOVA with Tukey's post hoc test. Significance was set at $p < 0.05$ unless otherwise stated.

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