

1 Histological imaging of gastric tumors by 2 scanning acoustic microscope

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10 ABSTRACT

Background: Human body tissues have their own speed-of-sound (SOS) by which sound travels through them. Harder materials exhibit greater SOS; thus, SOS through each tissue can provide information regarding its elasticity. For tumor discrimination in clinical medicine, palpation provides important information. However, manual palpation is subjective, whereas SOS data are objective and can be directly used to compare lesions.

Methods: A scanning acoustic microscope (SAM) uses ultrasound to image an object from SOS through tissues. We investigated the utility of SAM in imaging gastrointestinal lesions.

Results: We visualized the digestive tract as a layered structure and discriminated tumors to a degree comparable with that observed by light microscopy. Areas with greater SOS corresponded to those with higher collagen or muscle fiber concentrations. Cell-poor areas or regions with degeneration demonstrated less SOS than surrounding tissues. Gastric tumors displayed appropriate SOS similar to their original tissues and showed significant differences in SOS between scirrhous carcinomas and well-differentiated medullary carcinomas, scirrhous carcinomas and malignant lymphomas, and leiomyomas and leiomyosarcomas.

Conclusion: SAM offered the following benefits over LM: (1) images are acquired in a few minutes without special staining; (2) repeated observations of the same section are possible; (3) high-resolution digital imaging from SOS is comparable to LM; (4) SAM analysis could be helpful in understanding endoscopic ultrasonography imaging; and (5) digitized SOS data could be statistically compared among different stomach lesions.

14 *Keywords: scanning acoustic microscope, tissue elasticity, cancer imaging, gastric tumor,*
15 *fibrosis*

18 1. INTRODUCTION

20 Humans possess two major sensory organs, the eyes and ears, which facilitate vision and
21 hearing, respectively. Material discrimination by light microscopy (LM) has been useful in
22 elucidating various cell systems, although sound has been rarely used in medical science.
23 Some mammals, such as bats and dolphins, use high-frequency (>20 KHz) ultrasound (US)
24 for chasing game.
25

Human body tissues have their own speed-of-sound (SOS) by which sound travels through them. Harder materials exhibit greater SOS; thus, SOS through each tissue can provide information on its elasticity[1]. For tumor discrimination in clinical medicine, palpation provides important information: most sarcomas are softer than carcinomas, and scirrhous carcinomas are harder than medullary carcinomas. However, manual palpation is subjective and depends on experience, whereas SOS data is objective and can be directly used to compare lesions.

Endoscopic ultrasonography (EUS) has recently become a popular option for observing deep submucosal or deeper lesions, such as cancer invasion, submucosal tumors, and lymph node metastasis in the gastrointestinal (GI) tract[2, 3]. However, EUS is macroscopic and cannot be used to predict histology.

A scanning acoustic microscope (SAM) is a device that uses US frequency (>80 MHz) to microscopically image an object[4]. We recently reported that SAM could be used to generate useful information about lung[5] and lymph node[6] lesions.

Can SAM visualize GI normal structures? How are lesions detected by SAM? Does SOS really correspond to tissue elasticity? Is SAM superior to LM?

Here, we show representative SAM gastric images and discuss its utility for clinical diagnosis.

2. MATERIAL AND METHODS

Materials

All materials from anonymous donors were prepared from the Hamamatsu University Hospital archives. The research protocol using stored samples without link to patient identity was approved by the research ethics committee of Hamamatsu University School of Medicine. Formalin-fixed, paraffin-embedded (FFPE) blocks were flat-sectioned in 10- μ m thick sections. Because deparaffinized sections repelled water due to surface hydrophobicity and dried out easily, it was necessary to soak sections enough in distilled water to reduce hydrophobicity. So deparaffinized, unstained sections were soaked in distilled water for at least 3 h before observation. Continuous sections were stained with hematoxylin & eosin (H&E) for comparison of SAM images with corresponding light microscopy (LM) images.

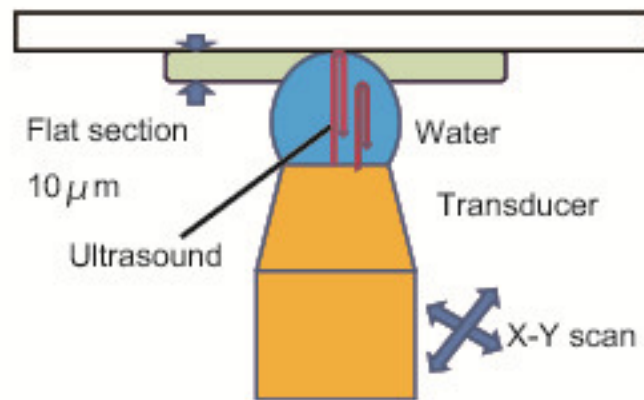
Tissue samples

We examined and selected normal and neoplastic stomach lesions, including adenocarcinomas (well or poorly differentiated), carcinoid tumors, GI stromal tumors (GISTs), leiomyomas, malignant lymphomas, leiomyosarcomas, and schwannomas.

SAM observation

The SAM was supplied by Honda Electronics (Toyohashi, Japan) and was equipped with a 120-MHz transducer, which has a resolution of approximately 13 μ m (Fig. 1). SAM functions by directing focused sound from a transducer to a small area of the target object on a glass

67 slide. The sound emitted by an acoustic transducer hits or penetrates the tissue and is
 68 reflected on the surface of the tissue or glass. It is then returned to the receiver, which is
 69 coincident with the transducer. The SOS through the tissue is automatically calculated by
 70 comparing the time-of-flight of the pulse from the surfaces of both tissue and glass.



71

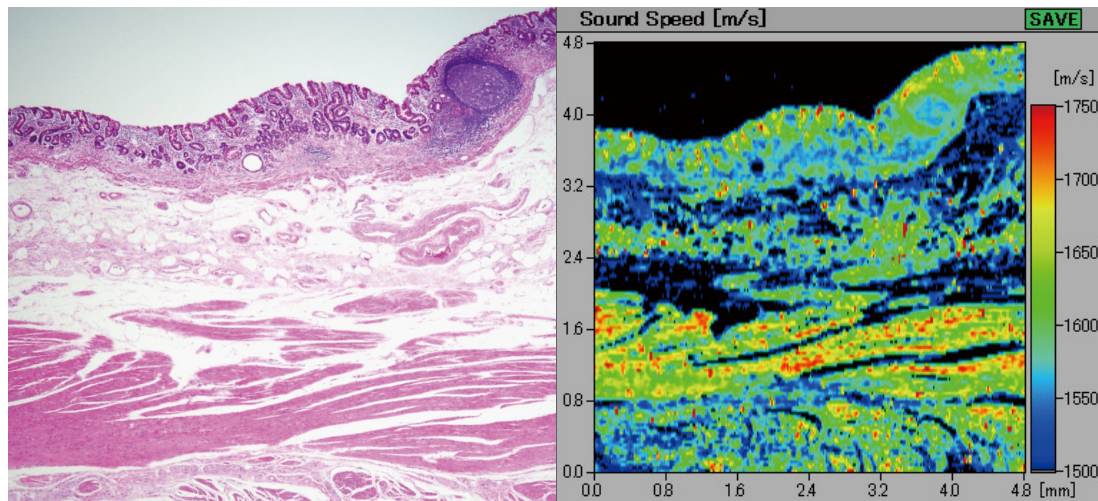
72 **Fig. 1. Principles of scanning acoustic microscopy (SAM)**

73 *Ultrasonic waves from the transducer reflect off both the glass slide and the section and*
 74 *return to the transducer. These waves pass through 10-μm sample sections with different*
 75 *ultrasonic properties. The transducer automatically scans the section to calculate the speed-*
 76 *of-sound through each area. The section is placed upside down on the transducer, and*
 77 *distilled water is applied between the transducer and the section as a coupling fluid. The*
 78 *control speed-of-sound (SOS) through water is 1500 m/s.*

79

80 To perform SAM imaging, the slide sections were placed upside-down on the stage above
 81 the transducer, and distilled water was applied between the transducer and the section as a
 82 coupling fluid; this was done because air interferes with sound transmission. After
 83 mechanical X–Y scanning, SOS from each point on the section was calculated and plotted
 84 on the screen to create two-dimensional, color-coded images (Fig. 2A). The vertical bar on
 85 the left and the horizontal bar at the bottom of each figure indicate the distance (mm) on the
 86 slide. The vertical colored column on the right side of the figure indicates the average SOS
 87 of each square area on the section. The region of interest (ROI: 1.2, 2.4, or 4.8 mm²) for
 88 acoustic microscopy was determined from the LM images. SOS values at 300 × 300 points
 89 were calculated and plotted on the screen to create the images, and sound data from 64
 90 cross points on the lattice screen were used for statistical analysis.

91 Other data such as thickness of the section and sound attenuation were also available from
92 each point and were shown on the screen (data not shown).



93

94 **Fig. 2A. Scanning acoustic microscopy imaging of normal stomach tissue**

95 *From top to bottom, the gastric wall consists of the mucosa, the lamina muscularis mucosae*
96 *(MM), the submucosa (SM), the muscularis externa (ME), and the serosa. The acoustic*
97 *image (right) clearly discriminates these layer structures comparable with its corresponding*
98 *H&E-stained light microscopy image (left). The speed-of-sound values of the thick ME is*
99 *highest among these layers.*

100

101 **Statistical analysis**

102 SOS data from each tissue element are presented as mean $\pm$ standard deviation (SD; m/s).
103 Student's t-tests were used to determine statistically significant differences among the
104 specimens. The level of statistical significance was set at $P < .01$.

105

106 **3. RESULTS AND DISCUSSION**

107

108 **Normal gastric structure**

109 From the inner to outer surfaces, normal gastric layers consist of mucosa, lamina muscularis
110 mucosae (MM), submucosa (SM), muscularis externa (ME), subserosa, and serosa. The
111 SAM system can effectively discriminate among these gastric layer structures (Fig. 2A). In
112 the mucosa, foveolar and glandular structures were well visualized by SAM (Fig. 2B).

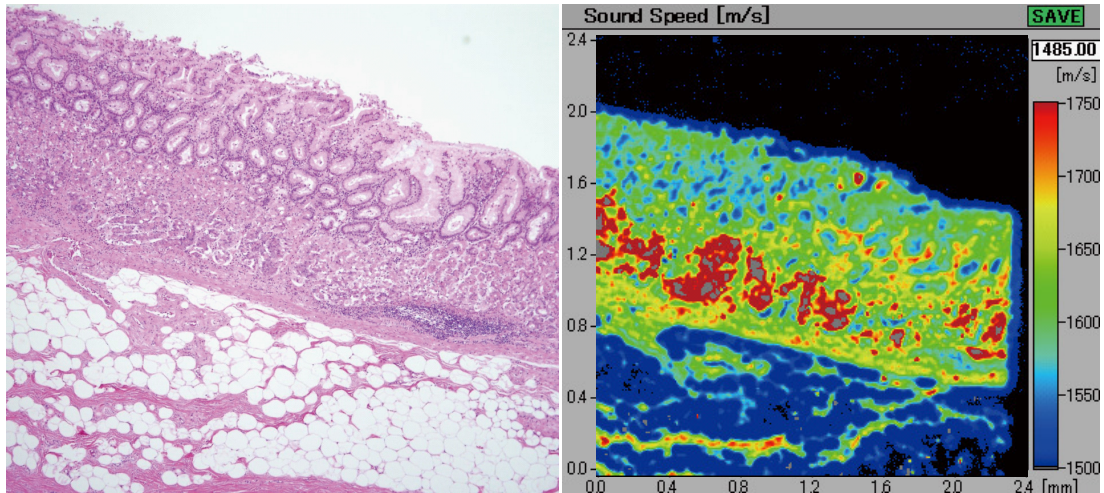


Fig. 2B. The acoustic image (right) of normal gastric mucosa and submucosa and its corresponding light microscopic image with H&E staining (left)

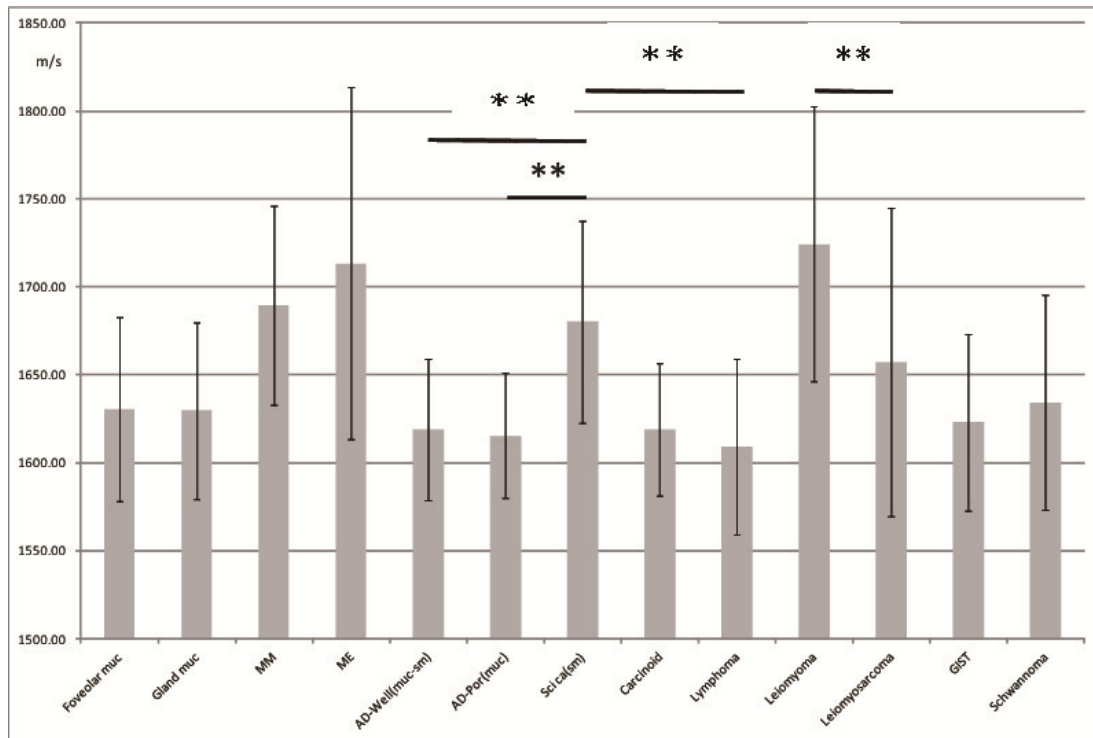
Foveolar and glandular structures are clearly visualized by scanning acoustic microscopy. The cell-rich area shows higher speed-of-sound (SOS) than the cell-deficient area. The muscularis mucosae exhibit a linear, thin-banded structure with higher SOS. In the submucosa, blood and lymph vessels show narrow tubular structures with greater SOS.

Cell-rich areas displayed greater SOS than cell-deficient areas. MM displayed linear band structure with greater SOS and showed a clear boundary with SM. Smooth muscle layers, including MM and ME, exhibited greater SOS than the mucosal layer (Table 1, Fig. 3).

Table 1. The mean speed-of-sound through gastric location (m/s) and SD

Location	n	AVE	SD
Foveolar muc	105	1630.51	52.14
Gland muc	53	1629.58	49.97
MM	57	1689.47	56.43
ME	90	1713.34	99.97
AD-Well(muc-sm)	155	1618.89	40.32
AD-Por(muc)	91	1615.36	35.67
Sci ca(sm)	210	1680.05	57.52
Carcinoid	59	1619.08	37.66
Lymphoma	196	1608.93	49.87
Leiomyoma	162	1724.30	78.38
Leiomyosarcoma	113	1657.07	87.72
GIST	171	1622.96	50.18
Schwannoma	68	1634.27	61.09

Foveolar muc, foveolar mucosa; Gland muc, glandular mucosa; MM, muscularis mucosae; ME, muscularis externa; AD-Well(muc-sm), well-differentiated adenocarcinoma (mucosa, submucosa); AD-Por(muc), poorly differentiated adenocarcinoma (mucosa); Sci ca (sm), scirrhous carcinoma (submucosal invasion); GIST, gastrointestinal stromal tumor.



129

130 **Fig. 3. The mean and SD (m/s) of speed-of-sound through gastric location**

131 *Foveolar muc, foveolar mucosa; Gland muc, glandular mucosa; MM, muscularis mucosae;*
 132 *ME, muscularis externa; AD-Well(muc-sm), well-differentiated adenocarcinoma (mucosa,*
 133 *submucosa); AD-Por(muc), poorly differentiated adenocarcinoma (mucosa); Sci ca (sm),*
 134 *scirrhous carcinoma (submucosa); GIST, gastrointestinal stromal tumor, ** P < 0.01*

135 **Carcinomas of the stomach**

136 Well and poorly differentiated adenocarcinomas arising from the mucosa formed tubular
 137 structures and signet-ring cells, respectively (Figs. 4A, 4B). The SOS of adenocarcinomas
 138 was low, which was identical to that of normal mucosa. No significant difference was
 139 observed between well and poorly differentiated carcinomas in the mucosa (Table 1, Fig. 3).

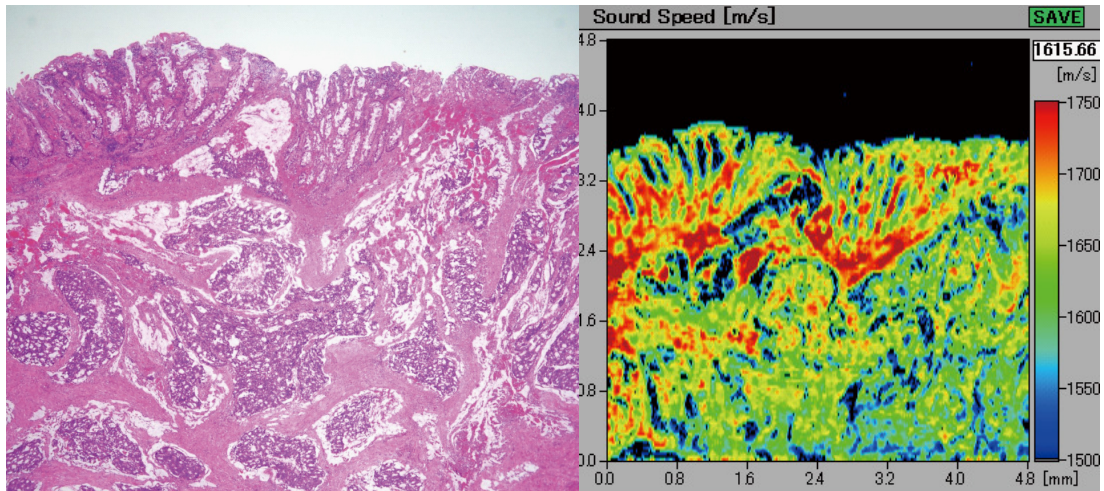


Fig. 4A. A well-differentiated adenocarcinoma with mucosal spread

Irregular tubular structures with fused glands are shown among normal foveolar tubules from the right upper to the left lower areas in the figure. Smooth muscles in the muscularis mucosae separate the clusters of cancer cells with conspicuous speed-of-sound values.

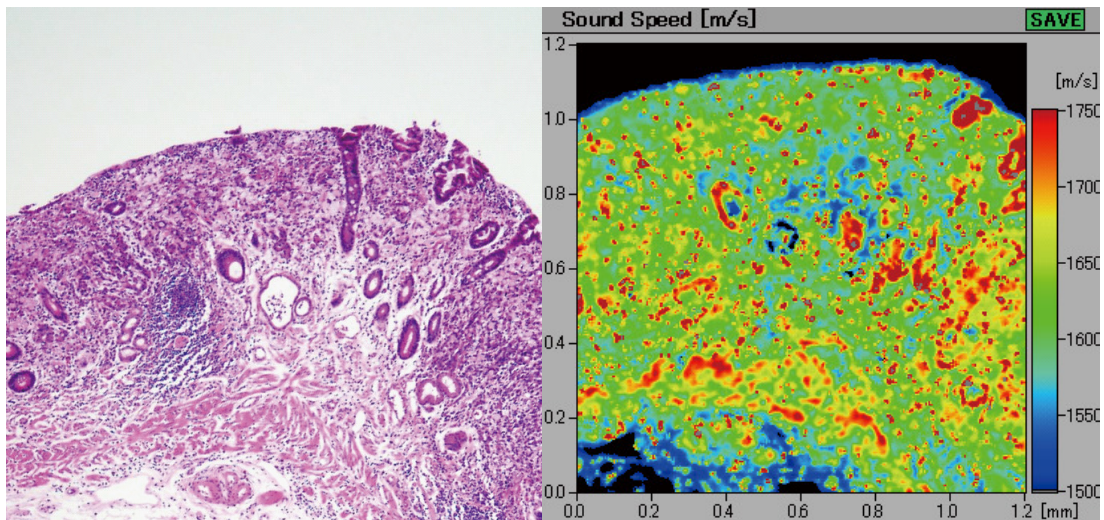
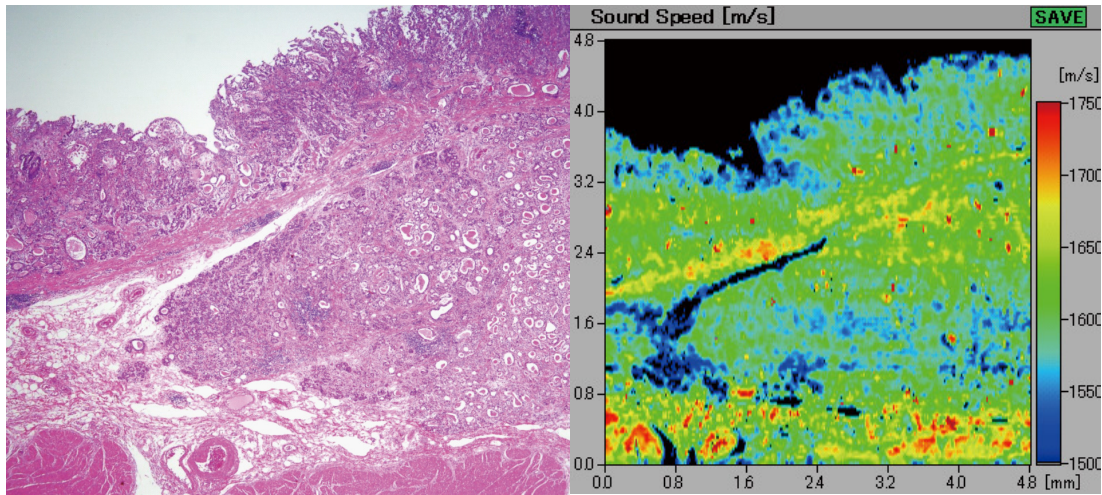


Figure 4B. A poorly differentiated adenocarcinoma spreading through the mucosa

The normal foveolar and glandular structures have disappeared and are replaced by signet ring cells that are represented as scattered dots with increased speed-of-sound values (right).

At sumucosal invasive sites, well-differentiated adenocarcinoma with medullary growth exhibited SOS which were the same as that of the original mucosa (Fig. 4C), while poorly differentiated scirrhous carcinoma exhibited greater SOS than normal mucosa (Figs. 4D and 4E). The SOS values of scirrhous carcinomas were significantly higher than well-differentiated carcinoma with medullary growth (Fig. 3, Table 1).

155

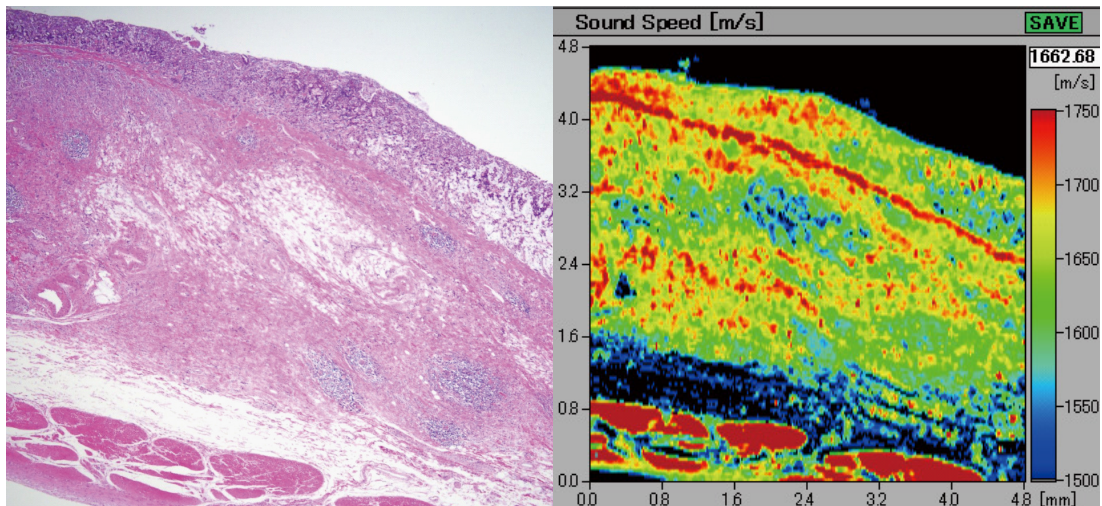


156

157 **Fig. 4C. A well to moderately differentiated adenocarcinoma invading from the mucosa to the**
158 **submucosa with medullary growth**

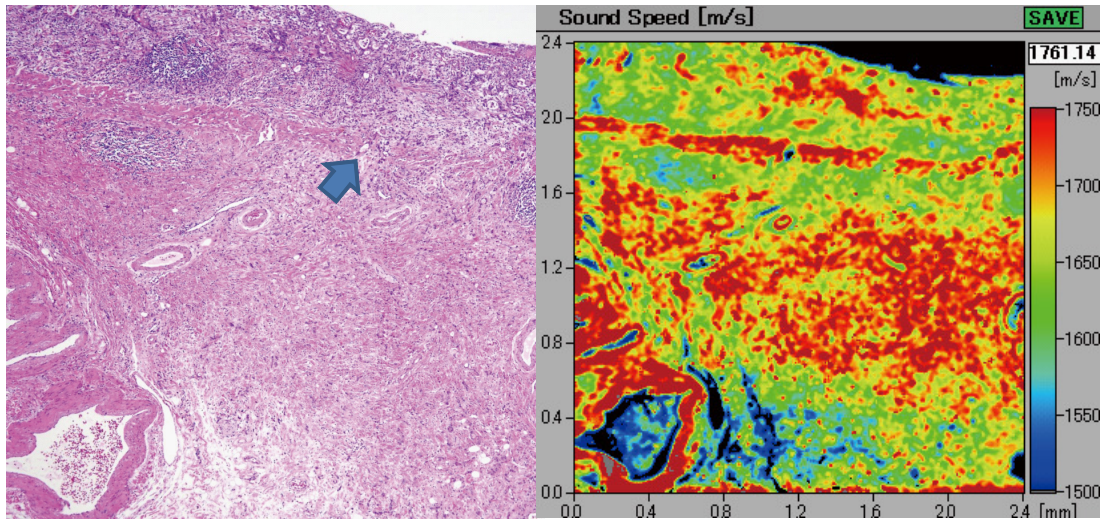
159 *The normal tubular architecture is replaced by cancer cells. The smooth muscle layer of*
160 *muscularis mucosae is split (arrow) by invading cancer.*

161



162 **Fig. 4D. A poorly differentiated adenocarcinoma with scirrhous submucosal invasion**

163 *Many signet ring cells spread in the mucosa and infiltrate beyond the muscularis mucosae*
164 *into the submucosa. In the submucosa, desmoplastic reactions with collagen fiber*
165 *proliferation are seen, and spicular structures exhibit greater speed-of-sound.*



166

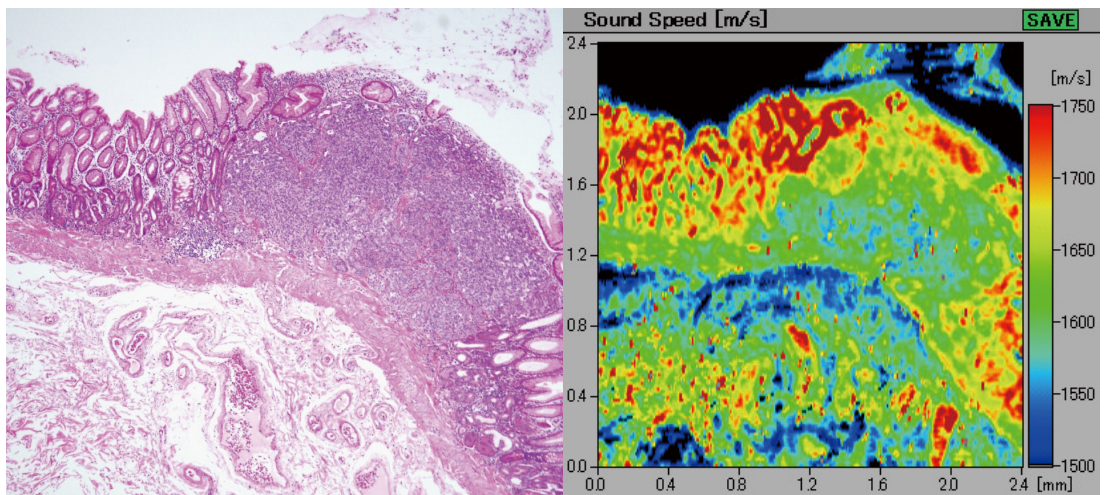
167 **Fig. 4E. A poorly differentiated adenocarcinoma with scirrhous spread**

168 *Many signet ring cells infiltrate from the mucosa to the submucosa with desmoplastic*
 169 *reactions, which demonstrate speed-of-sound similar to that observed in muscularis*
 170 *mucosae or perivascular smooth muscle. An arrow indicates split smooth muscles of the*
 171 *muscularis mucosae.*

172

173 **Carcinoid tumors of the stomach**

174 Carcinoid tumors consisting of trabecular structure with scarce stroma showed less SOS
 175 than the surrounding mucosa (Fig. 5).



176

177 **Fig. 5. A small gastric carcinoid tumor in the mucosa**

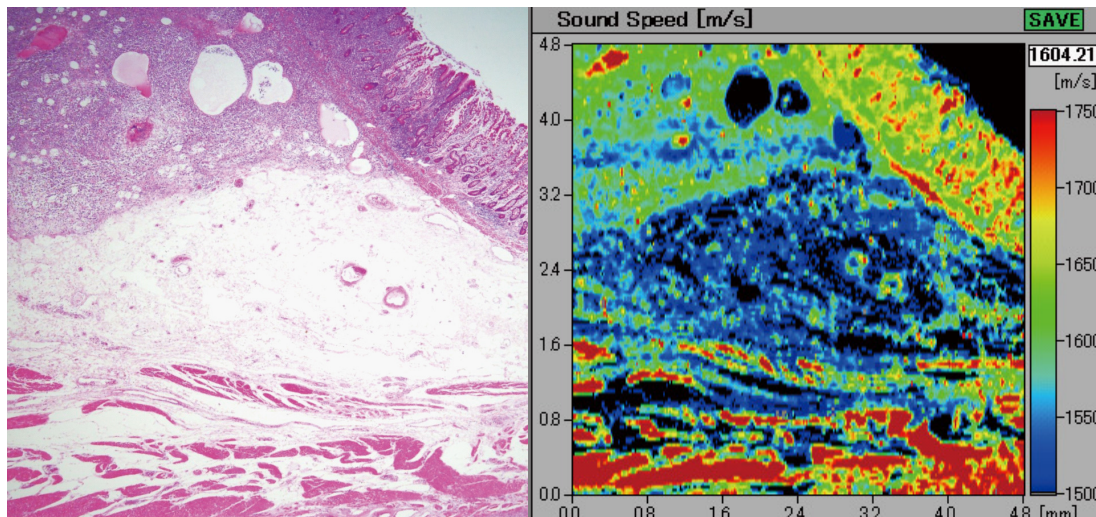
178 *The carcinoid portion consisting of trabecular structure with scant fibrous components*
179 *exhibits lower speed-of-sound than the surrounding mucosa (right).*

180

181 **Malignant lymphomas**

182 Malignant lymphoma displayed the least SOS of all assessed tumors (Fig. 6A and 6B, Table
183 1). The SOS values of the lymphomas were significantly lower than those measured in
184 scirrhous carcinoma samples (Fig. 3).

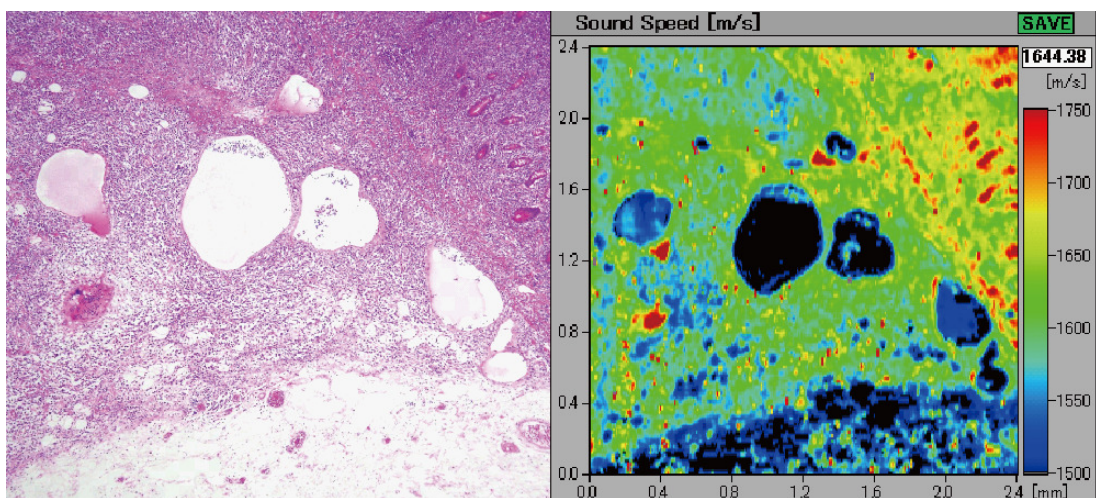
185



186 **Fig. 6A. A gastric malignant lymphoma infiltrating from the mucosa to the submucosa**

187 *The lymphoma shows reduced speed-of-sound and spreads among mucosal tubules (right).*

188



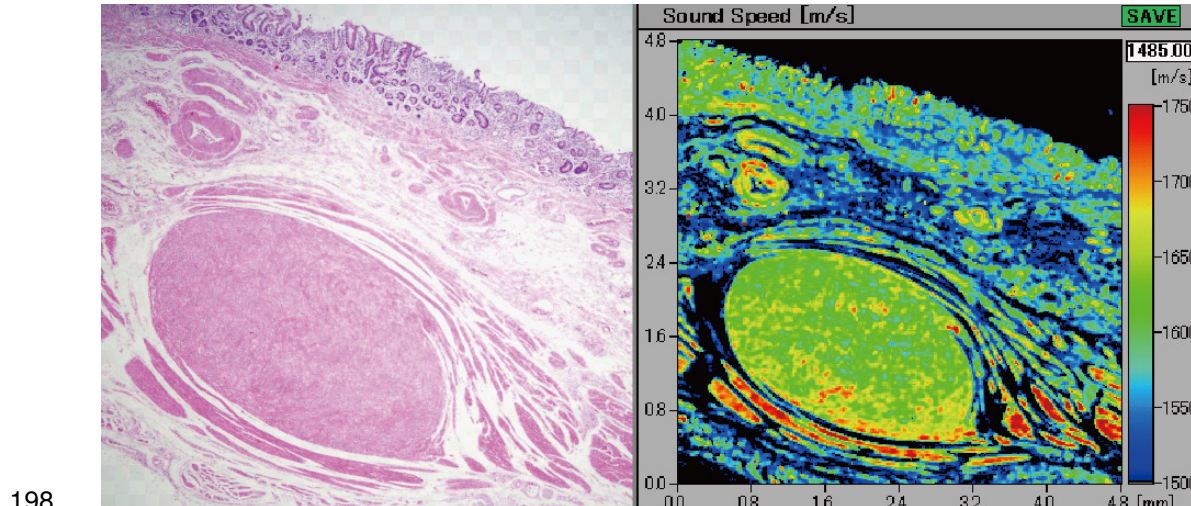
189 **Fig. 6B. Higher magnification of the gastric lymphoma shown in figure 6A**

190 The lymphomatous area exhibits the least speed-of-sound (SOS) among the mucosal
191 components. The dilated lymph vessels and the congested veins in the lymphoma show
192 lower and higher SOS, respectively.

193

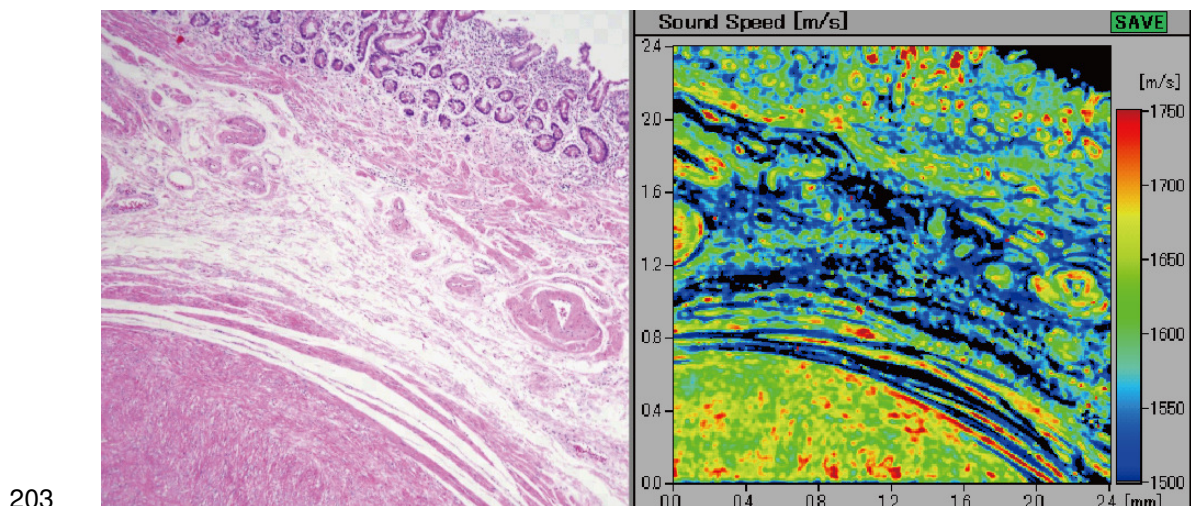
194 **Leiomyomas and leiomyosarcomas**

195 Leiomyomas appeared as sharp nodular structures and demonstrated SOS values as high
196 as those of smooth muscles of MM, ME, and blood vessel walls (Figs. 7A and 7B, Table 1). Thick
197 muscle bundles exhibited the greatest SOS values (Fig. 7C).



199 **Fig. 7A. A gastric leiomyoma in the ME**

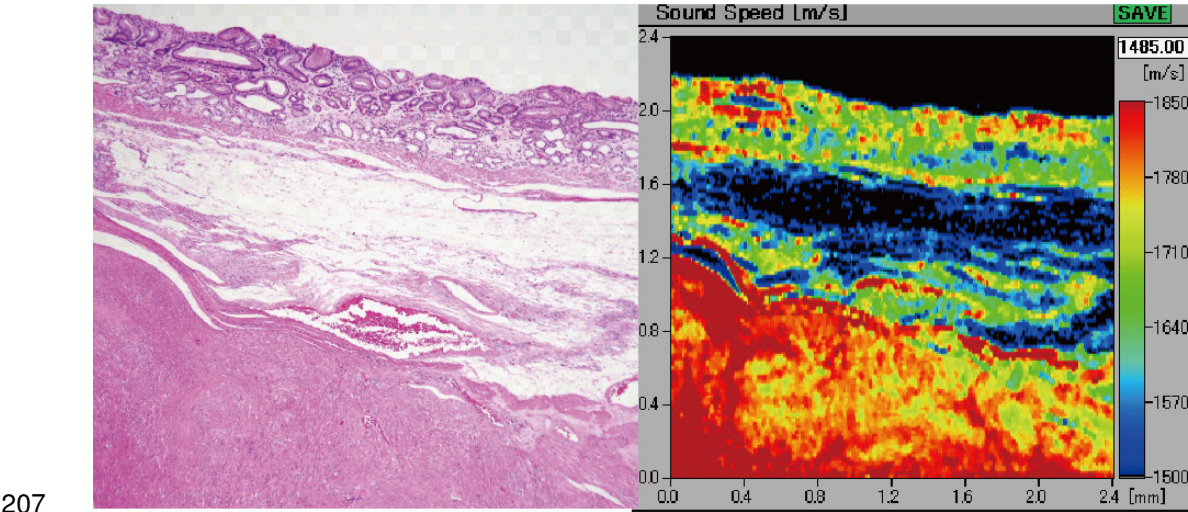
200 An elliptical leiomyoma with clear margins shows speed-of-sound (SOS) similar to that of the
201 smooth muscles of muscularis mucosae, ME, and blood vessel walls. The surrounding ME
202 displays higher SOS due to compression artifact.



203

204 **Fig. 7B. Higher magnification of the leiomyoma shown in figure 7A**

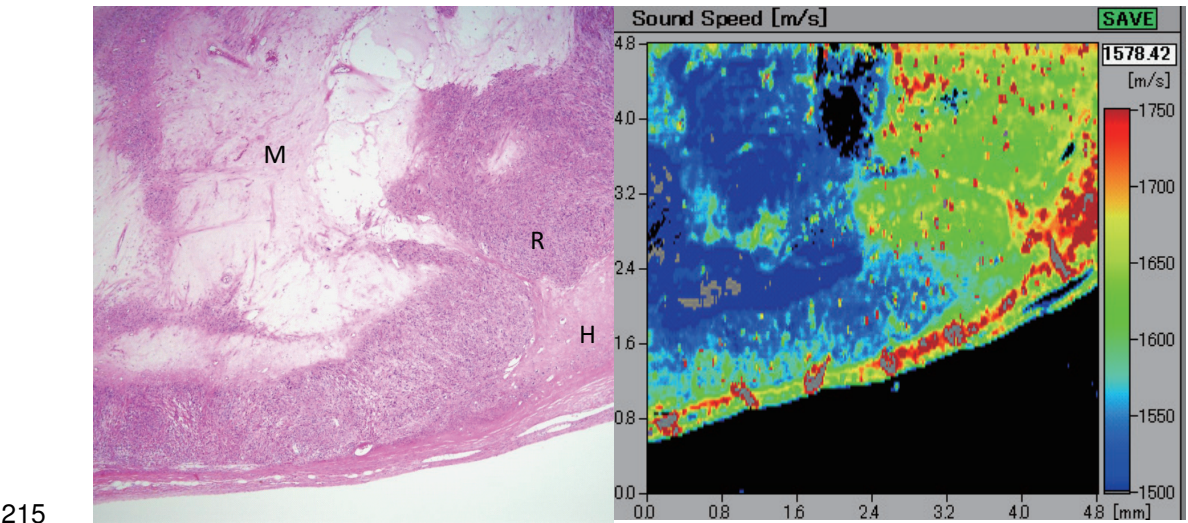
205 *The higher speed-of-sound areas in the right scanning acoustic microscopy image*
206 *correspond to dense fascicles of smooth muscle and interstitial blood vessels.*



208 **Fig. 7C. A leiomyoma with dense muscle bundles**

209 *The highest speed-of-sound values (right) were observed from the mucosa to the muscularis*
210 *externa.*

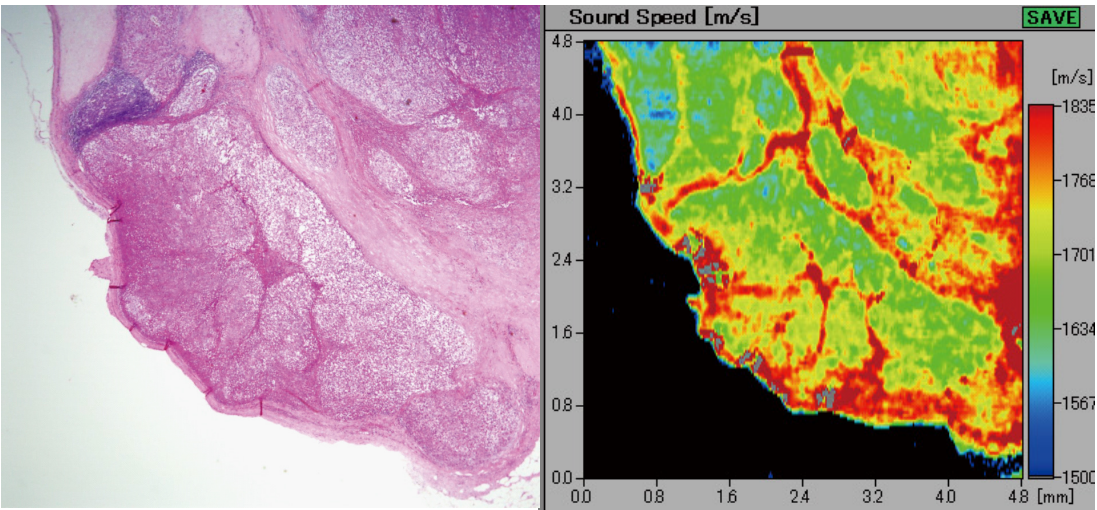
211 Leiomyosarcoma with great SOS area showed geographic myxoid degeneration with less
212 SOS area (Fig. 8A), and their transitions were abrupt and irregular. Multinodular leiomyomas
213 with high cellularity displayed less SOS compared with septal fibrous bands (Fig. 8B). The
214 SOS values of leiomyosarcomas were significantly lower than those of leiomyomas (Fig. 3).



216 **Fig. 8A. A leiomyosarcoma with myxoid areas**

217 The hyalinized area (H), tumor cell-rich area (R), and myxoid transition area (M) display the
218 highest, middle, and lowest speed-of-sound values, respectively. The hot spots in the cell-
219 rich area correspond to blood vessels.

220



221 **Fig. 8B. A gastric leiomyosarcoma comprising multinodular structures with irregular**
222 **outer contours**

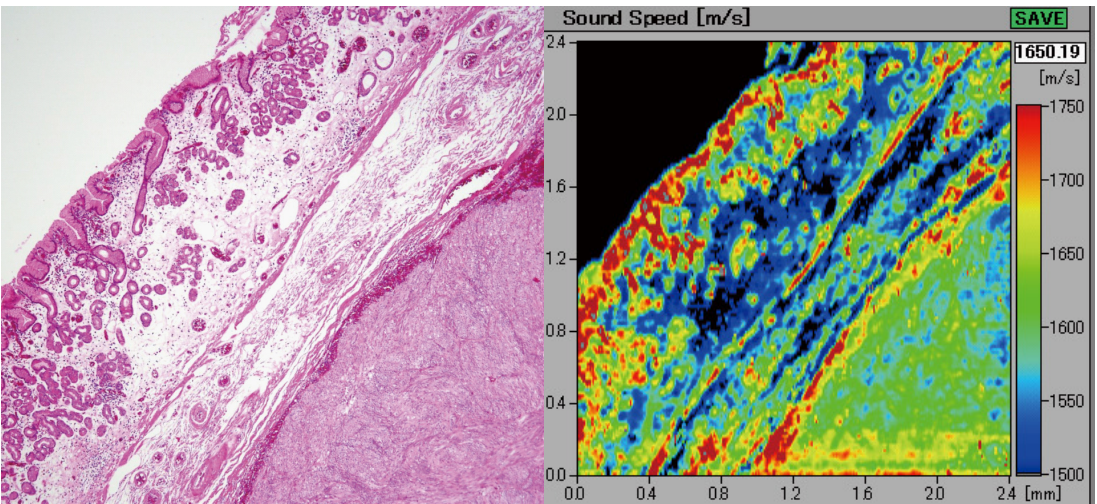
223 The nodules are made up of tumor cells that are separated by a fibrous band and exhibit
224 lower speed-of-sound than the fibrous band.

225

226 **GIST**

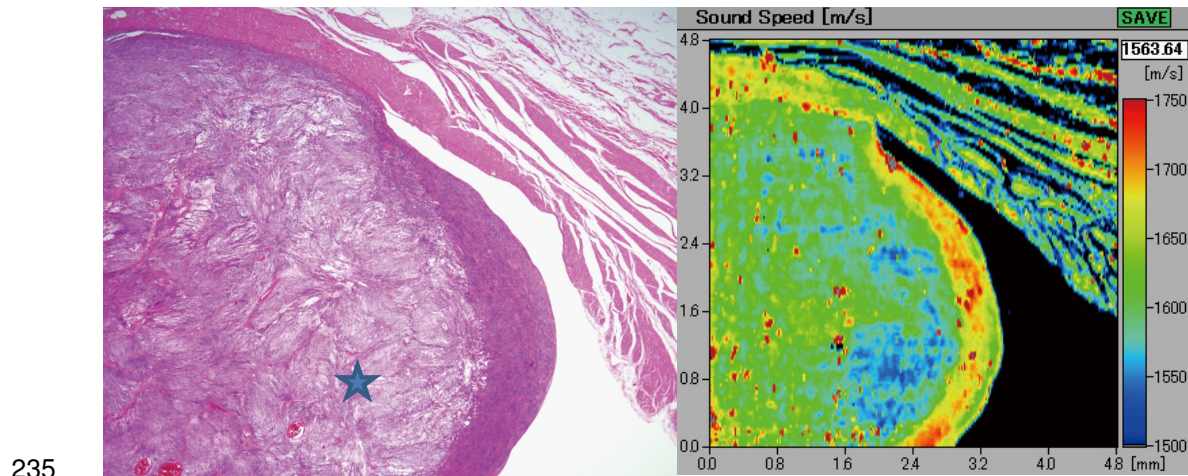
227 GISTs of the stomach formed large submucosal nodules, and their SOS values were almost
228 identical to those measured in the mucosa (Figs. 9A and 9B, Table 1). Focal areas with
229 myxoid change exhibited lower SOS values than cell-rich areas.

230



231 **Fig. 9A. A gastrointestinal stromal tumor in the submucosa**

232 *The tumor margin has numerous blood vessels and shows higher speed-of-sound (SOS),*
233 *whereas the tumor center displays almost the same SOS as that of the foveolar epithelium.*
234 *The mucosal surface shows greater SOS, which corresponds to rich mucousal content.*



236 **Fig. 9B. A gastrointestinal stromal tumor (GIST) protruding from the muscularis externa into**
237 **the serosa.**

238 *The GIST shows variable speed-of-sound (SOS) among different areas. The cell-rich serosal*
239 *surface displays higher SOS and the cell-deficient central area with myxoid change*
240 *(asterisk) exhibits reduced SOS. The spotty areas with increased SOS correspond to blood*
241 *vessels.*

242

243 **Schwannomas**

244 Schwannomas included both cell-rich and cell-deficient areas, which exhibited large and
245 small SOS values, respectively (Fig. 10).

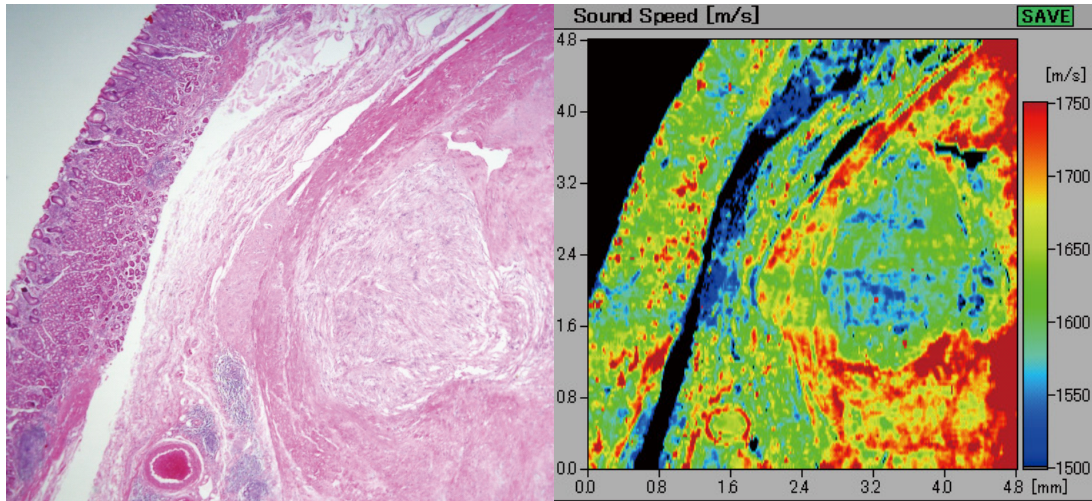


Fig. 10. A gastric schwannoma in the submucosa

The schwannoma mixed with cell-rich and cell-deficient areas demonstrate higher and lower speed-of-sound, respectively.

Discussion

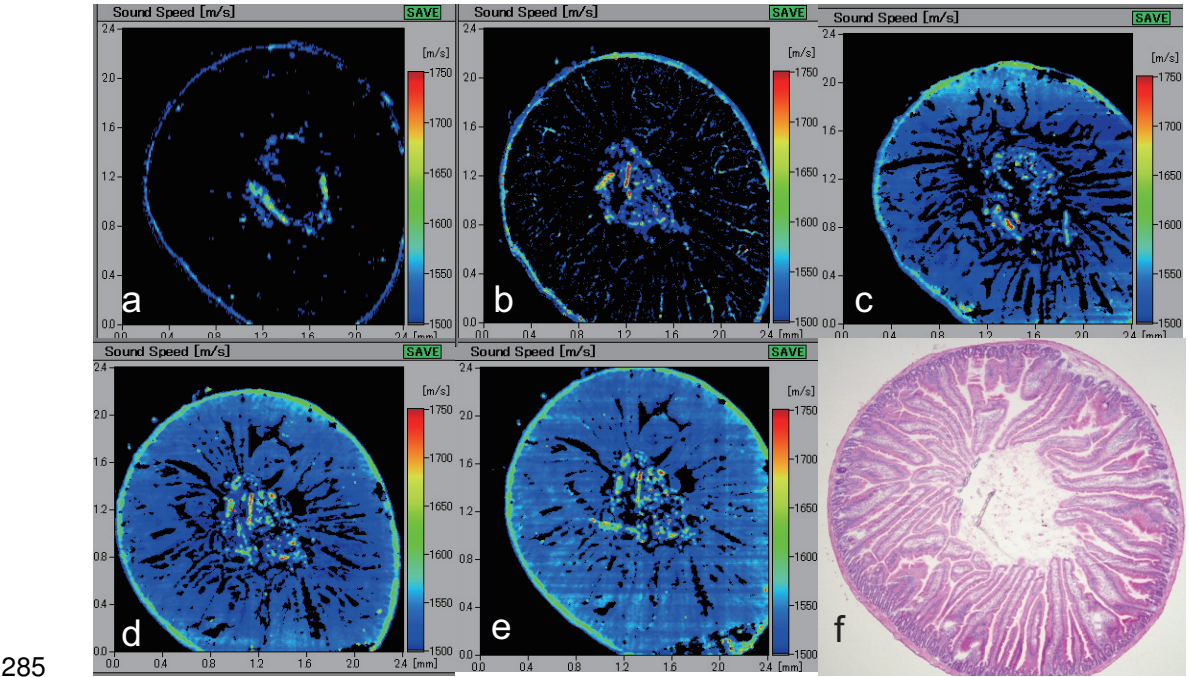
The biomedical application of SAM was first described by Lemons and Ouate in 1975[7]. SAM is a confocal imaging device in which thin specimens are scanned point-by-point using very strongly focused beams[4]. The attenuation of US in tissues is much greater than that in water and increases with elevation in the frequency.

The data on the SOS values in biological tissues in various organs can be found in reviews[8, 9]. Studies of the acoustic properties of tissues at low frequency suggest that SOS values in soft tissues differ only slightly from those in water and are virtually independent of frequency. Significant differences between SOS in water and objects were only observed for solid biological tissues, such as bones and tissues that contain large concentrations of fibrillar proteins[9]. Because the stomach has muscle layers and exhibits reactive fibrosis, data from high-frequency SAM may provide useful information to complement low-frequency EUS imaging.

The abovementioned SAM results show both normal and abnormal gastric images. Normal layer structure was destroyed by various causes, including neoplastic and inflammatory lesions. Each layer or lesion is characterized by their own SOS, which is useful for discriminating lesions. On EUS imaging, five distinct layers are seen[2, 3], which correspond to the following: layer 1, interface echo between the superficial mucosa and the acoustic coupling medium; layer 2, deep mucosa; layer 3, SM plus the acoustic interface between SM and ME; layer 4, ME without the acoustic interface between SM and ME; and layer 5, serosa and subserosal fat. In SAM, layer 1 corresponded to surface mucous contents; layer 2 was equivalent to MM; and layers 3 and 4 almost corresponded to SM and ME, respectively, although their interface was sometimes obscured owing to the presence of loose muscle fibers.

275 In a normal stomach, muscle layers such as MM and ME were harder on palpation than
276 mucosa or SM, which were well discriminated by SAM imaging. Higher cellularity and richer
277 fiber content owing to fibrosis and muscle bundles also exhibited greater SOS values[6].
278 Overall, the SOS values for each layer corresponded well to tissue elasticity.

279 All tissues have their own speed-of-sound (SOS) by which ultrasound travels through them.
280 The SOS is usually proportional to the tissue elasticity. Even after formalin fixation, the
281 specimens usually keep their elasticity in the same order as their original unfixed ones
282 (Supplemental Fig. 1). Table 1 and Figure 3 showed particular SOS of each lesion or
283 component of stomach, so that imaging from SOS reflected each tissue character of
284 objective elasticity compared with palpation hardness.



285
286 **Supplemental Fig. 1. Effect of formalin fixation**

287 *A fresh-frozen section of mouse small intestine before fixation (a) and at 3 min (b), 1 h (c),*
288 *12 h (d), 24 h (e) after 10% formalin fixation, and their corresponding H&E section (f). The*
289 *mucosa of unfixed section shows almost the same SOS as that of water and internal*
290 *structures are invisible. At 3 min, mucosal cytoskeleton appears as axes of villi structures.*
291 *After 1 h and over, mucosal cytoplasm displays almost uniform SOS and internal structures*
292 *become unclear.*

293 Most tumors displayed similar SOS values to their original tissues. Carcinomas or carcinoid
294 tumors in mucosa demonstrated the same SOS values as mucosal glands or foveolar
295 tubules, and leiomyomas displayed the same SOS values as MM or ME.

296 Tubular structure architecture was irregular or lost in well and poorly differentiated
297 adenocarcinomas, respectively. At the cancer invasive site through MM, the smooth muscle
298 layer appeared split or penetrated by cancer cells and exhibited a lower SOS value
299 compared with the surrounding intact MM.

300 In general, carcinomas are harder than sarcomas, and this was definitely supported by the
301 SAM data. Although carcinomas in themselves showed the same SOS values as that of their
302 original mucosa, they induced stromal desmoplastic reactions in invasion sites with
303 increased collagen fibers, which resulted in increased SOS. Scirrhou carcinomas exhibited
304 significantly greater SOS values than non-invasive carcinomas or non-scirrhou carcinomas.

305 Scirrhou carcinomas also demonstrated significantly higher SOS values than other
306 sarcomas, such as lymphomas and GISTs, which could be useful supplementary data for
307 making histological tumor diagnoses.

308 Compared with benign leiomyomas that are conspicuously hard, leiomyosarcomas are
309 usually softer upon palpation. The SAM data proposed significantly less SOS of
310 leiomyosarcomas than that of leiomyomas, which confirms is in accordance with the
311 aforementioned findings.

312 We used FFPE sections instead of fresh-frozen tissue. In the literature, low-frequency
313 studies (1–7 MHz) of acoustic parameters, including SOS, have demonstrated that fixation in
314 4% formalin solution changed these parameters only slightly[10]. FFPE kidney tissue
315 showed no significant differences from fresh-frozen kidney tissue[11]. Moreover, we
316 assessed the effect of fixation effect on SAM observations before performing this study. We
317 prepared fresh-frozen sections and soaked them in 10% formalin to observe changes in
318 SAM findings according to the fixation duration. Because mucosal imaging was unstable
319 before 1-h fixation (Supplemental Fig. 1), we used paraffin sections that had been fixed for
320 more than 1 h in this study. Other merits of using FFPE included the availability of archive
321 blocks and easy preparation of 10- μ m flat sections compared with fresh-frozen sections.

322 Malignant tumors are, in general, harder than surrounding normal tissues. Manual palpation
323 has been used to assist in diagnosis, but it is subjective and restricted to large and more
324 superficial structures. In the upper GI tract, EUS has recently been used to diagnose
325 subepithelial lesions, estimate the invasive depth of early GI cancers, and evaluate of
326 esophageal varices[12]. For definite diagnosis of subepithelial lesions, particularly deep SM
327 and ME layers, histological access, such as that achieved with EUS-guided fine needle
328 aspiration, is usually needed[3]. SAM results indicated that SM non-epithelial tumors
329 demonstrated similar SOS values. Irregular margins and geographic myxoid changes may
330 be suggestive of malignancy, and biopsy specimens should be obtained from these sites.

331 An ultrasonic imaging system typically needs to be able to resolve structures of $\sim$ 1 mm size
332 at depths of up to $\sim$ 150 mm in humans. US waves travel $\sim$ 1500 m/s through water. The
333 wavelength, which is one of the factors that determines spatial resolution, is optimal for
334 abdominal scanning, for example, 0.5 mm at 3 MHz[4]. Objects smaller than the wavelength
335 do not reflect US. The penetration length through tissues decreases proportional to the
336 wavelength. For histological imaging, we employed a 120-MHz transducer that had about
337 13- μ m spatial resolution, which barely allows it to detect single cells.

338 We previously reported SAM imaging of lungs[5] and lymph nodes[6]. In the latter,
339 statistically significant differences in SOS values were observed among scirrhou
340 carcinomas, lymphomas, and medullary carcinomas. The SOS values of these tumors
341 correlated with the elasticity on manual palpation. These results were also applicable to
342 gastric lesions. In the lung, the degree of pulmonary fibrosis was reflected with SAM and
343 was statistically comparable between lesions. In gastric lesions, tumor invasion-induced
344 stromal fibrosis was detected by SAM, and the SOS data revealed a statistical difference
345 between non-invasive and invasive carcinomas.

4. CONCLUSION

In conclusion, based on our experiences and the present observation, SAM offers the following benefits over LM: (1) images are acquired in only a few minutes without the need for special stains; (2) repeated observations of the same section are possible even after staining; (3) digital SOS imaging achieves high resolution, approaching that achieved with LM; (4) SAM analysis could be helpful to understand ultrasonic endoscopic images; and (5) digitized SOS data can be used to statistically compare different stomach lesions.

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COMPETING INTERESTS

The authors declare that they have no competing financial interests.

AUTHORS' CONTRIBUTIONS

Author KM designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author SY managed the analyses of the study. All authors reviewed the manuscript.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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