Thymidine kinase-1/CD31 double immunostaining for identifying activated tumor vessels

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Abstract

Although angiogenesis plays a crucial role in cancer growth and progression, no reliable method for assessing angiogenesis in tumor tissue sections currently is available. Using biomarkers with high specificity for proliferating endothelial cells could help quantify angiogenic activity. Thymidine kinase-1 (TK1) is an enzyme involved in the salvage pathway of DNA synthesis and its activity is correlated with cell proliferation. We investigated the use of double immunostaining for TK1 and CD31 for identifying activated tumor vessels. Differences in TK1/CD31 positive vessel rates (PVRs) between tumor and adjacent normal tissues were evaluated in 39 colorectal carcinoma (CRC) samples and compared with those of Ki67/CD31 double stained tissues. Mean TK1/CD31 PVR (23.6%) in CRCs was 13.9 fold greater than in adjacent normal tissues (1.7%)). By comparison, mean Ki67/CD31 PVR in CRCs was 20.0%, i.e., only 4.8 fold greater than in normal tissues (4.2%). Also, mean TK1/CD31 PVR in normal tissues was significantly less than mean Ki67/CD31 PVR. Our findings indicate that double immunostaining for TK1/CD31 detects activated tumor vessels more accurately than staining for Ki67/CD31 and could potentially identify tumors that will respond to anti-angiogenic therapy.

Key words: angiogenesis, CD31, colorectal carcinoma, double immunostaining, thymidine kinase 1

Angiogenesis plays an important key role in tumor growth by providing oxygen and nutrients to the tumor cells, and in metastasis by enhancing tumor cell entry into the circulation (Folkman 2002, Liotta and Stracke 1988, Pang and Poon 2006). Therefore, inhibition of tumor angiogenesis is a promising strategy for treatment of cancers. Molecular targeted therapies that use angiogenesis inhibitors have been applied to several cancer types including colorectal, non-small cell lung, breast and ovarian carcinomas, and glioblastoma (Braghiroli et al. 2012, Mountzios et al. 2014). Treatment of this type does not produce the same results in all patients and a more effective agent than endothelial cell markers is needed to aid clinicians in planning anti-angiogenic therapies (Hlatky et al. 2002).

The vascular status of human tumors can be assessed by microvessel density (MVD) using immunostaining of pan-endothelial cell markers such as CD31, CD34 and von Willebrand factor (factor VIII-related antigen), which stain both mature pre-existing vessels and new tumor vessels (Des Guetz et al. 2006, Hasan et al. 2002, Poon et al. 2003, Vermeulen et al. 1996). MVD, however, neither indicates the rate of ongoing angiogenesis nor the functional status of tumor blood vessels.

Evaluation of proliferating vessels in tumor tissues may provide more functional information concerning the tumor angiogenic activity than MVD (Hillen et al. 2006, Vermeulen et al. 1995). In addition, a tool for distinguishing new tumor caused vessels from normal pre-existing vessels would greatly facilitate assessment of tumor angiogenesis. The number of proliferating endothelial cells can be measured using double immunostaining of tumor tissue sections with antibodies against a cell

proliferation marker such as Ki67 and an endothelial cell marker such as CD31 or CD34 (Eberhard et al. 2000, Fox et al. 1993, Vartanian and Weidner 1994, 1995).

Thymidine kinase-1 (TK1, EC number 2.7.1.21) affects DNA synthesis by catalyzing phosphorylation that converts thymidine to deoxythymidine monophosphate (dTMP) in the salvage pathway (Kit 1985). The activity of TK1 is tightly controlled during the cell cycle; activity increases at the border of G_1/S-phases, peaks during late S-phase and is undetectable during M-phase (Sherley and Kelly 1988, Wang et al. 2001). TK1 is expressed in proliferating normal and malignant tissues (Shintani et al. 2010, Wang et al. 2001). A study in vitro suggests that TK1 is an important enzyme for modulating DNA synthesis and replication in endothelial cells (Junod et al. 1987).

Among the pan-endothelial cell markers, CD31 exhibits more sensitive and specific staining of microvessels in paraffin sections than other endothelial cell markers (Vermeulen et al. 1996). Taken together, double immunostaining for TK1 and CD31 may enable selective staining of activated tumor vessels.

We investigated the value of double immunostaining for TK1 and CD31 for distinguishing normal from tumor vessels. Our findings also were compared with those of double immunostaining for Ki67 and CD31. We found that TK1/CD31 double immunostaining is superior to Ki67/CD31 double immunostaining for identifying activated tumor vessels.

Material and methods

Tissue specimens

Our study was approved by the ethics committees of Kobe University Graduate School of Health Sciences and Fujita Health University School of Medicine. All patients provided informed consent for use of their tissue samples.

We used 39 colorectal carcinoma (CRC) samples from patients who had undergone surgery between 2003 and 2014 at Fujita Health University Hospital. All tissue samples had been fixed in 10% formalin and embedded in paraffin. After an initial review of all available hematoxylin and eosin (H & E) stained sections, one representative paraffin block was selected from each case. The corresponding adjacent normal tissues were also assessed. New sections were cut at 3 μm and mounted on aminopropyltriethoxysilane-coated slides.

Double immunostaining

The first staining was performed using the Histofine Simple Stain MAX PO system (Nichirei Bioscience, Tokyo, Japan). Sections were deparaffinized with xylene and rehydrated through ascending ethanols. Sections were incubated in 0.3% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase activity. Heat-induced antigen retrieval was performed in 10 mM Tris base containing 1 mM ethylenediaminetetraacetic acid (Tris-EDTA solution, pH 9.0) for 8 min at 120º C in a pressure cooker. After rinsing with 50 mM Tris-buffered saline containing 0.05% Tween 20, pH 7.6 (TBST), sections were incubated overnight at room temperature with anti-TK1 mouse

monoclonal antibody (clone F12; Bio-Rad, Hercules, CA) diluted 1:300 in 1% bovine serum albumin (BSA) in TBST. After rinsing with TBST, sections were incubated with an anti-mouse peroxidase-labeled polymer (Nichirei Bioscience**,** Tokyo, Japan) for 1 h at room temperature. Sections were then rinsed again with TBST. Color was developed using a diaminobenzidine solution (Dako, Glostrup, Denmark).

The second staining was performed using the Histofine Simple Stain AP system (Nichirei Bioscience). For antigen retrieval, sections were heated in Tris-EDTA solution for 3 min at 120º C in a pressure cooker. Sections were incubated overnight at room temperature with anti-CD31 rabbit monoclonal antibody (clone EP3095; Abcam, Cambridge, UK) diluted 1:200 in 1% BSA in TBST. After rinsing with TBST, sections were incubated with an anti-rabbit alkaline phosphatase-labeled polymer (Nichirei Bioscience) for 1 h at room temperature. The reaction products were detected using Fuchsin+ solution (Dako). After rinsing with tap water, sections were counterstained with Mayer's hematoxylin. Finally, sections were covered with an aqueous permanent mounting medium (CC/Mount; Diagnostic Biosystems, Pleasanton, CA), dried for 15 min at 55º C and mounted with Multi Mount 480 (Matsunami Glass, Osaka, Japan). Tumor and endothelial cells in each tissue section served as internal positive controls for TK1 and CD31, respectively. For negative controls, the sections were processed as above, except that the primary antibodies were replaced with TBST.

Evaluation of double immunostaining

The immunostained sections were reviewed by four investigators (S.O., T.O., K.N., and H.O.). We selected four high density vascular areas ("hot spots") from tumor and

adjacent normal tissues using an optical microscope at 100 x. We evaluated only hot spots close to tumor cell clusters in viable areas; necrotic areas were excluded. To minimize inter-observer error in vessel counting, we identified highly vascular areas by marking individual hot spots on the slide glass (2.54 mm^2/field) using a Self Inking Object Marker (Nikon, Tokyo, Japan). In these areas (0.95 mm^2/field), total CD31-positive vessels, TK1/CD31 double positive and Ki67/CD31 double positive vessels were counted at x 200. To obtain the TK1/CD31 positive vessel rate (PVR) or Ki67/CD31 PVR, numbers of TK1/CD31 or Ki67/CD31 double positive vessels were divided by number of total vessels.

Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics 25 software (International Business Machines Corporation, Armonk, NY). Comparisons of TK1/CD31 PVR and Ki67/CD31 PVR in tumor and adjacent normal tissues were performed using the Wilcoxon sign rank test. Values for $p \leq 0.05$ were considered significant.

Results

TK1 and Ki67 appeared as brown nuclear staining and CD31 appeared as red membrane staining. Tumors exhibited heterogeneous distribution of vascular density and TK1/CD31 or Ki67/CD31 positive vessels.

Table 1 and Fig. 1 show TK1/CD31 PVR or Ki67/CD31 PVR in CRCs and adjacent normal tissues. Means ± SD for TK1/CD31 PVR were for tumor tissues: 23.6% ± 9.0 (median: 23.5%, range: 6.0–45.0%) and normal tissues: 1.7% ± 1.9 (median: 1.4%, range: 0 −9.6%; $p < 0.0001$). Means ± SD for Ki67/CD31 PVR were for tumor tissues: 20.0% ± 10.9 (median: 17.9%, range: 3.0–49.0%) and normal tissues: 4.2% ± 5.6 (median: 2.4%, range: 0–24.5%; $p < 0.0001$). Therefore, mean TK1/CD31 PVR and Ki67/CD31 PVR in tumor tissues were 13.9 fold and 4.8 fold higher, respectively, than for normal tissue.

The TK1/CD31 PVR was significantly higher than Ki-67/CD31 PVR in tumor tissues ($p = 0.012$), but significantly lower than the Ki67/CD31 PVR in normal tissues ($p < 0.0001$), which indicates that TK1 is more specific for activated tumor vessels than Ki-67 in CRC. Representative TK1/CD31 or Ki67/CD31 double immunostaining images in CRC and adjacent normal tissue are shown in Fig. 2.

Discussion

Anti-angiogenic therapy could prolong survival time in patients with colorectal, non-small cell lung, breast and ovarian carcinomas and glioblastoma if added to standard chemotherapy (Braghiroli et al. 2012, Mountzios et al. 2014). This treatment, however, does not produve the same results in all patients. Therefore, companion diagnostic assays must be developed to assess a patient's angiogenic status to identify who will benefit most from anti-angiogenic therapy.

TK1 catalyzes phosphorylation of thymidine for salvage synthesis of deoxythymidine monophosphate (dTMP); therefore, it plays a key role in the DNA synthesis (Kit 1985). TK1 is thought to be a key enzyme associated with DNA synthesis in endothelial cells (Junod et al. 1987). Conversely, Ki67 protein, the most commonly used proliferation marker, is present during all active phases of the cell cycle, G_1, S, G_2, and M, but is absent during resting phase, G_0 (Scholzen and Gerdes 2000). Therefore, Ki-67 is considered a useful marker for proliferating tumor cells.

The literature includes several reports of assessment of proliferating vessels in tissue sections using double immunostaining techniques. Combinations of antibodies to a proliferation marker, e..g., Ki67, proliferating cell nuclear antigen (PCNA), 5-bromo-2′-deoxyuridine (BrdU), and an endothelial cell marker, CD31 or CD34, have been used frequently (Vermeulen et al. 1995, Hillen et al. 2006, Fox et al. 1993, Vartanian and Weidner 1994, 1995, Eberhard et al. 2000). To our knowledge, however, no immunohistochemical analysis of proliferating vessels has used TK1. We developed a TK1/CD31 double immunostaining technique and investigated its efficacy for

identifying activated tumor vessels. Ki67/CD31 double immunostaining was assessed also for comparison.

In CRCs, intratumor TK1/CD31 PVR (mean: 23.6%) was 13.9 fold greater than that observed in adjacent normal tissues (mean: 1.7%; $p < 0.0001$). Conversely, mean Ki67/CD31 PVR in CRC tissues was 20.0−4.8 fold greater than that in normal tissues (4.2%; $p < 0.0001$). In addition, the PVRs for TK1/CD31 in normal tissues were significantly lower than those for Ki67/CD31 ($p < 0.0001$). The difference of mean TK1/CD31 PVR between CRC and adjacent normal tissues was greater than that of mean Ki67/CD31 PVR (13.9 fold vs 4.8 fold). In addition, the PVRs for TK1/CD31 in normal tissues were significantly lower than those for Ki67/CD31. These findings indicate that TK1/CD31 double immunostaining can be used to detect activated tumor vessels more accurately than Ki67/CD31 double immunostaining.

Using Ki67/CD31 double immunostaining, Vermeulen et al. (1995) reported that the number of Ki67/CD31 positive vessels was higher in the vascular hotspots of CRC tissues than in the adjacent normal mucosa; the difference was 23-fold. These investigators reported that mean Ki67/CD31 PVR in CRC tissues was similar (21.0 vs. 20.0%), but lower in normal mucosa (1.5 vs. 4.2%) compared to our findings, The differences may be due to the numbers of tumors examined, or use of different primary antibodies, antigen retrieval methods or detection systems.

Our findings suggest that DNA synthesis via the salvage pathway, in which TK1 plays a key role, is active in tumor vessels of CRCs, but that it is suppressed in pre-existing normal vessels. The direct relation between TK1 and angiogenetic factors, however, including vascular endothelial growth factor, is unclear.

TK1 is a specific marker for activated tumor vessels. When used with CD31 in double immunostaining, TK1 distinguishes tumor and normal vessels and could potentially identify tumors that are likely to respond to anti-angiogenic therapy. Further investigation is required, however, to verify our findings and to validate their clinical use as a companion diagnostic assay for anti-angiogenic therapies.

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Fig. 1. PVR for TK1/CD31 and Ki67/CD31 in colorectal carcinomas and adjacent normal tissues. Bar = mean value.

Fig. 2. Representative examples of TK1/CD31 (A, B) or Ki67/CD31 (C, D) double immunostaining of colorectal carcinomas (A, C) and adjacent normal tissues (B, D). TK1/CD31 positive vessels (arrows) are more common in tumor tissues, but not in normal tissues; whereas Ki67/CD31 positive vessels (arrows) are common in tumor tissues, but are seen also in normal tissues. TK1 and Ki67 are expressed also in tumor cell nuclei.

Table 1. PVR for TK1/CD31 and Ki67/CD31 in colorectal carcinomas and adjacent normal tissues

	Tumor tissues			Normal tissues		
	Mean ± SD	Median	Range	Mean ± SD	Median	Range
TK1/CD31 PVR	23.6 ± 9.0	23.5	6.0–45.0	1.7 ± 1.9	1.4	0–9.6
Ki67/CD31 PVR	20.0 ± 10.9	17.9	3.0–49.0	4.2 ± 5.6	2.4	0–24.5

TK1/CD31 PVR in tumor vs. normal tissues: $p < 0.0001$. Ki67/CD31 PVR in tumor vs. normal tissues: $p < 0.0001$. TK1/CD31 PVR vs. Ki67/CD31 PVR in tumor tissues: $p = 0.012$. TK1/CD31 PVR vs. Ki67/CD31 PVR in normal tissues: $p < 0.0001$.

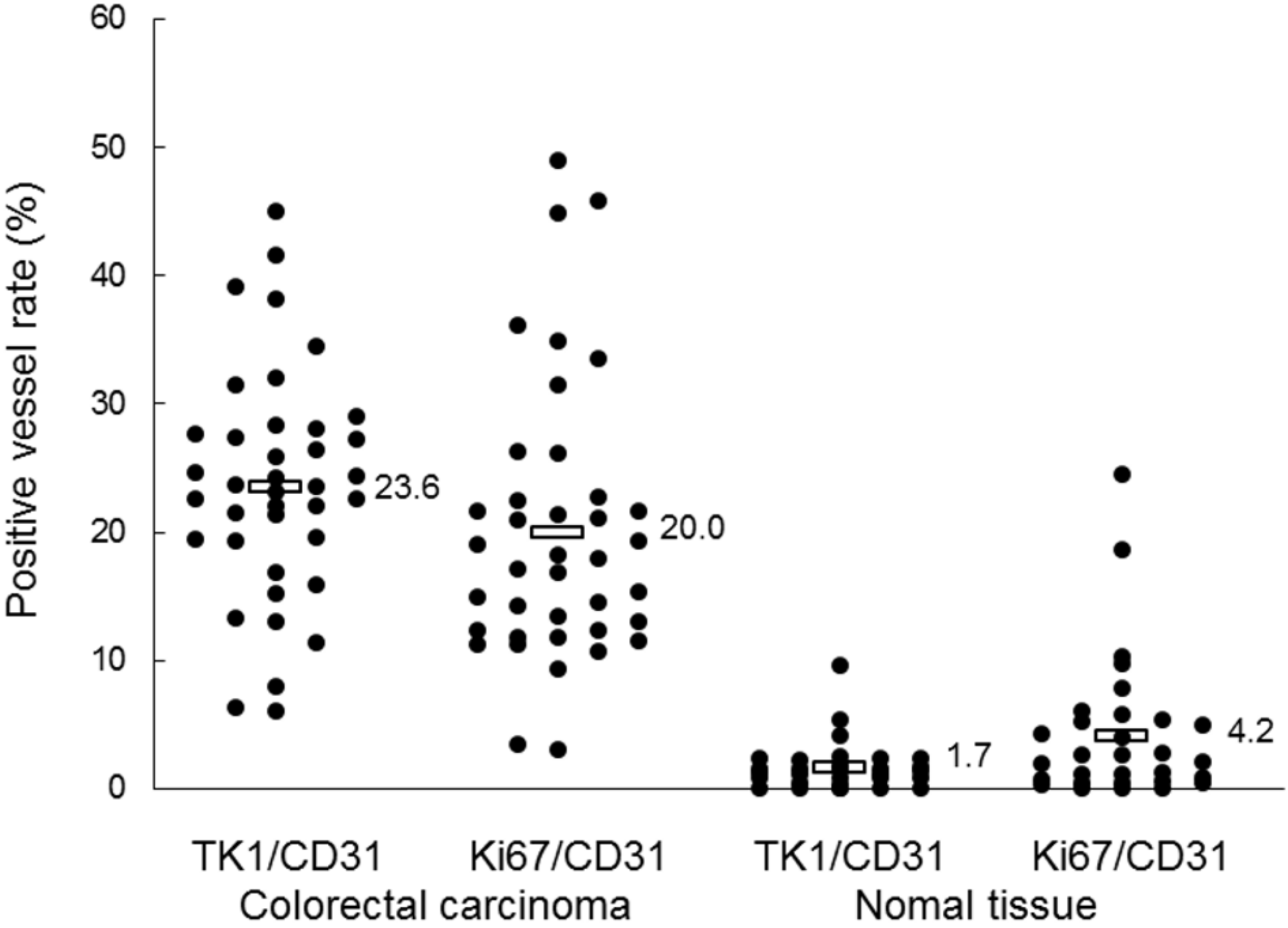
60
50
40
30
20
10
0
Positive vessel rate (%)
23.6
20.0
1.7
4.2
TK1/CD31
Ki67/CD31
TK1/CD31
Ki67/CD31
Colorectal carcinoma
Nomal tissue

Figure 1

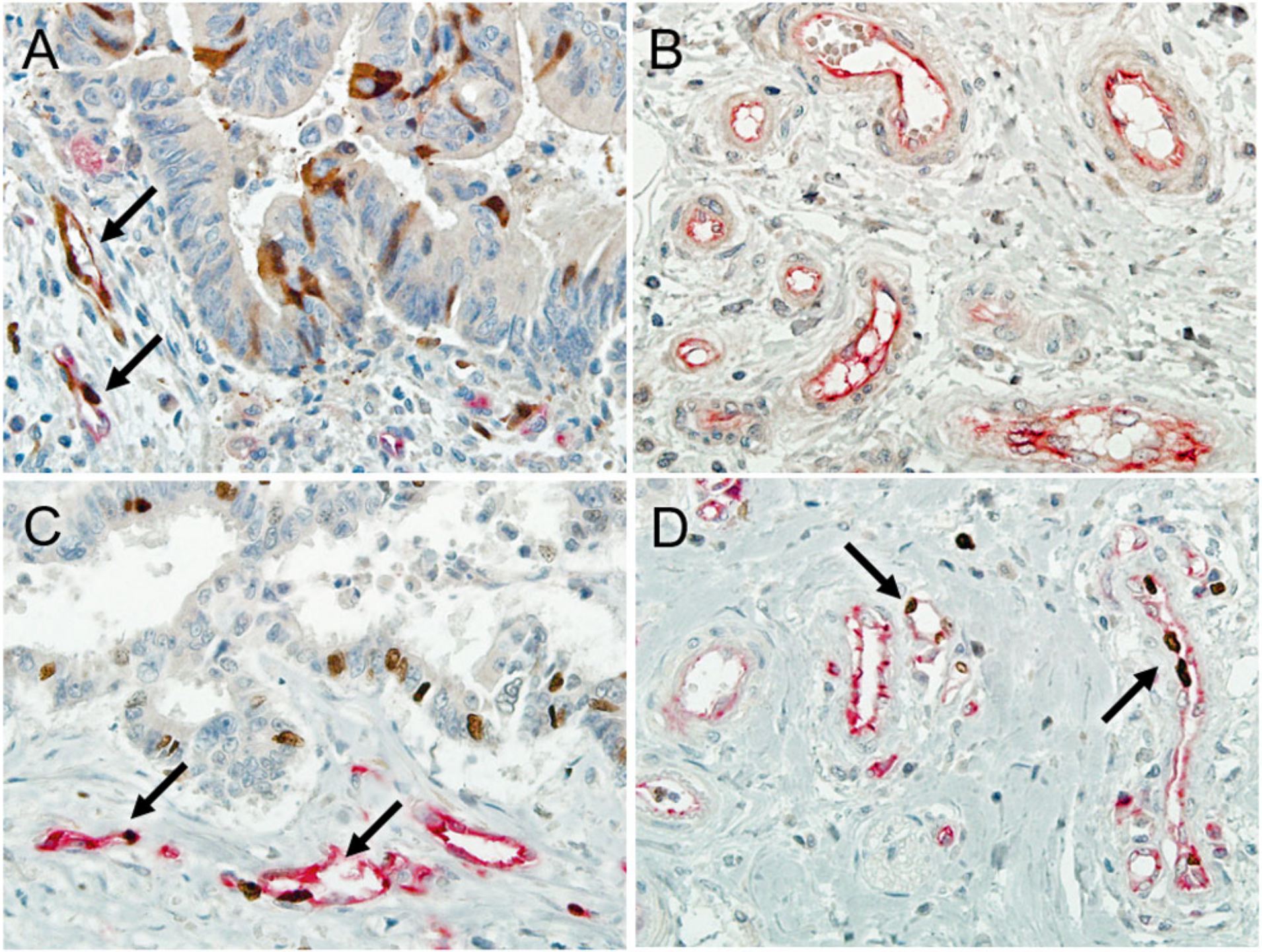

Figure 2