Unenhanced areas revealed by contrast-enhanced abdominal ultrasonography with Sonazoid™ potentially correspond to colorectal cancer

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Abstract. The present study investigated the potential utility of contrast-enhanced abdominal ultrasonography (CEUS), using Sonazoid™, in colorectal cancer (CRC). Three patients were subjected to CEUS with Sonazoid™. Surgical specimens were immunostained for CD31. Numbers of blood vessels positive for CD31 were analyzed in each of five fields at x400 magnification and averaged to determine blood vessel density. Blood vessel density was compared between non-tumorous and tumorous areas. Prior to the administration of Sonazoid™, CRC was illustrated as irregular-shaped wall thickening. One minute after the administration of Sonazoid™, the majority of the thickened wall was enhanced, while some parts of the thickened wall remained unenhanced. Blood vessel densities of non-tumorous and tumorous areas in patient two were 25.2±2.5 and 5.2±1.1 ($P<0.0001$). Blood vessel densities of non-tumorous and tumorous areas in patient three were 19.0±3.1 and 2.2±0.8 ($P<0.0001$). Tumorous areas of CRC were not enhanced 1 min after the administration of Sonazoid™. Blood vessel density was lower in tumorous areas compared with non-tumorous areas, as evidenced by immunohistochemistry for CD31. These findings suggest that CEUS may be useful for the determination of the extent of CRC.

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Introduction

Colorectal cancer (CRC) is commonly observed in clinical settings (1). To improve the prognosis in patients with CRC, prompt and accurate diagnosis is essential. Screening for CRC is performed using fecal occult blood testing, and is diagnosed with colonoscopy (2).

Abdominal ultrasound (US) is useful for the safe and easy diagnosis of patients (3-6). During US screening of the abdomen, CRC is sometimes encountered (7). A thickened colonic wall has been reported as a criteria for the diagnosis of CRC (8). Changes in stratification and contour illustrated with abdominal US are associated with the depth of invasion, in either the subserosa or the extra subserosa (7).

Contrast-enhanced ultrasonography (CEUS) enables the evaluation of tissue vascularity with low blood flow velocity (9). Primary or metastatic liver tumors are the most common indication examined by CEUS (10,11). Regarding the alimentary tract, guidelines put forth by the European Federation of Societies for Ultrasound in Medicine and Biology recommend use of CEUS in inflammatory bowel disease for diagnosis, activity assessment, and examining complications such as stenosis or fistula (9). In addition, CEUS is useful for the diagnosis of gastrointestinal bleeding (12).

Sonazoid™ consists of perfluorocarbon microbubbles with a median diameter ≤3 mm, which are stable during examination and act as a strong contrast agent (13). Sonazoid™ is primarily used for the management of hepatocellular carcinoma, while no reports yet exist regarding the use of Sonazoid™ in CRC (14,15).

In the present study, we analyzed CEUS images using Sonazoid™ to examine its usefulness in the diagnosis of CRC. Blood vessel density was compared between tumorous areas and non-tumorous areas and supplemented by immunostaining for cluster of differentiation (CD)31, a pan-endothelial cell marker (16).

Materials and methods

Patients. Between August 2011 and May 2015, 13 patients were diagnosed with CRC using US screening at the National Hospital Organization Shimoshizu Hospital (Yotuskaido, Japan). Of those, three patients were subjected to CEUS with Sonazoid™ to examine for liver metastasis. In all cases, CRC was observed prior to examination for liver metastasis. The study was approved by the National Hospital Organization Shimoshizu Hospital Ethics Committee, and written informed consent was obtained from all three patients. All procedures followed were in accordance with ethical standards put forth by the responsible institutional and national committees on human experimentation, and with the Helsinki Declaration of 1964 and later versions. Patient characteristics are listed in Table I. Clinical parameters analyzed by blood tests were white blood cell count, hemoglobin, C-reactive protein, carcinoembryonic antigen and carbohydrate antigen 19-9.

Patients two and three consented to further investigation, and agreed to provide surgical specimens for analysis.

Abdominal US and administration of Sonazoid™. Abdominal US was performed by Senior Fellows of the Japan Society of Ultrasonics in Medicine using a SSA-700A US system (Toshiba Medical Systems Corporation, Ohtawara, Japan) with a 3.75-MHz curved-array probe (PVT-375BT; Toshiba Medial Systems Corporation) or an 8.0-MHz linear-array probe (PLT-805AT; Toshiba Medical Systems Corporation). Sonazoid™ (Daiichi Sankyo Co., Ltd., Tokyo, Japan) was administered intravenously at 0.015 ml/kg following the manufacturer's instruction.

Criteria for the diagnosis of CRC. The diagnostic criteria for CRC used were localized irregular wall thickening or a hypoechoic mass mixed with hyperechoic lesions (a pseudokidney sign) (8). The former is a common finding in patients with CRC (17), and the latter represents tumor tissue with air in the residual lumen (18).

Pathological analysis and immunostaining. The depth of invasion by the CRC was determined by pathologists analyzing specimens obtained via surgical resection, using standard histological methods. Immunostaining proceeded as follows. Serial sections were cut from formalin-fixed paraffin-embedded surgical samples. The sections were deparaffinized, and autoclaved in 0.05 M citrate buffer at pH 6.0. Endogenous peroxidase was inactivated by incubating with 0.1% hydrogen peroxide in 100% methanol for 30 min at 4˚C. To prevent non-specific antibody binding, the sections were incubated with 2% normal goat serum (Cappel, Aurora, OH, USA) in phosphate-buffered saline for 30 min at 4˚C. Staining for CD31 was used as a marker of vascular endothelial cells (19). After a 4˚C overnight incubation with mouse anti-human CD31 antibody at a 1:100 dilution (3528S; Cell Signaling Technology, Inc., Danvers, MA, USA), sections were incubated at 4˚C for 2 h with alkaline phosphatase-labeled goat anti-mouse IgG with a 1:1,000 dilution (S3721; Promega Corporation, Madison, WI, USA). Subsequently, Vector Red Substrate (Vector Laboratories, Inc., Burlingame, CA, USA) was applied to the sections as a chromogen. The nuclei were counterstained with hematoxylin (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) for 10 sec. Specimens were observed and photographed under an AX80 microscope (Olympus Corporation, Tokyo, Japan). To determine blood vessel density, the number of blood vessels staining positive with CD31 were counted per field at x400 magnification under the microscope. The number of positive blood vessels across five fields for each patient was examined and the average determined.

Statistical analysis. Blood vessel densities were compared between tumorous areas and corresponding non-tumorous areas by using a one-factor analysis of variance. Statistical analysis was performed using JMP 10.0.2 software (SAS Institute, Cary, NC, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Contrast-enhanced ultrasonography of colorectal cancer. Prior to the administration of Sonazoid™, a thickened colonic wall with an irregular shape was observed in patients one, two and three, as shown in Fig. 1A-C, respectively. One minute after administration, most of the thickened wall was enhanced in patients one, two and three, as shown in Fig. 1D-F, respectively, with some parts of the thickened wall remaining unenhanced (indicated by arrows). Ten minutes after the administration of Sonazoid™, the enhanced areas of the thickened wall returned to a hypoechoic state in patients one, two and three, depicted in Fig. 1G-I, respectively. The shapes of the unenhanced areas resembled those of tumorous areas identified in post-surgical samples from patients two (Fig. 1J) and three (Fig. 1K). These results suggest that areas remaining unenhanced 1 min after the administration of Sonazoid™ may be tumorous. It might be postulated that the blood vessel density was lower in tumorous areas compared with surrounding non-tumorous areas.

Vascular structure of colorectal cancer. To compare the blood vessel densities between tumorous areas and the surrounding non-tumorous areas, immunostaining with an antibody to CD31 was performed. Varieties of blood vessel sections were observed (Fig. 2A). Specimens remained negative in the absence of incubation with anti-CD31 antibody (Fig. 2B). All positive signals observed were thought to be blood vessels. Surgical specimens from patients two (Fig. 2C and D) and three (Fig. 2E and F) were subjected to anti-CD31 immunostaining. Non-tumorous areas (Fig. 2C and E) exhibited more positive signals compared with tumorous areas (Fig. 2D and F). Measurements of blood vessel densities for non-tumorous areas and tumorous areas in patient two (25.2±2.5 and 5.2±1.1, respectively) were found to be significantly different ($P<0.0001$; Fig. 2G). Similarly, measurements of blood vessel densities for non-tumorous areas compared with tumorous areas in patient three (19.0±3.1 and 2.2±0.8, respectively) were found to be significantly different ($P<0.0001$; Fig. 2H). These results clearly indicate that blood vessel densities were significantly lower in tumorous areas compared with non-tumorous areas.

Discussion

During CEUS, non-tumorous areas are enhanced by using Sonazoid™. In the liver, non-tumorous areas become diffusely enhanced while liver abscesses and metastases remained

Table I. Patient characteristics.

	Normal range	Patient 1	Patient 2	Patient 3
Age		74	73	84
Gender		Male	Female	Female
Location		Sigmoid	Ascending	Ascending
Pathology		Moderate	Well	Well
Size (cm)		3.7	5.5	5.5
Depth		pSS	pSS	pSS
WBC, /μl	3500-8500	19000	5200	4400
Hb, g/dl	13.5-17.0	13.7	6.4	5.9
CRP, mg/dl	0.00-0.30	2.8	0.17	0.16
CEA, ng/ml	0.0-5.0	44.2	2.9	48.4
CA19-9, U/ml	0.0-37.0	47.3	7.6	27.3

WBC, white blood cell count; Hb, hemoglobin; CRP, c-reactive protein; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; Sigmoid, sigmoid colon; Ascending, ascending colon; Well, well-differentiated tubular adenocarcinoma; Mod, moderately differentiated tubular adenocarcinoma; pSS, invasion to subserosa.

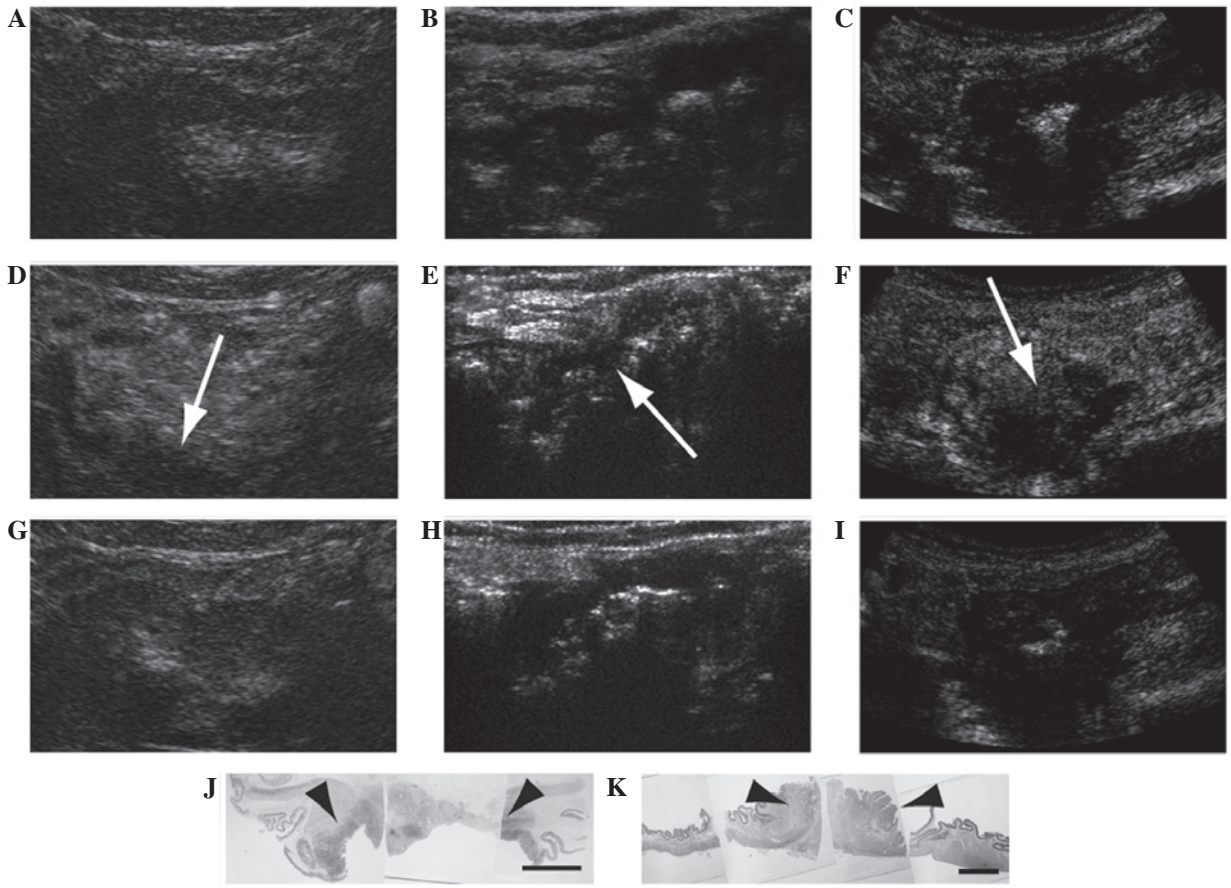

Figure 1. Contrast-enhanced ultrasonography of colorectal cancer. (A-C) Colonic wall showing thickening, irregular shape and low echo, and (D-F) 1 min after and (G-I) 10 min after administration of Sonazoid™. Patients (A, D, G) one, (B, E, H) two and (C, F, I) three showed the same enhancement trend. (B, E, H) The thickened wall was enhanced with Sonazoid™ 1 min after its administration. (D, E, F) Part of the thickened wall remained unenhanced (indicated by arrows). (D, F) Shapes of tumorous areas (indicated by arrowheads) resembled those of the unenhanced areas in patient (J) two and (K) three. Scale bar, 10 mm.

unenhanced (20,21). In the present study, CEUS revealed enhancement of non-tumorous areas, while CRC tissue remained unenhanced while using Sonazoid™. Previous studies have shown the bowel to be diffusely enhanced under examination with CEUS (22). Tumorous areas of CRC have been reported to not be enhanced with CEUS (23). The results of previous studies are in accordance with this previously published literature (20-23). Previous reports and the results from the present

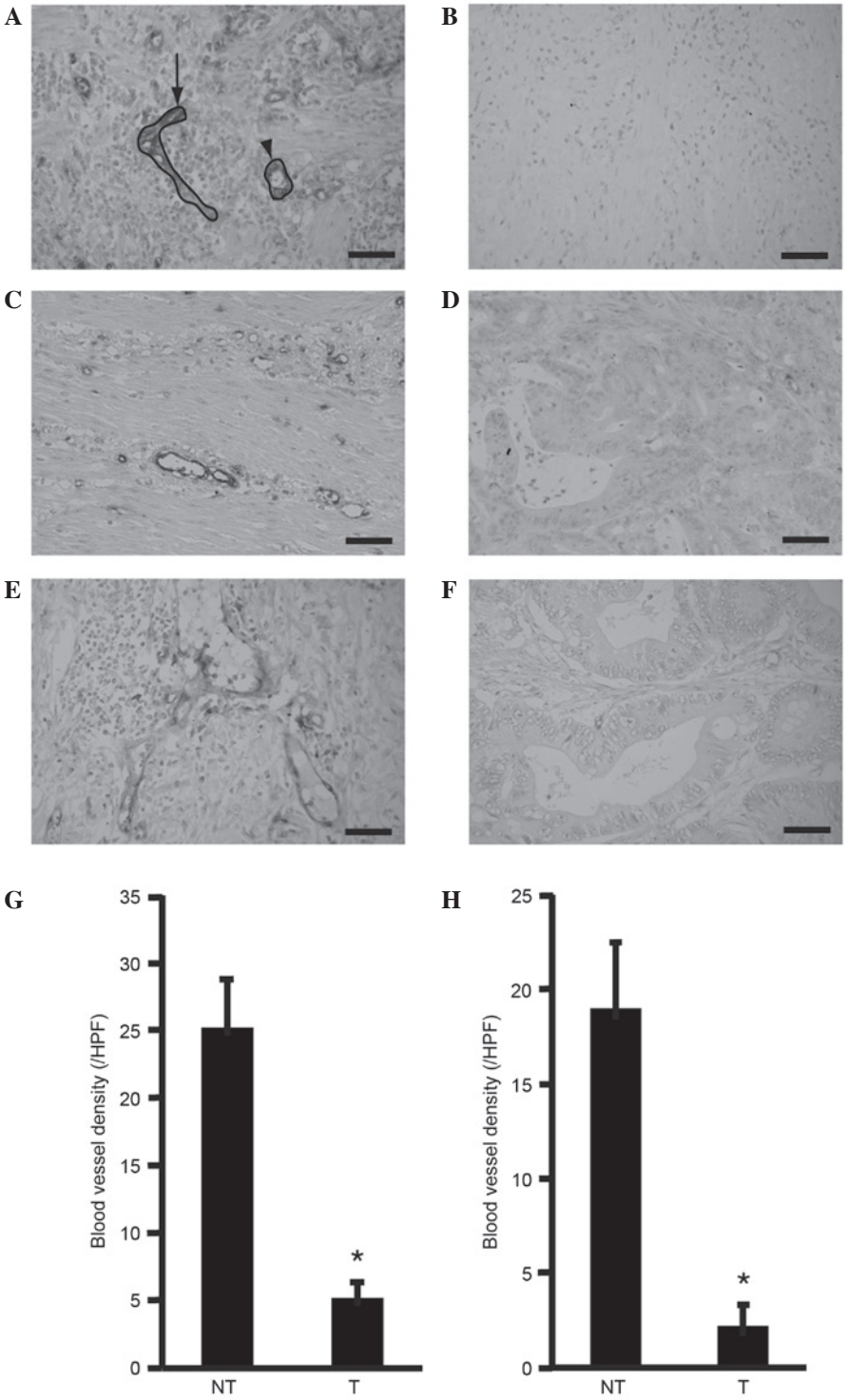

Figure 2. Blood vessel density in tumorous and non-tumorous tissues. Surgical specimens were immunostained for CD31. Longitudinal (arrow) and cross (arrowhead) sections of blood vessels were observed, and (A) examples outlined. Each section was counted as one blood vessel. (B) A control section without staining for CD31 showed no signal. Sections from patients (C, D) two and (E, F) three were examined. (C, E) Non-tumorous areas exhibited more blood vessels than (D, F) tumorous areas. The number of blood vessels was counted in each of five fields at x400 magnification, and then averaged to determine the blood vessel density. The blood vessel density was compared between non-tumorous and tumorous areas in patients (G) two and (H) three. Original magnification: 400x. Scale bar, 50 μm. Error bar, standard deviation. *P<0.05 vs. NT. n=5. CD31, cluster of differentiation 31; HPF, high power field; NT, non-tumorous; T, tumorous.

study clearly suggest that under examination with CEUS using Sonazoid™ non-tumorous areas are enhanced, while tumorous areas remain unenhanced in CRC. In the present study, vascular structure was not evaluated. Vascular structure is often irregular in CRC and this can be evaluated using CEUS (24).

In the present study, blood vessel densities were found to be lower in tumorous areas as compared with non-tumorous areas. A number of prior studies have investigated blood vessel density in tumorous areas (25). Blood vessel density was demonstrated to not correlate with histological grade in

CRC (26). Additionally, the number of blood vessels positive for CD31 was found to be lower in tumorous areas compared with non-tumorous areas (23). The results of the present study are consistent with these prior reports, and may be supported by the fact that intensity of enhancement in CEUS positively correlates with the density of blood vessels (27).

The use of Sonazoid™ in CEUS may be useful for the diagnosis of cancers other than primary or metastatic liver tumors. For example, metastasis has been successfully diagnosed in axillary lymph nodes of patients with breast cancer using Sonazoid™ (28). Clinical trials are currently being performed, aiming to differentiate benign and malignant focal lesions in the breast (29). The present study shows a possible application for Sonazoid™ in CRC. In the future, Sonazoid™ may be used for the diagnosis of tumors other than those in the liver.

One major limitation of this study was that it was based on a small number of patients. The next step would be to increase the number of patients under investigation.

In conclusion, during examination by CEUS, tumorous areas of CRC were not enhanced 1 min after the administration of Sonazoid™. In addition, blood vessel density was lower in tumorous areas compared with non-tumorous areas as evidenced by immunohistochemistry with CD31. These findings suggest that CEUS may be useful for the determination of the extent of CRC.

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