Analysis of the amount of tissue sample necessary for mitotic count and Ki-67 index in gastrointestinal stromal tumor sampling

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Abstract. There are no established opinions concerning whether the amount of tissue affects the accuracy of histological analyses in gastrointestinal stromal tumors (GISTs). The aim of the present study was to investigate the appropriate amount of tissue sample needed for mitotic count based on the risk classification of GISTs and the Ki-67 index using the following three methods: endoscopic ultrasound-guided fine-needle aspiration (FNA), a novel sampling method called tunneling bloc biopsy (TBB), and biopsy forceps followed by TBB (Bf). Forty-three samples (12 FNA, 17 TBB and 14 Bf) diagnosed as GISTs by immunohistological analysis were utilized. The major and minor axes and overlay area of one piece of specimen (OPS) from the three sampling methods were measured using digital imaging software and were analyzed comparatively regarding the acquisition of histological data. The mean major and minor axes (mm) and overlay areas (mm²) were in the order of TBB > Bf > FNA. The evaluable yield for GI subepithelial tumors.

Introduction

Gastrointestinal subepithelial tumors (SETs) include mesenchymal tumors, such as gastrointestinal stromal tumors (GISTs), myogenic and neurogenic tumors, which collectively account for 54% of all SETs, followed in frequency by heterotopic pancreases, cysts, lipomas, carcinoid tumors, lymphangiomas and hemangiomas (1). GISTs are the most common type of SET, and they exhibit malignant potential, thus requiring a multidisciplinary approach to optimize their management (2). Minimally invasive local resection techniques, such as endoscopic full-thickness resection (EFTR), have been developed for the treatment of intramural GISTs (3).

However, it is important to diagnose SETs preoperatively, since doing so can help to avoid unnecessary resection and enable optimal surgical resection, thereby reducing the number of unselectable or metastatic GIST cases. According to major guidelines such as the National Comprehensive Cancer Network (NCCN), the European Society for Medical Oncology (ESMO), the definitive final diagnosis of SETs should be based on immunohistochemistry (4-8). Immunohistochemical staining of various cellular proteins can be performed on tissue samples to provide diagnostic information. The most important markers used to evaluate hypoechoic intramural masses are CD-117 (c-kit), CD-34, smooth muscle actin and S-100 (9,10). c-kit is a transmembrane receptor with tyrosine kinase activity that is highly sensitive and specific for GISTs. CD-34 is also expressed in ~80% of GISTs. Positive staining for smooth muscle actin suggests the presence of a leiomyoma, and the presence of S-100 suggests a neural origin or a schwannoma. A preliminary study suggested that Ki-67 index (a marker of cell proliferation) immunohistochemical staining improves the ability to diagnose malignant GISTs (11). Moreover, the clinical behavior of GISTs is quite variable and can be difficult to predict on the basis of available clinical and histologic features. Nonetheless, a consensus conference proposed a
strategy for predicting the malignant behavior of GISTs based on the size (<2, 2-5, >5-10, or >10 cm) and mitotic count on histology [<5, 6-10, or >10/50 high-power fields (HPFs)], with the understanding that no GIST can be defined as benign on the basis of the currently available diagnostic testing (9).

Recently, several tissue sampling methods have been proposed for the diagnosis of SETs, with endoscopic ultrasound (EUS)-guided fine-needle aspiration (FNA) emerging as a standard method. However, the diagnostic yield of EUS-FNA, including spindle cell neoplasms (‘suspicious’), has generally been suboptimal (66-83.9%) and has partially depended on the location, size and characteristics of the target tissues, as well as certain technical and procedural factors (12-14). In particular, the immunohistological (IH) analysis needed for a definitive final diagnosis has revealed the low diagnostic rate of EUS-FNA (34-61.6%) (13,14). We previously developed a bloc biopsy method involving submucosal endoscopy with a mucosal flap (SEMF) (15), called tunneling bloc biopsy (TBB), to obtain core biopsy specimens under direct vision from growing endoluminal SETs (16). To date, no studies have investigated whether the amount of tissue sample can affect the accuracy of histological diagnostic methods, including IH analysis, mitotic count and histological Ki-67 staining.

The aim of the present study was to investigate the amount of GIST tissue needed for histological data: mitotic count and Ki-67 index by analyzing samples acquired using FNA and applying our TBB method.

Materials and methods

Materials. Between November 2008 and May 2014, 43 samples acquired by the following three tissue sampling methods: FNA, TBB and the use of biopsy forceps followed by TBB (Bf), which were diagnosed definitely as GISTs by IH staining were utilized. The 43 samples consisted of 12 FNA, 17 TBB and 14 Bf samples (Fig. 1). The present study was designed as a retrospective study and was conducted at a single academic medical center, Kagawa University Hospital, Japan. The present study was approved by the Clinical Ethics Committee of Kagawa University Hospital. The clinical application of TBB and Bf sampling methods was previously approved by the above ethics committee on November, 2011. All of the patients provided written informed consent to undergo the tissue sampling methods.

Analysis methods. The length of the major axis, the length of the minor axis, and the overlay area of each one piece of specimen (OPS) from the three tissue sampling methods were measured using digital imaging software (CellSens Standard; Olympus, Tokyo, Japan) on hematoxylin and eosin (H&E)-stained or IH tissue sections. The CellSens Standard software introduces interactive measurement capabilities for distances and polygons. The overlay area of each OPS was calculated by polygon measurement. Additionally, associations between sampling methods and histological data were analyzed comparatively.

Evaluation items. First, we calculated the mean of each parameter (major and minor axes, overlay area) of each OPS acquired using three sampling methods (FNA, TBB and Bf).

Materials and methods

Evaluation of histological findings. The degree of mitotic index, indicating the average number of mitotic cells in 50 HPFs (x40 objective and x10 ocular lens), was estimated by the visual impressions of two expert pathologists (Y.K. and R.H.) without counting the actual numbers of tumor cells. The cell blocks documented an equivalent morphology, which was characterized by monotonous sheets and groups of spindle-shaped cells with oval nuclei and well-defined cellular borders. Immunohistochemical procedures were performed on the 3-μm serial sections, utilizing the following commercially obtained antiserum: CD117 [w.d. (working dilution), 1:50], smooth muscle actin (SMA; w.d., 1:50), vimentin (w.d., 1:200), S-100 (w.d., 1:400), Ki-67 (MIB-1; w.d., 1:50), desmin (w.d., ready to use) (all obtained from DakoCytomation, Copenhagen, Denmark) and CD34 (w.d., ready to use) (Leica Biosystems, Newcastle, UK). The growth fraction, determined by Ki-67 abundance as the MIB-1 labeling-index, was low, showing <10% positively labeled nuclei.

Techniques of tissue sampling methods

Endoscopic ultrasound-guided fine-needle aspiration. With patients in the left lateral decubitus position under conscious sedation, EUS-FNA was performed using a conventional convex scanner echo endoscope (UCT-240-AL5; Olympus) connected to an ultrasound scanner (ProSound SSD-α10; Aloka, Tokyo, Japan). Tissue samples were obtained with disposable 19-, 22- or 25-gauge aspiration needles (Expect™ standard type; Boston Scientific, Tokyo, Japan) (FNA
sample) (Fig. 2A). Color flow and Doppler sonography was performed to exclude intervening vascular structures and to select a vessel-free needle track to avoid vessel puncture. EUS-FNA was performed as previously described (17). Briefly, after advancing the needle into the lesion under EUS visualization, the central stylet was removed, a 10-ml syringe with extension tubing was attached to the hub of the needle, and suction was applied as the needle was moved backward and forward within the lesion. During each puncture session, the needle was moved in various directions >10 times within the lesion, before being retracted into the catheter and the entire catheter being removed. Saline containing the aspirated material was transferred to a Petri dish and was examined macroscopically by an on-site cytopathologist to determine whether the tissue sample was cytologically adequate; if deemed inadequate after two punctures, an additional puncture was performed with a larger needle. All of the EUS-FNA procedures were performed by an endosonographer and an experienced endoscopist (H. Kamada), who has successfully performed more than 200 EUS-FNA procedures.

**Tunneling bloc biopsy.** Patients were administered intravenous midazolam (0.05 mg/kg) and pethidine (50 mg) prior to TBB, which consisted of five major procedures (16). Briefly, in the first step, after placing several dots around the tumor at a margin of ~5 mm, with one dot at the top of the tumor, a small incision was made to create a 10-mm opening flap, followed by submucosal injection of 0.4% hyaluronate sodium solution (MucoUp; Johnson & Johnson K.K., Tokyo, Japan) with a needle knife (KD-441Q; Olympus). In the second step, SEMF (15), a short 10-mm tunnel through the opening flap was created by additional submucosal dissection to approach the tumor. In the third step, bloc biopsy, the tumor was visually identified and exposed, and a bloc specimen measuring ~5 x 5 x 2 (major axis x minor axis x depth, mm) (TBB sample) (Fig. 2B) was obtained using the needle knife on the electrosurgical unit (VIO300D; ERBE Elektromedizin, Tübingen, Germany) in EndoCut mode (effect 2, duration 3) while minimizing tissue crushing. Separation of the bloc specimen from the tumor required a 2-mm-deep spindle-shaped incision. In this step, a bloc specimen was simultaneously acquired using biopsy forceps (Radial Jaw™ 4 Standard Capacity; Boston Scientific) (Bf sample) (Fig. 2C). In the fourth step, tissue collection, the specimen was detached from the tumor with grasping forceps (FG-6U-1) or hemostatic forceps (FD-410 LR) (both from Olympus) and was collected into the transparent cap that was longer at the tip (Elastic Touch F-030; ToP Corporation, Tokyo, Japan). All of the procedures were performed by an experienced endoscopist (H. Kobara), who has successfully performed more than 200 gastric endoscopic submucosal dissection (ESD) cases. Bleeding was controlled in all of the procedures using hemostatic forceps (FD-410 LR).

**Statistical analysis.** Summary statistics (mean, range) were calculated for each tissue sampling method. The results were compared using Fisher’s exact test, as appropriate. The cut-off
values between ‘Successful Samples’ and ‘Unsuccessful Samples’ were chosen using the likelihood ratio test by logistic regression analysis, calculating the balanced error rate (BER), odds ratio (OR), receiver operating characteristic (ROC) curve, and area under the ROC curve (AUC). All of the data analyses were performed using STATA software, version 7.0 (StataCorp, College Station, TX, USA), and a P-value <0.05 was considered to indicate a statistically significant result.

**Results**

*Assessment of mean parameters.* The parameters of the 43 specimens adequate for IH staining, obtained with the FNA needles, TBB and Bf, were calculated as follows. The mean major axis lengths were 1.598 mm (range, 0.16-3.8), 3.765 mm (range, 1.7-6.4) and 1.829 mm (range, 1.1-2.6), respectively. The mean minor axis lengths were 0.486 mm (range, 0.1-0.8), 2.382 mm (range, 1.4-4.2) and 1.214 mm (range, 0.6-2.1), respectively. The mean overlay areas by polygon measurement were 0.907 mm² (range, 0.098-3.11), 4.864 mm² (range, 1.4-12.1) and 1.478 mm² (range, 0.35-3.1), respectively (Table I). Representative digital slides obtained using the three sampling methods are presented in Fig. 3A-D.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Mean major lengths, n (mm)</th>
<th>Mean minor lengths, n (mm)</th>
<th>Mean overlay areas by polygon measurement (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FNA (n=12)</td>
<td>1.598</td>
<td>0.486</td>
<td>0.907</td>
</tr>
<tr>
<td>TBB (n=17)</td>
<td>3.765</td>
<td>2.382</td>
<td>4.864</td>
</tr>
<tr>
<td>Bf (n=14)</td>
<td>1.829</td>
<td>1.214</td>
<td>1.478</td>
</tr>
<tr>
<td>Total (n=43)</td>
<td>2.397</td>
<td>1.361</td>
<td>2.416</td>
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</table>

The major and minor axes and overlay area of one piece of specimen from the three sampling methods were measured using digital imaging software. The mean major and minor axes (mm) and overlay areas (mm²) were in the order of TBB > Bf > FNA. FNA, EUS-guided fine-needle aspiration; TBB, tunneling bloc biopsy; Bf, use of biopsy forceps followed by TBB.

*Associations between sampling methods and histological parameters.* We analyzed the evaluable rates by mitotic...
The evaluable rates by mitotic counts and Ki-67 index were respectively, 75% (9/12) and 83.3% (10/12) for FNA samples, 100% (17/17) and 100% (17/17) for TBB samples, and 100% (14/14) and 100% (14/14) for Bf samples. There were no significant differences between the three sampling methods in regards to the evaluable rates (FNA vs. TBB vs. Bf; P>0.05, Fisher’s exact test).

Identification of appropriate tissue amounts for histological analysis.

The mean major and minor axes (mm) and overlay areas (mm$^2$) of the ‘Successful Samples’ were 2.688, 1.562 and 2.847 (n=40), while those of the ‘unsuccessful Samples’ were 0.423, 0.29 and 0.127 (n=3), respectively. Three FNA samples were judged unevaluable due to too small specimens in overall diagnosis including mitotic count and Ki-67.

Table II. The evaluable rates of tissue samples according to the sampling method and histological parameters in the gastrointestinal stromal tumors.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Mitotic counts/ 50 HPF, % (n)</th>
<th>Ki-67 (M1B) index, % (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FNA (n=12)</td>
<td>75 (9)</td>
<td>83.3 (10)</td>
</tr>
<tr>
<td>TBB (n=17)</td>
<td>100 (17)</td>
<td>100 (17)</td>
</tr>
<tr>
<td>Bf (n=14)</td>
<td>100 (14)</td>
<td>100 (14)</td>
</tr>
<tr>
<td>Total (n=43)</td>
<td>93 (40)</td>
<td>93 (40)</td>
</tr>
</tbody>
</table>

The evaluable rates by mitotic counts and Ki-67 index were respectively, 75% (9/12) and 83.3% (10/12) for FNA samples, 100% (17/17) and 100% (17/17) for TBB samples, and 100% (14/14) and 100% (14/14) for Bf samples. There were no significant differences between the three sampling methods in regards to the evaluable rates (FNA vs. TBB vs. Bf; P>0.05, Fisher’s exact test). HPF, high-power field; FNA, EUS-guided fine-needle aspiration; TBB, tunneling bloc biopsy; Bf, use of biopsy forceps followed by TBB.

Table III. Comparison of sufficient specimens needed for overall histological data (immunohistological staining, mitotic count and Ki-67) for each sampling method.

<table>
<thead>
<tr>
<th>Mean major x minor axis lengths (mm), overlay areas (mm$^2$), (n)</th>
<th>Successful Samples</th>
<th>Unsuccessful Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=40)</td>
<td>(n=3)</td>
</tr>
<tr>
<td>FNA (n=12)</td>
<td>1.99 x 0.55, 1.17</td>
<td>0.42 x 0.29, 0.123</td>
</tr>
<tr>
<td>(n=9)</td>
<td></td>
<td>(n=3)</td>
</tr>
<tr>
<td>TBB (n=17)</td>
<td>3.77 x 2.38, 4.86</td>
<td>-</td>
</tr>
<tr>
<td>(n=17)</td>
<td></td>
<td>(n=0)</td>
</tr>
<tr>
<td>Bf (n=14)</td>
<td>1.83 x 1.21, 1.48</td>
<td>-</td>
</tr>
<tr>
<td>(n=14)</td>
<td></td>
<td>(n=0)</td>
</tr>
<tr>
<td>Total (n=43)</td>
<td>2.69 x 1.56, 2.85</td>
<td>0.42 x 0.29, 0.123</td>
</tr>
<tr>
<td>(n=40)</td>
<td></td>
<td>(n=3)</td>
</tr>
</tbody>
</table>

We defined one piece of specimen as a ‘Successful Sample’ when the sample was suitable for histological analysis of the mitotic count and Ki-67 index. The mean major and minor axes (mm) and overlay areas (mm$^2$) of the ‘Successful Samples’ were 2.688, 1.562 and 2.847 (n=40), while those of the ‘Unsuccessful Samples’ were 0.423, 0.29 and 0.127 (n=3), respectively. Three FNA samples were judged unevaluable due to too small specimens in overall diagnosis including mitotic count and Ki-67. FNA, EUS-guided fine-needle aspiration; TBB, tunneling bloc biopsy; Bf, use of biopsy forceps followed by TBB.

Figure 4. Identification of appropriate tissue amounts for histological analysis. Comparing ‘Successful Samples’ with ‘Unsuccessful Samples’ yielded cut-off values (BER, OR and AUC) of 0.17 mm$^2$ (0, ∞, 1) for the overlay area (logistic regression analysis by likelihood ratio test). BER, balanced error rate; OR, odds ratio; AUC, area under the ROC curve.

Identification of appropriate tissue amounts for histological analysis. The mean major and minor axes (mm) and overlay areas (mm$^2$) of the ‘Successful Samples’ were 2.688, 1.562 and 2.847 (n=40), while those of the ‘Unsuccessful Samples’ were 0.423, 0.29 and 0.127 (n=3), respectively (Table III). Representative digital slide of a small specimen obtained by EUS-FNA is shown in Fig. 3A.

Comparing ‘Successful Samples’ with ‘Unsuccessful Samples’ yielded cut-off values (BER, OR and AUC) of 0.17 mm$^2$ (0, ∞, 1) for the overlay area (logistic regression analysis by likelihood ratio test) (Fig. 4).
Concordance rate between pre- and post-operative samples in the GISTs. In the 16 resected GIST cases, the concordance rates regarding mitotic count were 50% (5/10) in FNA, 92.3% (12/13) in TBB and 90.9% (10/11) in Bf. Comparing the three
sampling methods, TBB was significantly better than FNA (TBB vs. FNA; P=0.035, Fisher's exact test). The concordance rates regarding the Ki-67 index were 60% (6/10) in FNA, 92.3% (12/13) in TBB and 90.9% (10/11) in Bf. There were no significant differences among the three sampling methods in regards to Ki-67 concordance (FNA vs. TBB vs. Bf; P>0.05, Fisher's exact test) (Fig. 5).

The discords between pre- and post-operative samples were refined as follows. Three FNA samples were too small for evaluation by mitotic count/50 HPF (sample number, major x minor axes, overlay area: sample 1, 0.54 x 0.4 mm, 0.16 mm²; sample 2, 0.16 x 0.1 mm, 0.11 mm²; sample 3, 0.35 x 0.37 mm, 0.098 mm²). Two samples obtained by FNA, one sample obtained by TBB, and one sample obtained by Bf had discrepancies with the post-operative sample. In all of these four samples, mitotic count/50 HPF <1 was converted to >5, Ki-67 index <5% was converted to >10%. The representative digital slides of TBB and FNA samples are shown in Fig. 6A-D.

**Discussion**

An optimal tissue sampling strategy for the diagnosis of SETs is needed to determine the most appropriate management plan (e.g., surgical resection or observation). Since each type of SET lesion can have a different prognosis, different therapeutic options are required. The acquired tissue samples should be appropriate materials for immunohistochemical staining for proteins such as CD-117 (c-kit), CD-34, smooth muscle actin and S-100, to differentiate between various SETs. Additionally, in GIST cases, the material should enable pathologists to evaluate mitotic count by histology, based on major consensus conferences (NCCN, ESMO) (4,6), and the risk classification of GIST (9,18).

The present study, by analyzing the acquired samples with EUS-FNA, TBB and Bf, has presented findings regarding the appropriate amounts of tissue samples required for IH diagnosis, cell count and Ki-67.

**Differences in tissue amounts between sampling methods.**

The mean major and minor axis lengths (mm) and overlay area (mm²) by polygon analysis were in the order of TBB > Bf > FNA (major axis, 3.765 vs. 1.829 vs. 1.598; minor axis, 2.382 vs. 1.214 vs. 0.486; area, 4.864 vs. 1.478 vs. 0.907). Sakamoto et al previously reported that the mean maximum size of fragments obtained with 25- and 22-gauge FNA needles and with a 19-gauge Trucut needle were 0.4 mm (range, 0.2-1.1 mm); 0.7 mm (range, 0.3-1.4 mm); and 2.7 mm (range, 0.8-3.6 mm), respectively (19).

The outer diameters of 25-, 22- and 19-gauge FNA needles (Expect™ standard type) are generally 0.52, 0.72 and 1.10 mm, respectively. The outer diameter of the needle will be equivalent to the minor axis length in samples such as ours. In the present study, the mean major lengths obtained with the 25-, 22- and 19-gauge FNA needles were 0.38 (n=4), 0.56 (n=6) and 0.49 mm (n=2), which did not show a tendency toward similarity with the outer diameter of each size of needle. Additionally, since the major axis lengths obtained with FNA needles depend on the depth of the puncture needle, based on the size of the targeted tumor, the caliber of the FNA needle would not affect the length of the acquired specimens.

Therefore, there were no significant differences in the major axis lengths among the 25-, 22- and 19-gauge needles (2.19 vs. 1.30 vs. 1.16 mm). In contrast, the mean lengths of the specimens obtained by TBB were 3.77 mm on the major axis and 2.38 mm on the minor axis, and these were the largest specimens among the 3 tissue sampling methods. The mean lengths of specimens obtained with the use of the Bf method were 1.83 mm (major) and 1.21 mm (minor). Since the cup size of the biopsy forceps (Radial Jaw™ 4 Standard Capacity) used in the present study was ~2.36 x 1.83 mm, the size of the Bf specimens was slightly smaller than expected from the caliber of the devices.

**Histological analysis of the acquired tissue samples.**

Regarding the assessment of the mitotic count/50 HPF and Ki-67 index, the evaluable rate of the FNA samples was 75% (9/12), which was lower than the 100% observed with both TBB (17/17) and Bf (14/14). Three samples in the FNA group were judged unvaluable since they were too small. Accordingly, although these samples were diagnosed definitively as GISTs by IH staining, they could not be given a risk classification of GIST. This finding suggested that the FNA samples had some limitations in diagnosing the risk classification of GIST.

Notably, two samples obtained by FNA, one sample obtained by TBB, and one sample obtained by Bf had discrepancies between pre- and post-operative histological findings regarding the mitotic count/50 HPF and the Ki-67 index. This finding demonstrated that cell proliferation of GISTs could be expressed differently at each site within the tumor. Therefore, we must recognize that pre-operative diagnosis by tissue sampling methods with regard to GIST risk classification may be rarely discordant with the final definitive diagnosis.

**Appropriate tissue amounts for histological analysis.**

Comparing ‘Successful Samples’ with ‘Unsuccessful Samples’, we calculated the cut-off value for the overlay area of OPS as 0.17 mm², suggesting the appropriate amount of OPS needed for IH diagnosis, cell count and Ki-67. The mean overlay areas of OPS acquired by TBB and Bf were 4.86 and 1.48 mm². Consequently, the cut-off value revealed that the amounts of tissue acquired by Bf can be sufficient for the assessment of mitotic count and Ki-67 without the need for the amounts of tissue obtained by TBB.

**Strengths and limitations of each sampling method.**

Although EUS-FNA, which has the advantages of being rapid and convenient, has emerged as a standard method, appropriate tissue samples can occasionally not be acquired due to too little material and technical issues. The first prospective study of the diagnostic yield of EUS-FNA with a commercially available needle in patients with gastric SETs was reported in 2009 (20). However, the diagnostic yield of EUS-FNA was not satisfactory (63%; 31/49) since the tissue samples obtained were too small to determine their mitotic indices reliably. Diagnostic accuracy <60% has been reported by others (21,22). Therefore, further developments in needle devices and technical skills are required to minimize sampling errors.

In contrast, a key advantage of TBB and Bf is its use of a lateral approach with submucosal endoscopy with a mucosal flap safety valve (SEMF), making it easy to create a platform
that provides an operative field and to manage hemostasis while obtaining a core specimen of sufficient size (~5 mm) for IH analysis under direct vision (23). Owing to this technical advantage, TBB and Bf demonstrated higher rates of overall diagnosis including cell counts and Ki-67 than FNA. However, these methods have a limitation of indicating for primarily intraluminal growing GISTs excluding extraluminal growing GISTs. According to growth pattern of SETs, appropriate sampling methods have to be introduced.

In conclusion, while the amounts of tissues obtained by TBB and Bf are excessively unnecessary for the histological assessment of the mitotic count and Ki-67 index, developments of the FNA method are needed to minimize sample error. Considering the technical aspects, as well as the size of specimens, could help to guide therapeutic planning and improve the diagnostic yield for GI subepithelial tumors.

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References