Detection of sentinel lymph node using contrast-enhanced agent, Sonazoid (TM) , and evaluation of its metastasis with superb microvascular imaging in oral and oropharyngeal cancers: a preliminary clinical study

著者	Wakisaka Naohiro, Endo Kazuhira, Kitazawa Tomohiro, Shimode Yuzo, Kato Koroku, Moriyama-Kita Makiko, Koda Wataru, Ikeda Hiroko, Ishikawa Kazuya, Ueno Takayoshi, Nakanishi Yosuke, Kondo Satoru, Sugimoto Hisashi, Yoshimura Kenichi, Tsujii Hiroyuki, Kawashiri Shuichi, Omoto Kiyoshi, Yoshizaki Tomokazu
著者別表示	脇坂 尚宏, 遠藤 一平, 加藤 広禄, 喜多 万紀子, 香田 渉, 池田 博子, 石川 和也, 上野 貴雄, 中西 庸介, 近藤 悟, 杉本 寿史, 吉村 健一, 川尻 秀一, 吉崎 智一
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Title page

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Detection of sentinel lymph node using contrast-enhanced agent, Sonazoid™, and evaluation of its metastasis with Superb Microvascular Imaging in oral and oropharyngeal cancers: A preliminary clinical study

Authors

Naohiro Wakisaka[1*], Kazuhira Endo[1], Tomohiro Kitazawa[2], Yuzo Shimode[3], Koroku Kato[4], Makiko Moriyama-Kita[1], Wataru Koda[5], Hiroko Ikeda[6], Kazuya Ishikawa[1], Takayoshi Ueno[1], Yosuke Nakanishi[1], Satoru Kondo[1], Hisashi Sugimoto[1], Kenichi Yoshimura[7], Hiroyuki Tsuji[3], Shuichi Kawashiri[4], Kiyoka Omoto[8], Tomokazu Yoshizaki[1]

Institutions

[1] Department of Otolaryngology, and Head & Neck Surgery, Kanazawa University, Kanazawa, Ishikawa, Japan,

[2] Canon Medical Systems, Ohtawara, Tochigi, Japan,

[3] Department of Head and Neck Surgery, Kanazawa Medical University,

Uchinada, Ishikawa, Japan,

[4] Department of Oral and Maxillofacial Surgery, Kanazawa University, Kanazawa, Ishikawa, Japan,

[5] Department of Radiology, Kanazawa University, Kanazawa, Ishikawa, Japan,

[6] Division of Pathology, Kanazawa University, Kanazawa, Ishikawa, Japan,

[7] Department of Biomedical Statistics, Innovative Clinical Research Center, Kanazawa University, Kanazawa, Ishikawa, Japan,

[8] Department of Laboratory Medicine, Saitama Medical Center, Jichi Medical University, Saitama, Japan

Corresponding author*

Naohiro Wakisaka, M.D., Ph.D.

Department of Otolaryngology, and Head & Neck Surgery, Kanazawa University, Takara-machi 13-1, Kanazawa 920-8640, JAPAN

Phone: +81-76-265-2413

Fax: +81-76-234-4265

E-mail: wakisaka@med.kanazawa-u.ac.jp

Abstract

Background: In sentinel lymph node (SLN) biopsy for head and neck cancers, the radioisotope method has been the gold standard. However, this method has several problems, such as unavoidable radiation exposure and requirements of expensive equipment.

Aims/Objectives: To overcome these problems, we evaluated the contrast-enhanced ultrasonography (CEUS)-guided SLN-detection method, and predicted the SLN metastatic status using novel ultrasound technology, Superb Microvascular Imaging (SMI).

Methods: Ten patients (6 with oral, and 4 with oropharyngeal cancers) without neck lymph node metastasis were enrolled in this study. Ultrasound contrast agent, Sonazoid™, was infiltrated into the mucosa at the primary site to observe the lymphatic ducts and SLNs in the neck field. The detected SLNs were examined for blood flow using SMI to categorize the SLNs metastases-positive or negative.

Results: SLNs were successfully detected in 8 out of 10 cases. In 7 out of the 8 cases, in whom SLNs were successfully detected, metastatic status of SLNs

were correctly diagnosed with SMI.

Conclusions and significance: Although more clinical data are needed based on a larger cohort, establishing the CEUS-guided SLN-detection and criteria for the accurate diagnosis of SLN-metastases using SMI would be valuable as an alternative to radioisotope method, in oral and oropharyngeal cancers.

Keywords

Contrast-enhanced ultrasonography, oral cancer, oropharyngeal cancer, sentinel lymph node, Sonazoid™, Superb Microvascular Imaging.

Introduction

A randomized controlled trials and a meta-analysis of five randomized controlled trials showed higher overall and disease-free survival rates following elective neck dissection (ipsilateral neck dissection at the time of the primary surgery) than therapeutic neck dissection (watchful waiting followed by neck dissection for nodal relapse) in early-stage T1 or T2 oral squamous cell carcinoma (OSCC) patients with clinically node-negative (cN0) disease[1,2]. Currently, elective neck dissection of lymph node levels I

to III, also known as supraomohyoid neck dissection, is commonly used in the management of cN0 patients with OSCC because levels IV and V rarely harbor nodal metastases [3].

Regarding therapeutic neck dissection for oropharyngeal cancers, Lim et al. showed that the incidence rate of metastasis at level IV (37%) was higher than at level I (10%). In addition, patients with level IV metastasis showed a significantly lower survival rate compared with those with other levels. Therefore, for oropharyngeal squamous cell carcinoma (OPSCC) with cN0, they recommended elective neck dissection for levels II to IV [4].

Lymphatic metastases develop in 20 to 30% of cN0 patients with early oral and oropharyngeal cancers after watchful waiting and are associated with decreased survival [5,6]. Most surgeons favor the resection of regional lymphatics for cN0 disease, based on these data; however, 70 to 80% of patients ultimately are pathologically N0 and would theoretically be over-treated. However, it causes quantifiable morbidity, such as shoulder dysfunction due to accessory nerve injury, lower lip paresis, and chylous leakage.

Sentinel lymph node (SLN) biopsy has been used as an alternative or

additional staging procedure [5,7]. The concept of SLN biopsy is based on the fact that, in head and neck squamous cell carcinoma (SCC), the process of lymphatic metastasis generally follows an orderly and predictable pattern of progression, beginning with the SLN, before progression to other lymph nodes in the nodal basin. Thus, the SLN status predicts the presence of metastasis in the remainder of the nodal basin. The technique to identify SLN uses a radiolabeled colloid with or without colored dye as a tracer injected into the primary site, and the gamma probe for identifying SLN is now well standardized in head and neck SCC[7,8]. Although SLN biopsy is still an invasive procedure, it causes less morbidity than elective neck dissection [7,9]. However, several problems exist regarding its applicability. While using radiolabeled particles, the exposure of patients and medical staff to radioisotopes is unavoidable. The gamma probe is simple to operate and can identify SLNs, but the gamma probe-guided method requires expensive equipment and facilities, which has hampered the increasing use of this method. In addition, due to the nature of radiolabeled colloids, they are retained for prolonged periods within the injected site, which will contribute to the phenomenon of the shine-through effect that has been associated with

lower identification rates of SLN associated with oral tumors, especially for tumors located in the floor of the mouth [7,8]. Another limitation of the SLN biopsy procedure is that because we have no reliable technique to analyze the SLN. Therefore, in case of a positive SLN, it is a two-step procedure [10]. Blue dye is a routinely used non-radioactive tracer for SLN biopsy [7]. As the SLNs are stained within 10 to 15 minutes after injection of the dye at the primary site, surgeons must be skillful enough to detect the SLNs within this limited time.

To overcome these drawbacks, here, we investigated a simpler, easier, and yet reliable and accurate method using contrast-enhanced ultrasonography (CEUS) with Sonazoid™ (Daiichi-Sankyo Pharmaceuticals, Tokyo, Japan), a new-generation contrast agent for ultrasonography (US), to identify SLN associated with oral and oropharyngeal cancers. Sonazoid™ is a perflubutane microbubble that is stabilized using hydrogenated egg phosphatidyl serine sodium. Omoto et al. first reported an SLN detection method using CEUS by the subareolar injection of Sonazoid™ as a tracer, in breast cancer patients. In a preliminary study, they could observe contrast-enhanced SLNs in 14 of 20 patients [11]. In the next series of 32

breast cancer patients, all SLNs were identified using the same protocol [12]. In the current study, we detected SLNs and evaluated adverse events, as a primary endpoint, by the mucosal injection of Sonazoid™ around the oral and oropharyngeal cancers. Furthermore, we observed the blood flow distribution in the detected SLNs, using Superb Microvascular Imaging (SMI), to evaluate the metastatic status. SMI is an innovative Doppler ultrasound technique specifically for imaging very low flow states, which uses a unique algorithm that allows visualization of minute vessels with a slow velocity without using a contrast agent.

Methods

Patients

This study was approved by the Bioethics Committee of Kanazawa University (Nos.2016-037 and 2017-015). The clinical study protocol was explained in detail to patients eligible for the study. Written consent was obtained from all patients who agreed to participate.

Between April 2017 and August 2018, 10 patients (median age: 61 years, range: 29-76 years) with a pathological diagnosis of oral or

oropharyngeal cancer by tissue biopsy from the primary sites were enrolled. It was confirmed that they did not have distant metastases by contrast enhanced computed tomography findings. All cases were in the clinically N0 category of the UICC tumor-node-metastasis (TNM) classification [13]. Clinical positivity was determined by physical examination, and CT findings according to the following criteria: a dimeter of > 15 mm for level I and II lymph nodes; a diameter of > 10 mm for level III, IV, and V lymph nodes; a retropharyngeal node diameter of > 8 mm; central necrosis; a maximum diameter on the affected side at least twice that on the unaffected side; extracapsular extension presenting as an unclear border of the lymph node; and the presence of > 3 fused lymph nodes [8]. The clinical and pathological findings are summarized in Table.

Imaging examination

On the day before surgery, divided injections of 2 mL of Sonazoid™ were gently administered into 4 mucosal regions around the primary tumor in the oral cavity or oropharynx using a 22G-needle. In case 1, Sonazoid™ was directly injected into the tumor, because normal mucosa surrounding the

T4 tumor could not be found. The ultrasound equipment used in this study was an Aplio i700 (Canon Medical Systems, Tochigi, Japan). Contrast-enhanced scanning was performed using code-phase inversion harmonic ultrasound with mechanical indices (MIs) of 0.17-0.20, at 16 frames per second and a single focus zone at a depth of 10-20 mm from the surface. The transducer was placed lightly on the neck skin, and the lymphatic ducts and SLNs were observed. We marked the skin directly above the detected SLNs. The SLNs were then examined for blood flow using SMI to evaluate the metastatic status of the SLNs, which were categorized as positive for metastasis if they exhibited more than two vascular supplies except for that from the hilum [12].

In the operation, while patients were under general anesthesia, 2 mL of indigo carmine blue dye was injected into the same 4 regions as for Sonazoid™. After injection of the blue dye, skin incision was made and the skin flap was elevated, we detected SLNs by following the lymphatic route stained by indigo carmine. Although some SLNs did not stain blue, SLNs were detected with reference of informations from ultrasonography, such as depth from the skin, morphology, and size. All SLNs were subjected to

intraoperative pathological examination. SLNs were cut into 2-mm blocks, and 4-μm sections from each block were used for intraoperative frozen section analysis. When the SLNs were metastasis-negative, we performed elective neck dissection from levels I to III for oral cancers, or from levels II to IV for oropharyngeal cancers. When positive SLNs were found in a frozen section, neck dissections from levels I to V (or IV) for oral cancers, and from levels I (or II) to V were performed for oropharyngeal cancers.

Results

Identification of SLNs using CEUS with intra-mucosal administration of Sonazoid™

SLNs were identified in 8 of the total of 10 patients (6 with oral cancers and 4 with oropharyngeal cancers) by the CEUS-guided method using the topical administration of Sonazoid™ as a tracer. Images of two representative cases (Cases 4 and 7), in which SLNs were successfully detected, are shown in Figures 1A and 1B, respectively. In these cases, contrast-enhanced SLNs were identified with a concomitant lymphatic duct draining the SLNs. In two patients (Cases 1 and 9), detection was

unsuccessful. Figure 1C shows an image of Case 9, in which a lymphatic duct was identified without the enhancement of lymph nodes. Case 1 was our first case in this preliminary study. In the case, Sonazoid™ was injected directly into the tumor, because normal mucosa surrounded the locally advanced oral T4 tumor, an optimal site for the infiltration of Sonazoid™ could not be found. In all eight cases in which SLNs were detected, no metastatic lesion was found pathologically in either frozen or permanent sections. A summary of SLN detection is presented in Table.

No adverse events, the primary endpoint of the current study, related to Sonazoid™ infiltration were found.

Evaluation of vascularities in SLNs using SMI

In 7 of eight cases, in whom SLNs were successfully detected, no blood flow was detected other than vascular supply from the hilum (Figure 2A). In these SLNs, no metastatic lesion was found, being compatible with the categorization as metastasis-negative with SMI. In one case (Case 8), at least two blood flows, other than vascular supply from the hilum, were detected (Figure 2B). In this case, the SLN was categorized as metastasis-positive,

but no metastatic lesion was noted on pathological diagnosis. The data concerning vascular supply evaluated by SMI are summarized in Table.

Discussion

In this preliminary clinical trial to overcome the problems associated with the SLN detection method using radiolabeled isotopes, we evaluated the CEUS-guided method using a non-isotopic contrast agent, Sonazoid™, which is readily available on the market, in oral and oropharyngeal cancers. In addition, SMI facilitated the visulalization of microvessel flow in SLNs by ultrasound, allowing prediction of the metastatic status of SLNs. Our final aim is to establish non-invasive procedures to detect SLNs and categorize the metastatic status in head and neck cancers.

In an experimental animal model, a mixture of Sonazoid™ and indocyanine green was infiltrated into the mucosa of the pharynx for the detection of SLNs [14]. In the model, neither mucosal edema nor inflammatory cell infiltration was noted. In the current study, no adverse events related to Sonazoid™ injection were observed, suggesting the safety of its topical use in patients.

In two (Cases 1 and 9) of ten cases, SLNs were not identified by our CEUS-guided method. Beasley et al., on examining head and neck primary tumor specimens, showed that tumor emboli were mainly present within peritumoral lymphatic vessels, but they were not observed within intratumoral ones [15]. They concluded that intratumoral lymph vessels are probably not a major conduit for nodal metastasis in head and neck cancers. Therefore, in Case 1, Sonazoid™ directly injected into the tumor tissue might not have drained through lymphatic ducts interconnecting with SLNs, resulting in SLN identification failure. In all the following 9 cases, Sonazoid™ was infiltrated into peritumoral mucosa, and then lymphatic ducts were clearly identified. However, in Case 9, SLNs were not detected, although lymphatics duct were clearly visible (Figure 1C). Particles of Sonazoid™ are about 2 to 3 μm in diameter, while the diameter of lymph ducts in submucosal tissue is normally 0.2-0.5 mm. Therefore, Sonazoid™ can easily move into lymphatic ducts. We previously showed that dilatation of the lymphatic sinus in SLNs occurs before metastasis (pre-metastatic lymphvascular niche) in OSCC patients [16]. In Case 9, the pre-metastatic niche might not have formed, in which the lymphatic sinus would be

insufficient for the diffusion of Sonazoid™ in SLNs, although lymphatic ducts were clearly visible with CEUS. Following this theory, undetected SLNs drained by identified lymphatic ducts would probably be metastasis-negative. Thus, in such cases with identified lymphatic ducts, but undetected SLNs, neck dissection can be omitted. Actually, in Case 9, no metastatic lymph node was found in the permanent pathological specimens from elective neck dissection (levels II to IV).

The number of detected SLNs in the current study was 12 from 10 patients (median: 1; range: 0-2). In our previous study using Technetium 99m (^{99m}Tc) phytate as a tracer, a total of 196 SLNs were detected from 57 patients (median: 3; range: 1-7) with clinical late-T2 or T3 OSCC [8]. Particles of Sonazoid™ are about 2 to 3 μm in diameter, which is larger than that of ^{99m}Tc-phytate (about 0.2 to 0.3 μm). Sato et al. stated that the larger the size of the tracer, the longer the particle remains in the lymph node [17]. Kogashiwa et al. showed that lymphocytes and macrophages phagocytize Sonazoid™, which explains why Sonazoid™ does not flow into secondary LNs and remains in SLNs for a long time [14]. The reason why a lower number of SLNs are detected with the CEUS-guided method should be

elucidated, and the false-negative ratio of the method also needs to be evaluated in a large series.

In the current study, we elucidated the application of SMI to evaluate SLN vascularity and the diagnostic performance of SMI in differentiating metastatic from non-metastatic nodes, according to the criteria for categorizing positive metastatic nodes used by Matsuzawa et al. [12]. Among eight cases evaluated, SLN was diagnosed as metastasis-positive in one case (Case 8), according to the criteria. One specific finding in the case was that the pathological diagnosis of the primary tumor was a mucoepidermoid carcinoma, in which an intra-tumoral abscess had formed due to concordant bacterial infection. We may have identified inflammatory findings in the SLN of Case 8. We do not know whether the tumor pathology affected the accuracy of SLN-categorization by SMI. To date, clinical research using SMI technology for microvascular evaluation has been limited [18,19]. For the application of this novel technology to differentiate SLNs, accumulating clinical data from a large series to generate reliable criteria for categorizing SLNs is necessary.

Methodological considerations / limitations

This study evaluated CEUS-guided SLN identification by the topical infiltration of Sonazoid™ at the primary site in oral and oropharyngeal cancers. SLNs were detected in 8 out of 10 cases. We analyzed and speculated on the reason why SLN-detection failed in two cases. No adverse event was observed.

Although more experiences and clinical data are needed based on a larger cohort, establishing a method for evaluating SLN metastasis using the combination of CEUS-guided SLN-detection and accurate diagnosis of SLN-metastasis using SMI would be valuable as an alternative to conventional SLN-detection using radiolabeled isotopes and biopsy, in oral and oropharyngeal cancers.

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Declaration of interest statement

The authors have no conflict of interest to disclose.

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Figure legends

Figure 1. Contrast-enhanced ultrasonography with Sonazoid™ in Cases 4 (a), 7 (b), and 9 (c). In each figure, the left half is a contrast-enhanced image, and the right half is the B-mode. In Cases 4 (a) and 7 (b), contrast-enhancement of sentinel lymph nodes (SLNs) (arrowheads) was observed concomitant with lymphatic ducts (arrows) draining the nodes. In Case 9 (c), lymphatic ducts (arrow) were clearly visible; however, no SLN was identified.

Figure 2. Detection of vasculatures in sentinel lymph nodes of Cases 10 (a) and 8 (b) using Superb Microvascular Imaging (SMI). In each figure, the left half is B-mode, and the right half is SMI. In Case 10 (a), SMI revealed that only one blood flow was observed, originating from a single vessel in the hilum (arrowhead). In Case 8, at least two vascular supplies were observed (arrows) except for that derived from the hilum (arrowhead).

Table. Patient characteristics and summary of SLN-detection and vascularity

Primary site	Case number	Age	Sex	Pathology of primary tumor	T-status	Detection of lymphatic duct	Number of CE-SLNs	Size of SLNs (mm)	Number of vascularity except for that from hilum	SLN metastasis
Oral cavity										
	1	76	Male	SCC	T4	not detected	0	N.A.	N.A.	N.A.
	2	47	Male	SCC	T3	detected	1	6.4	0	Negative
	3	60	Male	SCC	T2	detected	2	5.0, 7.2	0	Negative
	4	75	Female	SCC	T2	detected	1	10.4	0	Negative
	5	35	Female	SCC	T2	detected	2	7.4, 5.0	0	Negative
	6	62	Male	SCC	T2	detected	1	8.0	0	Negative
Oropharynx										
	7	67	Male	SCC	T1	detected	2	9.4, 12.0	0	Negative
	8	29	Male	MEC	T4	detected	1	14.8	2	Negative
	9	73	Male	SCC	T2	detected	0	N.A.	N.A.	N.A.
	10	56	Male	SCC	T2	detected	2	12.9, 7.4	0	Negative

CE-SLN,contrast-enhanced sentinel lymph node; MEC, mucoepidermoid carcinoma; N.A., not applicable; SCC, squamous cell carcinoma; SLN, sentinel lymph node.

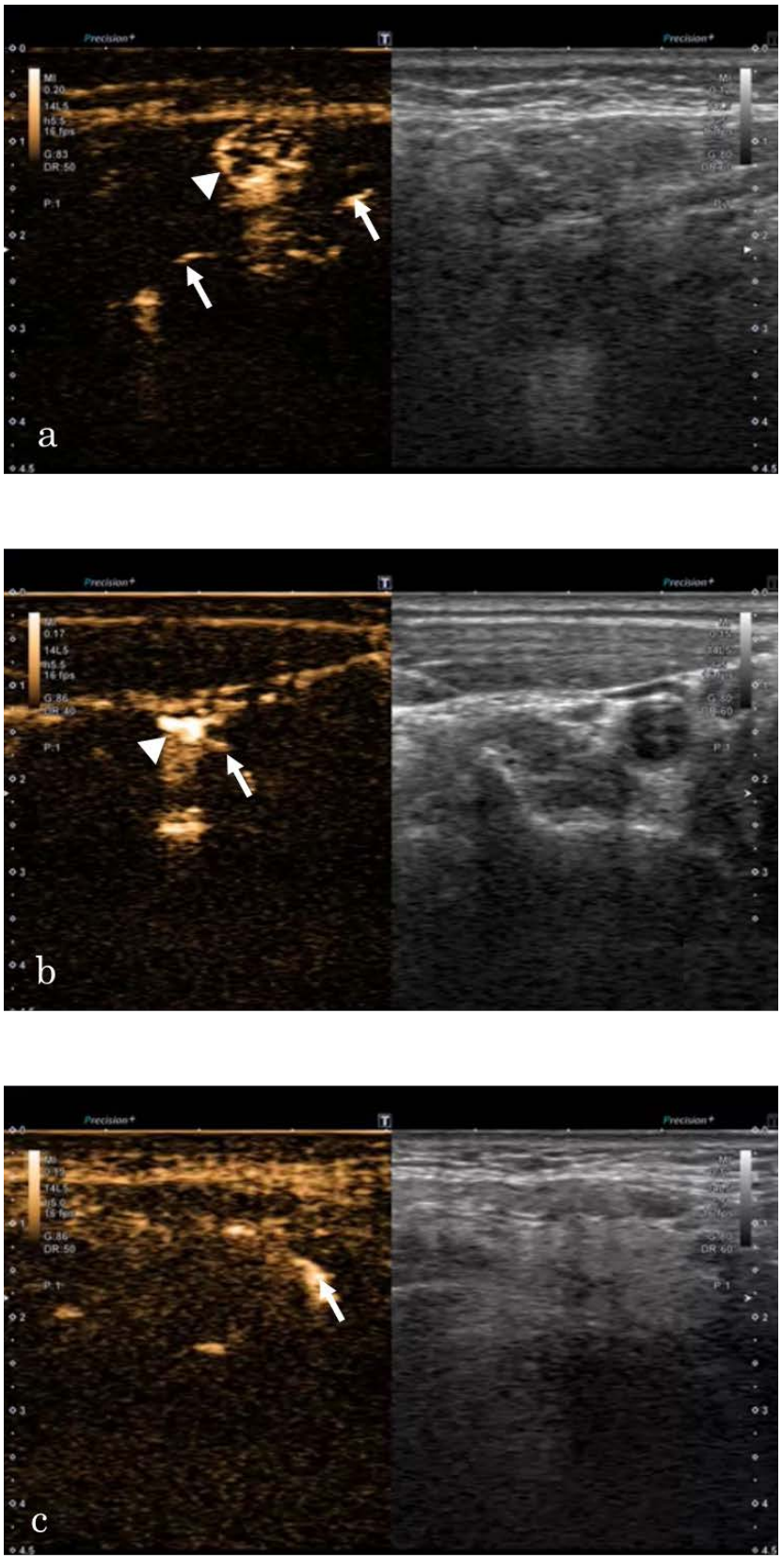
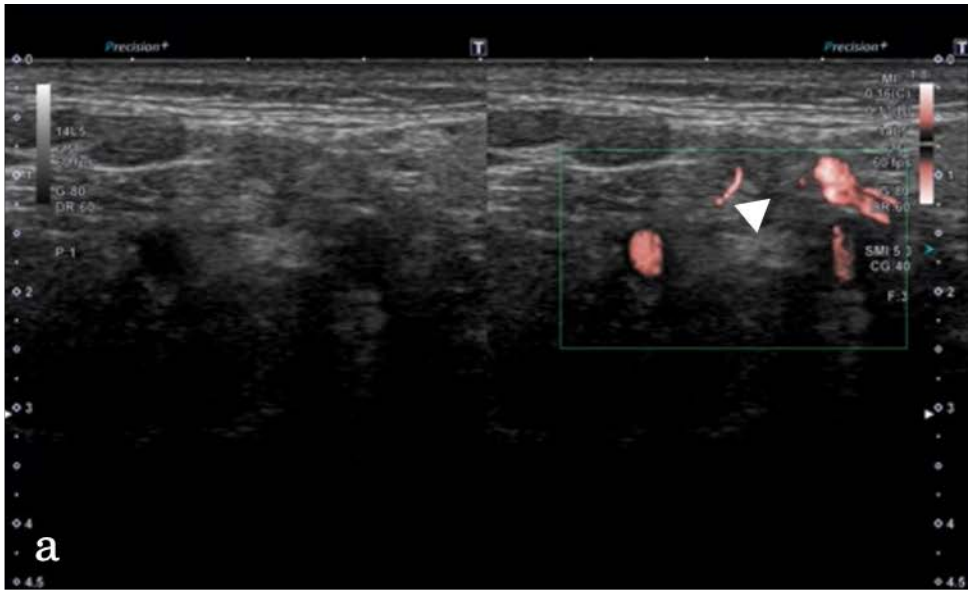


Figure 1



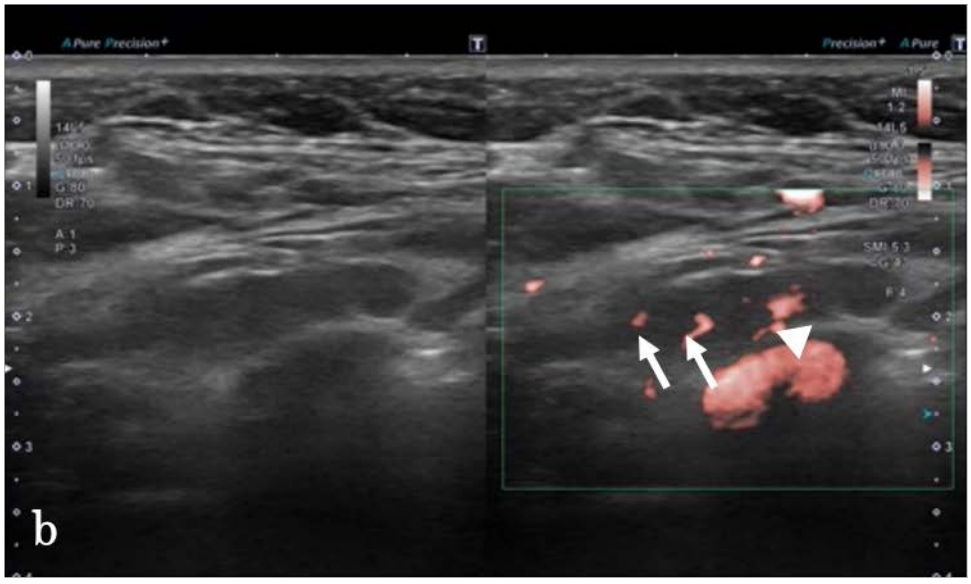


Figure 2