初発・再発を問わず，除菌療法が治療の第1選択である。除菌治療により維持療法なしに潰瘍再発が抑制されることは世界的にコンセンサスが得られている。活動性潰瘍では，除菌治療後プロトンポンプ阻害薬などの酸分泌抑制薬の投与を行うことが望ましい。

2．慢性胃炎

内視鏡検査と組織検査により診断する必要があり，1）症状改善を期待し本人および親が希望する，2）胃粘膜の萎縮が証明される，3）胃がんの家族歴を有する場合，除菌療法が考慮される。しかし，*H. pylori* 慢性胃炎と腹部症状との関連について確立した見解はなく，除菌により症状の消失ないし改善が得られる保証はない。

3．蛋白漏出性胃症

除菌治療により血清蛋白値や内視鏡・病理所見の正常化がみられることがある。*H. pylori* 感染以外に原因が見出されない場合に実施する。

4．鉄欠乏性貧血

小児では，*H. pylori* 感染と鉄欠乏性貧血との関連が多く報告されている。とくに，10歳以降の年長児の原因不明・再発をくり返す鉄欠乏性貧血には感染診断を行い，感染があれば除菌治療を考慮する。

5．血小板減少性紫斑病

成人（日本人）の *H. pylori* 陽性慢性 ITP 患者の約半数が除菌後に血小板増加を認められることが明らかとなってきた。成人では慢性 ITP の確定診断後早期に感染診断を実施し，*H. pylori* 陽性例に対しての first-line 治療として除菌治療をすることは，EBM として確立されてきている。小児の効果についてはさまざまな報告があるが，効果があったとする症例報告も散見され，治療抵抗性の症例あるいは無治療で経過観察中の症例（血小板数が正常化しない）における治療選択の一つと考えられる。

表2　小児の除菌療法に用いられる主な薬剤と一般的な用量

	用量 (mg/kg/日)	最大量 (mg/日)
プロトンポンプ阻害薬		
ランソプラゾール	1.5	60
オメプラゾール	1.0	40
抗菌薬		
アモキシシリン	50	1,500
クラリスロマイシン	10～20	800
メトロニダゾール	10～20	500

プロトンポンプ阻害薬はいずれか1剤，抗菌薬は1次除菌療法としてアモキシシリン，クラリスロマイシンの2剤を使用。ペニシリンアレルギーではクラリスロマイシン，メトロニダゾールの2剤を用いる。1次除菌が失敗した場合の2次除菌としてアモキシシリン，メトロニダゾールの2剤を用いる。いずれも分2投与とし，治療期間は原則7日間。

（加藤ら[6] 2005 を一部改変）

V．除菌治療法（表2）

1．一次除菌

まず選択される除菌薬剤は，プロトンポンプ阻害薬とアモキシシリン，クラリスロマイシンの3剤併用療法（PPI/AC）である。薬剤アレルギーに注意し，ペニシリンアレルギーがある場合はアモキシシリンをメトロニダゾールに変更する。投与期間は7日間が原則であるが，小児では14日間投与を推奨する意見もある[6]。副作用として下痢，味覚異常，悪心，発疹などがみられる。近年，除菌成功率は低下していると報告されており，主な原因はクラリスロマイシン耐性である。

2．二次除菌

一次除菌療法で除菌治療に失敗した場合，抗菌薬をクラリスロマイシンからメトロニダゾールに変更し，アモキシシリン・メトロニダゾール・プロトンポンプ阻害薬の3剤併用療法（PPI/AM）を7日間行う。

3．除菌判定法（表3）

除菌判定は治療終了後4週以降に実施する。判定時期を遅らせるほど診断精度は高くなる。除菌判定は，感染診断と同じ方法を用いるが，抗

表 3　小児の除菌判定

1）除菌判定は治療終了後 4 週以降に実施する。
2）判定時期を遅らせるほど診断精度は高くなる。
3）除菌判定は，生検組織を用いる診断法（培養法，迅速ウレアーゼ試験，鏡検法）ないし生検組織を用いない診断法（尿素呼気試験，便中抗原検査）で行う。
4）抗体測定法は除菌判定には用いない。
5）生検組織を用いた診断法では偽陰性に注意する。
6）尿素呼気試験では偽陽性が問題となるため，とくに低値の陽性ではただちに再除菌をせずに他の診断法の追加や追跡検査を行う。
7）最終的には検査を行ったすべての診断法が陰性の場合に除菌成功とする。

H. pylori 抗体測定は陰性化するまでに長期間を要するため除菌判定には用いない。複数の検査を併用することで感染診断の精度が高くなるため，2 法以上で除菌判定を行うことが望ましい。

Ⅵ. *H. pylori* 除菌治療の保険適用

（2012 年 4 月現在）

保険適用の対象疾患は *H. pylori* 感染がある胃潰瘍，十二指腸潰瘍，胃 MALT リンパ腫，特発性血小板減少性紫斑病，早期胃がんに対する内視鏡術後胃である。一次除菌治療としてプロトンポンプ阻害薬，アモキシシリン，クラリスロマイシンの 3 剤併用療法 7 日間，一次除菌治療に失敗した場合，二次除菌治療としてクラリスロマイシンをメトロニダゾールに変更した 3 剤併用療法 7 日間が適用となる。ただし，「小児に対する安全性は確立していない」と記載されていること，鉄欠乏性貧血や慢性胃炎などでは適用外であり，診療に際しては現時点の情報を本人と保護者に十分説明したうえで治療方針を決定することが重要である。

おわりに

小児の *H. pylori* 感染率の激減により胃十二指腸潰瘍は，小児期ではまれな疾患となってきた。一方，*H. pylori* 感染と胃がんとの関連が明らかになっており，胃がん予防として小児期の感染をどのようにコントロールするかは，今後の課題になると考える。

Key Points

❶ 小児の *H. pylori* 感染診断は 6 種の診断法からそれぞれの特徴を考慮して選択するが，複数であれば精度が高くなる。

❷ 小児の除菌治療は「小児に対する安全性は確立していない」と記載されており，適用外疾患も多く十分説明したうえで治療方針を決定することが重要である。

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EDITORIAL

Management of bleeding and artificial gastric ulcers associated with endoscopic submucosal dissection

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Abstract

Endoscopic submucosal dissection (ESD), an endoscopic procedure for the treatment of gastric epithelial neoplasia without lymph node metastases, spread rapidly, primarily in Japan, starting in the late 1990s. ESD enables en bloc resection of lesions that are difficult to resect using conventional endoscopic mucosal resection (EMR). However, in comparison to EMR, ESD requires a high level of endoscopic competence and a longer resection time. Thus, ESD is associated with a higher risk of adverse events, including intraoperative and postoperative bleeding and gastrointestinal perforation. In particular, because of a higher incidence of intraoperative bleeding with mucosal incision and submucosal dissection, which are distinctive endoscopic procedures in ESD, a strategy for endoscopic hemostasis, mainly by thermo-coagulation hemostasis using hemostatic forceps, is important. In addition, because of iatrogenic artificial ulcers that always form after ESD, endoscopic hemostasis and appropriate pharmacotherapy during the healing process are essential.

Key words: Artificial ulcer; Endoscopic hemostasis; Endoscopic submucosal dissection; Gastric epithelial neoplasia; Hemostatic forceps

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INTRODUCTION

Endoscopic submucosal dissection (ESD) is a novel endoscopic procedure developed in the 1990s[1,2], and is characterized by the use of electrosurgical knives for mucosal incision and submucosal dissection[3-15]. In ESD, the resected size and shape of tumors can be controlled, and even lesions difficult to resect by endoscopic mucosal resection (EMR) can be resected en bloc by ESD. As this technique permits en bloc resection of tumors, ESD has the advantages of enabling accurate pathological assessment and reducing the risk of local recurrence[2,16-19].

However, ESD requires a higher level of endoscopic competence than EMR. In addition, as a result of ESD being used to treat larger lesions and lesions with ulcerative findings, operation time is longer, with a higher risk

of adverse events such as bleeding and gastrointestinal perforation[20-29]. The incidence of procedure-related bleeding is higher with ESD than with EMR, and to permit safe completion of ESD, control of bleeding is very important. In this article, we discuss the characteristics of ESD-related bleeding (intraoperative and postoperative bleeding) and endoscopic hemostasis. Furthermore, to prevent postoperative bleeding, we also discuss the pharmacotherapy of artificial ulcers after ESD.

ENDOSCOPIC HEMOSTASIS USING HEMOSTATIC FORCEPS

Endoscopic hemostatic methods for peptic ulcers include various techniques, such as local injection of hypertonic saline-epinephrine (HSE) and ethanol, mechanical hemostasis using endoscopic hemoclips, and thermo-coagulation hemostasis[30,31]. Local injection of HSE alone is inferior to combination therapy with other hemostatic methods, but the clear superiority of any one method has not been definitively established[32]. Thermo-coagulation devices include contact thermal devices such as heater probes and hemostatic forceps, and non-contact thermal devices such as an argon plasma coagulator[33,34].

For hemostasis of ESD intraoperative bleeding, Enomoto *et al*[35] reported the usefulness of a method of thermo-coagulation hemostasis using monopolar hemostatic forceps in combination with an endoscope equipped with a water-jet system. Hemostatic technique in ESD, which differs from hemostasis for usual gastrointestinal bleeding, is often characterized by the need for repeated hemostasis during both mucosal incision and submucosal dissection. In addition, precise hemostatic maneuvers are required, in order not to interfere with the subsequent procedure after hemostatic treatment[36,37]. Therefore, hemostatic forceps, which enable reliable hemostasis when, with re-holding of the ruptured vessels permissible several times before coagulation, bleeding points can be accurately grasped, are useful for hemostasis in ESD-related bleeding[38,39] (Figure 1).

With wider use of ESD, hemostasis using hemostatic forceps has become routine at medical centers, and its usefulness for bleeding from exposed vessels at the base of peptic ulcers has also been reported[40,41]. Moreover, the usefulness not only of monopolar, but also of bipolar hemostatic forceps, has been reported[42].

MANAGEMENT OF BLEEDING DURING AND AFTER ESD

ESD-related bleeding includes intraoperative bleeding associated with procedures such as mucosal incision and submucosal dissection, and delayed bleeding, which occurs postoperatively from exposed vessels at ulcer bases. Appropriate management of each type of bleeding is required.

Endoscopic hemostasis for intraoperative bleeding

In ESD, the incidence of intraoperative bleeding, which is to some degree unavoidable given the nature of techniques such as incision and dissection, is as high as 22.6%[16]. In particular, with ESD for lesions in the upper third of the stomach, because of abundant vessels in the submucosa, the incidence of intraoperative bleeding is relatively high[43]. To predict intraoperative bleeding, identification of the submucosal vascular structure by preoperative endoscopic ultrasonography can be useful[44].

Of the series of techniques in ESD, bleeding is inevitable with submucosal local injection and mucosal incision because they are blind procedures in the vascular-rich submucosal tissue. To produce higher hemostatic ability, a small amount of epinephrine to a concentration of 0.0005% is added to the submucosal cushion (glyceol, Chugai Pharmaceutical Co., Tokyo Japan). On the other hand, during submucosal dissection, bleeding can be avoided at all sites by making every effort to visually identify vessels and not perform dissection blindly. Oyama *et al*[45] noted that identification of vessels prior to submucosal dissection and prophylactic thermo-coagulation are most important in preventing ESD intraoperative bleeding. Toyonaga *et al*[13,46] stated that knowing the correct layer of the submucosa containing fewer vessels and existing fibrous tissue, is important in reducing ESD intraoperative bleeding.

When bleeding occurs during ESD, by washing out the blood with the water-jet system and using a transparent attachment hood, a clear visual field can be maintained, and bleeding points can be rapidly identified[35]. For bleeding from vessels smaller than the electrosurgical knife tip or arm, hemostasis by thermo-coagulation with the knife is usually possible. For bleeding from vessels larger than the electrosurgical knife tip or arm, or bleeding for which hemostasis with the knife is difficult, hemostatic forceps are used (Figure 2). Fujishiro *et al*[47] reported that hemostatic forceps for vessels smaller than 2 mm in diameter, and hot biopsy forceps for vessels larger than 2 mm in diameter, are useful. When hemostasis by thermo-coagulation cannot be achieved, hemostasis using endoscopic hemoclips is necessary, so that subsequent procedures are not hindered.

Hemostasis for delayed bleeding

Delayed bleeding after ESD occurs in 0%-9% of cases[6,16,18,28,48-54] (Table 1). For resected lesions located in the middle and lower third of the stomach, the incidence is higher. Bleeding occurs when vessels at ulcer bases rupture due to physical stimulation by peristalsis or due to chemical stimulation, for example, by bile reflux[48]. Delayed bleeding often occurs within 24 h postoperatively and is related to lesion location, size, and ulcer-findings[48,55]. For delayed bleeding, in almost all cases, hemostasis is achieved with urgent endoscopic hemostasis[56]. However, cases requiring vascular embolization because endoscopic hemostasis could not be achieved[57], and cases complicated by disseminated intravascular coagulation the day after delayed bleeding[58] have been reported, so caution is necessary.

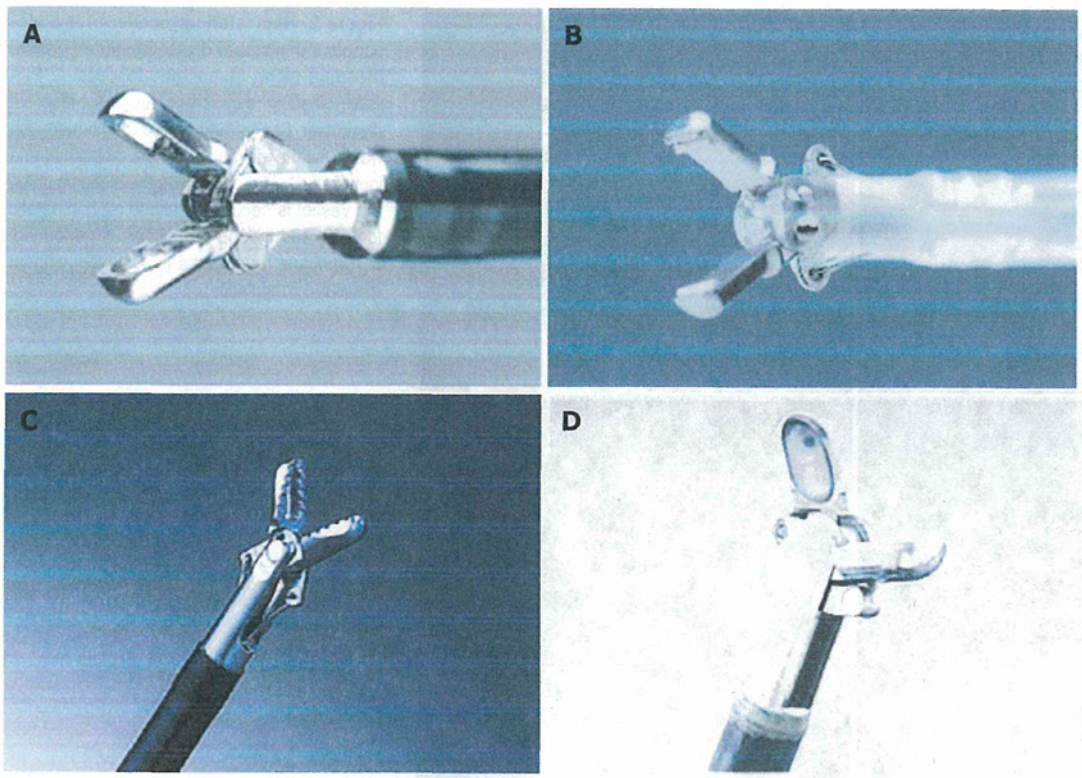

Figure 1 Hemostatic forceps tips. A: Monopolar hemostatic forceps (HDB2422W; Pentax, Tokyo, Japan); B: Bipolar hemostaticforceps (H-S2518; Pentax, Tokyo, Japan); C: Hemostatic forceps (Coagrasper: FD-410LR; Olympus, Tokyo, Japan); D: Hot biopsy forceps (FD-1L-1; Olympus, Tokyo, Japan).

Table 1 Delayed bleeding rate of endoscopic submucosal dissection for gastric epithelial neoplasia

Author	Year	Total cases	Delayed bleeding (%)	En bloc resection rate (%)
Oda *et al*[48]	2005	945	6	93
Kakushima *et al*[49]	2006	383	3.4	91
Imagawa *et al*[18]	2006	196	0	93
Onozato *et al*[50]	2006	171	7.6	94
Oka *et al*[16]	2006	195	6.2	83
Hirasaki *et al*[51]	2007	112	7.1	96
Ono *et al*[6]	2008	161	8.7	99
Hoteya *et al*[52]	2009	572	4.9	95
Isomoto *et al*[53]	2009	510	1.8	95
Tsuji *et al*[54]	2010	398	5.8	NA
Akasaka *et al*[28]	2011	1188	3.1	95

NA: Not analyzed.

To prevent delayed bleeding, prophylactic coagulation of exposed vessels at the bases of artificial ulcers that occur after ESD lesion resection is very useful. According to Takizawa *et al*[59], the cause of delayed bleeding is due more to insufficient prophylactic thermo-coagulation than insufficient primary hemostasis during ESD[60], because the site of delayed bleeding is not the site of endoscopic hemostasis during surgery. In addition, a study has been conducted on the prevention of delayed bleeding by evaluation of blood flow at ulcer bases using endoscopic Doppler ultrasound (US). Uedo *et al*[61], based on blood flow detected using Doppler US, reported that, by coagulation of vessels seen at artificial ulcer bases after ESD lesion resection, delayed bleeding is reduced, and unnecessary thermo-coagulation of vessels without blood flow can be avoided. On the other hand, Choi *et al*[62] reported that prophylactic closure of gastric EMR-induced ulcers with metal hemoclips prevent delayed bleeding.

In 2008, a survey of treatment methods for peptic and artificial ulcer bleeding was conducted at nine departments of high-volume center hospitals in Japan[63]. For endoscopic hemostasis of peptic ulcer bleeding, the number one method used was clipping (32.9%), followed by coagulation forceps (23.5%). In contrast, for artificial ulcer bleeding, coagulation forceps (77.8%) were used significantly more. In addition, the proportion of patients who underwent second-look endoscopy, compared to peptic ulcers, was significantly lower for artificial ulcers (86% and 71%, respectively).

The effectiveness of second-look endoscopy after hemostasis of peptic ulcer bleeding has previously been shown[64,65]. However, according to Goto *et al*[66], for artificial ulcers, no significant difference in the incidence of delayed bleeding before and after second-look endoscopy was found. This suggests that delayed bleeding after ESD, irrespective of whether second-look endoscopy is performed, may develop. However, for artificial ulcers located in the lower third of the stomach, compared to ulcers located in the upper and middle third of the stomach, because delayed bleeding occurs earlier, careful follow-up observation or early second-look endoscopy may be useful[54,66].

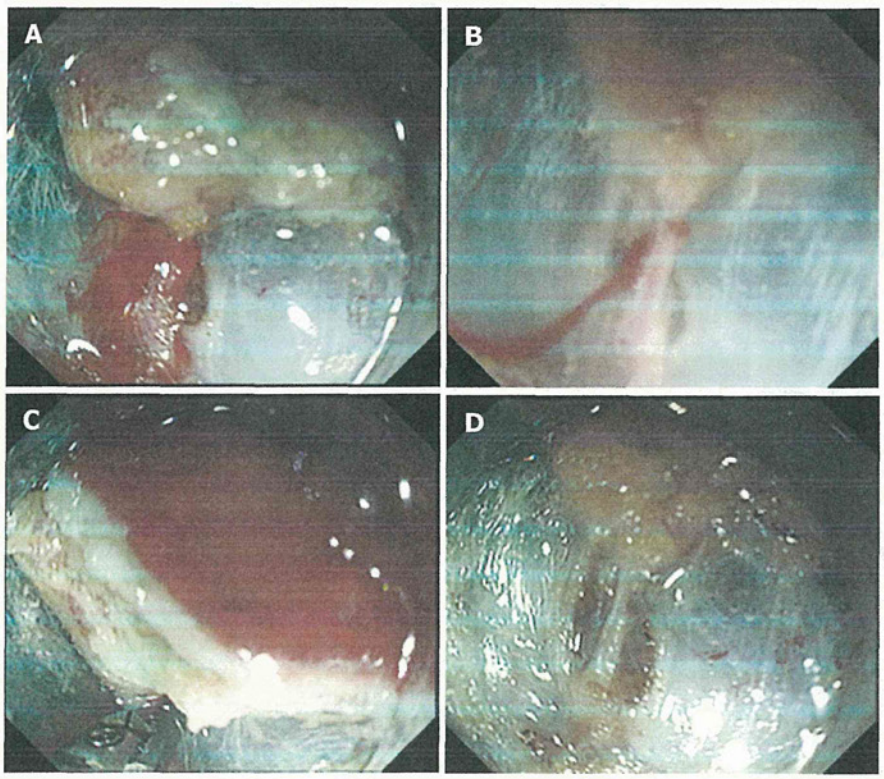

Figure 2 Hemostatic procedure for endoscopic submucosal dissectionintraoperative bleeding using hemostatic forceps. A: Pulsatile bleeding is observed during submucosal dissection; B: By filling the tip attachment with water, the bleeding point can be pinpointed and identified; C: After identifying the bleeding point, the vessel is securely grasped by hemostatic forceps, and thermo-coagulation is performed; D: Complete hemostasis is achieved, without excessive coagulation.

MANAGEMENT OF ARTIFICAL GASTRIC ULCERS AFTER ESD

Pharmacotherapy of artificial ulcers that develop after ESD lesion resection is also important to prevent delayed bleeding. However, management must take into account the differences in etiology between peptic ulcers and artificial ulcers after ESD.

Comparison of peptic ulcers and artificial ulcers

Currently, proton pump inhibitors (PPIs) are the drugs of first choice for treatment of peptic ulcers, and when a PPI cannot be used, an H2-receptor antagonist (H2RA) is selected. Treatment is generally for 8 wk. A meta-analysis of ulcer healing rates reported significantly higher ulcer healing rates with PPIs than with H2RAs[67,68]. In addition, in a meta-analysis of the efficacy of preventing recurrence of bleeding gastric ulcers, no differences in rebleeding rates, surgical intervention rates, or mortality rates between the two classes of drugs were reported[69].

The etiology of artificial ulcers after gastric ESD and peptic ulcers also differs greatly[70]. First, peptic ulcers develop, at least in part, due to hyperacidity, whereas artificial ulcers form in a hypoacidic environment in which there is severe mucosal atrophy. Second, peptic ulcers develop at sites where there is breakdown of gastric mucosal defense mechanisms, whereas artificial ulcers occur iatrogenically at sites where mucosal defense mechanisms are intact. Third, peptic ulcers include ulcers deeper than the submucosa, and inflammation spreads in the ulcer periphery, whereas artificial ulcers, because they basically occur due to submucosal dissection, are relatively shallow ulcers down to the submucosa, and the inflammation is localized. Despite these differences, treatment of an artificial ulcer after gastric ESD, based on treatment for a peptic ulcer, is empiric, with an anti-acid drug for 8 wk[63] (Table 2).

Anti-acid drugs for artificial ulcers

For artificial ulcers that develop after ESD for gastric mucosal lesions without preoperative ulcer findings, Kakushima *et al*[71] reported that healing occurred within 8 wk with PPI administration for 8 wk, irrespective of ulcer size or location. In addition, factors that influence artificial ulcer healing such as artificial ulcer size, location, *Helicobacter pylori* infection status, and extent of gastric mucosal atrophy had no effect. However, with fibrosis deeper than the submucosa of lesions prior to ESD, healing may be delayed[72,73]. According to Huang *et al*[74], although the recurrence rate of ESD artificial ulcers is lower than that of peptic ulcers, *Helicobacterpylori* infection and lesion ulcer findings are risk factors for recurrence. In contrast, Oh *et al*[75] reported that, because the extent of healing of artificial ulcers 4 wk after ESD is determined by the size of the ulcer initially formed, the duration of PPI treatment should be decided based on this parameter.

For artificial ulcers after EMR, Lee *et al*[76] compared PPIs in 1-wk and 4-wk treatment groups. They found that, after 4 wk, ulcer size, stage, subjective symptoms, and use of other mucosal-protective antiulcer drugs did not significantly differ between the groups. Niimi *et al*[77] reported that administration of PPI for 2-wk for artificial ulcers after ESD may be sufficient to help them heal. These results suggest that, for artificial ulcers, unlike peptic ulcers, the importance of acid secretion inhibition

Table 2 Healing process of gastric artificial ulcers after endoscopic submucosal dissection

Author	Year	Total cases	Drugs administration	Weeks	Ulcer healing rate (%)		Average ulcer size	
					4 wk	8 wk	Maximal diameter (mm)	Resected area (mm^2)
Kakushima *et al*[71]	2004	70	PPI + sucralfate	8	NA	100	34.7	NA
Lee *et al*[76]	2004	26	OPZ 20 mg	1	12	NA	NA	503
		34	OPZ 20 mg	4	15	NA	NA	575
Yamaguchi *et al*[78]	2005	29	OPZ 20 mg	8	NA	NA	27.8	NA
		28	Famotidine 40 mg	8	NA	NA	22.4	NA
Uedo *et al*[79]	2007	73	RPZ 20 mg	8	NA	83	41	NA
		70	Cimetidinde 800 mg	8	NA	89	40.5	NA
Asakuma *et al*[80]	2009	28	RPZ 20 mg + ES 3.0 g	8	40.7	96.3	NA	1306
		28	RPZ 20 mg	8	11.5	76.9	NA	1274
Kato *et al*[81]	2010	31	RPZ 10 mg + rebamipide 300 mg	4	68	NA	35	NA
		31	RPZ 10 mg	4	35	NA	31	NA
Fujiwara *et al*[82]	2011	30	RPZ 20 mg + rebamipide 300 mg	8	NA	86.7	41	1453
		31	RPZ 20 mg	8	NA	54.8	42.8	1521
Niimi *et al*[77]	2011	55	RPZ 10 mg	2	NA	80.0	32.7	NA

NA: Not analyzed; PPI: Proton pump inhibitor; OPZ: Omeprazole; RPZ: Rabeprazole; ES: Ecabet sodium.

in the ulcer healing process may be low.

Yamaguchi *et al*[78] compared PPI-treatment and H2RA-treatment groups in patients with artificial ulcers after EMR. They reported no differences in the incidence of delayed bleeding or ulcer size at 30 d and 60 d postoperatively. They did state that artificial ulcers healed more easily than peptic ulcers, and they concluded that, for artificial ulcers with severe bleeding within 24 h after surgery, treatment with H2RA drugs, whose onset of inhibition of gastric acid secretion is more rapid than that with PPIs, is appropriate.

Uedo *et al*[79] compared PPI-treatment and H2RA-treatment groups in patients with artificial ulcers after ESD. There were no differences in the incidence of delayed bleeding or ulcer healing rates between the groups. However, the cumulative non-bleeding rate using the Kaplan-Meier method was significantly higher in the PPI group. Moreover, on multivariate analysis, PPI treatment was an independent factor in reducing the rate of delayed bleeding. Their results suggested that PPIs are more effective than H2RAs for preventing ESD delayed bleeding.

For post-EMR ulcers and post-ESD ulcers, in terms of formation by endoscopic resection, with the exception of size, the pathophysiology is the same. However, in studies to date, with regard to ulcer healing and prevention of delayed bleeding when artificial ulcers are treated with acid secretion inhibitors, there is no agreement in the results. Regarding the need for and duration of treatment with acid secretion inhibitors for artificial ulcers, there is still room for debate.

Mucosal-protective antiulcer drugs in artificial ulcers

In the treatment of peptic ulcers, there is no evidence that combined therapy with a PPI and a mucosal-protective antiulcer drug is superior to a PPI alone. However, in artificial ulcers, an additive effect of mucosal-protective antiulcer drugs has been reported (Table 2). Asakuma *et al*[80] compared combined therapy with a PPI (rabeprazole 20 mg/d) and ecabet sodium (3.0 g/d) *vs* the PPI alone for artificial ulcers after ESD. At 4 wk and 8 wk, ulcer healing rates were significantly higher in the combined treatment group. In addition, Kato *et al*[81] compared combined therapy with a PPI (rabeprazole 10 mg/d) and rebamipide (300 mg/d) *vs* the PPI alone for artificial ulcers after ESD. At 4 wk, the ulcer scarring rate was significantly higher in the combined treatment group. Similarly, Fujiwara *et al*[82] compared combined therapy with a PPI (rabeprazole 20 mg/d) and rebamipide (300 mg/d) *vs* the PPI alone for artificial ulcers after ESD. At 8 wk, the ulcer scarring rate was significantly higher in the combined treatment group.

Thus, among the mucosal-protective antiulcer drugs, there are drugs that accelerate ulcer healing. This may be attributable to differences in the etiology between artificial ulcers and peptic ulcers, as previously mentioned, but further evidence must be accumulated.

CONCLUSION

With the increasing use of ESD for gastric epithelial neoplasia, management of ESD-related bleeding and artificial ulcers after lesion resection has become an important issue not only in Japan, but throughout the world. Therefore, more effective endoscopic hemostatic methods and appropriate pharmacotherapy of artificial ulcers, taking into account their etiology, are becoming increasingly important. Moreover, safer and more reliable ESD techniques must be developed.

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Image of the Month

Microcoil Slipping Out of the Gastric Varices

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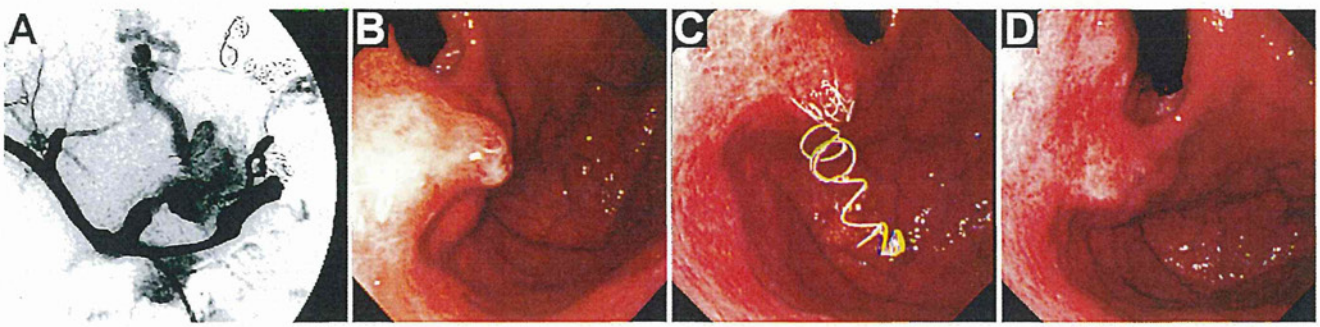

A 67-year-old man with alcoholic cirrhosis developed a gastric varix with a gastrorenal shunt. He underwent the interventional radiology of balloon-occluded retrograde transvenous obliteration and percutaneous transhepatic obliteration using ethanolamine oleate and microcoils (Figure *A*). Three years after the radiologic procedures, a part of the coil was found to be exposed at the apex of the gastric varix with ulceration (Figure *B*). Although ulceration was aggravated after 5 months, no sign of bleeding was observed. To evaluate blood flow to the varices, angiography was performed again. Negligible blood flow to the varices strongly indicated that the chance of massive bleeding was unlikely, therefore, the clinical course was carefully followed. After a follow-up period of 5 months, the exposed coil protruded in a spring pattern into the gastric lumen (Figure *C*). After an additional follow-up period of 10 months, the exposed coil completely migrated, passed per stomach wall, and the ulcer healed with a scar (Figure *D*). All through the clinical course, neither symptoms such as hematemesis or tarry stools, nor the progression of anemia was observed.

Hemorrhagic peptic ulcers and varices are treated by embolization with an embolic agent including coils using interventional radiology when their endoscopic treatment is difficult. In addition, there is an increase in cases treated by angiographic intervention, which is minimally invasive and helpful in patients with severe complications and a high risk for surgery. Investigators reported patients with hemorrhage gastroduodenal ulcer or aneurysms treated using coil embolization in whom a part of the coil was exposed to the gastrointestinal lumen after the procedure.[1,2]

We present a rare case of coil embolization for gastric varices, in which the clinical course from microcoil exposure to spontaneous migration into gastric lumen could be followed up. There is a possibility that microcoils, even after their placement in gastric varices, could become displaced and migrate into the gastrointestinal lumen, and, therefore, a careful follow-up evaluation of the coil in each case is necessary.

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Conflicts of interest

The authors disclose no conflicts.

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CASE REPORT

A case of chronic pancreatitis in which endoscopic ultrasonography was effective in the diagnosis of a pseudoaneurysm

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Abstract

Endoscopic ultrasonography (EUS) was performed on a patient being treated for chronic pancreatitis because a submucosal tumor was observed in the stomach during gastrointestinal endoscopy. As internal pulsatile blood flow on Doppler was present, the diagnosis of an aneurysm was made. The pseudoaneurysm of the left gastric artery was embolized with histoacryl and lipiodol and the splenic artery was embolized with coils at the location of the pseudoaneurysm to prevent hemorrhage. Follow up EUS confirmed the cessation of blood flow from the pseudoaneurysm. Clinicians encountering a gastric submucosal tumor-like protrusion in a patient with chronic pancreatitis should use EUS to investigate the possibility of a pseudoaneurysm, which must be treated as quickly as possible once identified.

INTRODUCTION

Pseudoaneurysms are a known complication of chronic pancreatitis. Untreated, pseudoaneurysms may rupture, and can be fatal.

We herein describe a patient with chronic pancreatitis who was diagnosed with a pseudoaneurysm of the left gastric artery while undergoing endoscopic ultrasonography (EUS) for a gastric submucosal tumor-like protrusion.

CASE REPORT

The patient, a 39-year-old male, presented with the primary complaints of chest tightness and upper abdominal pain. Previously, the patient had been repeatedly admit-

ted and discharged for alcoholic pancreatitis. An approximately 4 cm, left mediastinal, cystic lesion continuing from the tail of the pancreas was seen on multidetector row computed tomography (MDCT) at the time of presentation. An area of high density was observed within the cyst, and a severely atrophied pancreas with a calcified body was observed (Figure 1). As a pseudocyst complicating an acute exacerbation of chronic pancreatitis and hemorrhage in the pseudocyst was suspected, it was suggested that the patient be admitted for a detailed examination. However, the patient, refused to be admitted for a detailed examination as recommended, and returned home. Later, when his symptoms progressively worsened and his stool had been black for 1 wk, he was rushed to the hospital.

At the time of admission, his blood pressure was 105/60 mmHg, his pulse was regular at 90 bpm, and his temperature was 37.2 °C. The patient's abdomen was soft, flat, and slightly distended, with mild tenderness in the upper abdomen. The laboratory findings were as follows: marked anemia with hemoglobin of 7.2 g/dL, amylase of 262 IU/L, mildly elevated pancreatic enzymes with lipase of 109 IU/L, and an inflammatory response with C-reactive protein of 4.76 mg/dL. Following admission, 4 units of packed red blood cells were transfused to treat anemia. Endoscopic retrograde pancreatography (ERCP) was performed to further investigate and treat the pseudocyst. Pancreatography revealed stenosis of the principal pancreatic duct at the head, dilation of the duct at the tail, and a communication between the tail duct and the pseudocyst (Figure 2). Therefore, the pancreatic duct was stented (stent size, 7 Fr, 7 cm). Although no substantial bleeding in the upper gastrointestinal tract was seen during ERCP, upper gastrointestinal endoscopy was performed to investigate the marked anemia which was present on admission. Endoscopy revealed a 2 cm protrusion resembling a submucosal tumor in the lesser curvature of the middle of the body of the stomach (Figure 3). EUS using the GF-UE260-AL5 (Olympus, Tokyo, Japan) and Prosound α10 (Aloka, Tokyo, Japan) was performed for diagnosis. On EUS, a 1 cm submucosal anechoic region whose entire periphery was hypoechoic was seen. The pulsating anechoic mass with Doppler signal enhancement identified in the gastric submucosa was diagnosed as an aneurysm with hematomas around the periphery (Figure 4A). Angiography proceeded, and a 1 cm pseudoaneurysm of the left gastric artery and a large pseudoaneurysm of the splenic artery measuring 2 mm in diameter were detected. Hemorrhage was prevented with transluminal embolization using lipiodol and histoacryl because a small aneurysm was observed in the left gastric artery upon angiography. This was embolized with coils as a pseudoaneurysm measuring 2 mm was further observed in the splenic artery (Figure 5).

Cessation of blood flow to the pseudoaneurysm was confirmed on EUS performed 1 wk later (Figure 4B). Since there was no subsequent bleeding, follow-up MDCT was performed 1 mo later. The left mediastinal pseudocyst had shrunk markedly.

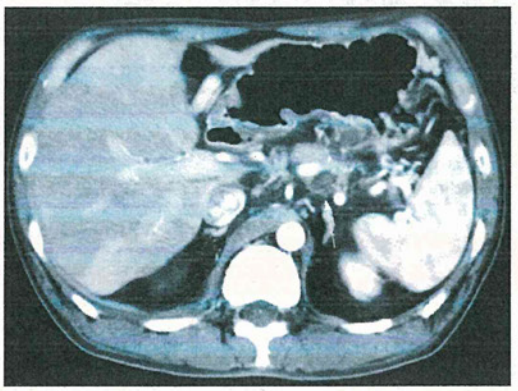

Figure 1 Abdominal computed tomographic findings. A severely atrophied pancreas with a calcified body was noted. The pseudocyst (arrow) ranged from the back of the pancreas to the left mediastinum and was adjacent to the splenic artery.

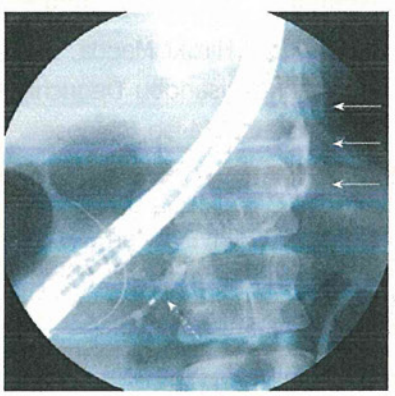

Figure 2 Endoscopic retrograde pancreatography findings. A: Endoscopic retrograde pancreatography showed stenosis of the principal pancreatic duct at the pancreatic head (dotted arrow) and a dilated tail duct communicating with the left mediastinal pseudocyst (solid arrows).

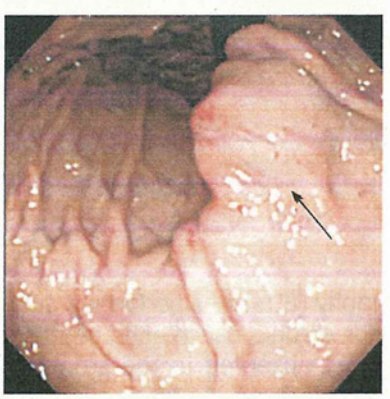

Figure 3 Upper gastrointestinal endoscopy findings. Upper gastrointestinal endoscopy showed a 2 cm, submucosal tumor-like protrusion with a red, eroded upper region located in the lesser curvature of the middle of the body of the stomach (arrow).

DISCUSSION

Hemorrhage in the pseudocyst was seen on MDCT at the time of presentation and ERCP performed after ad-

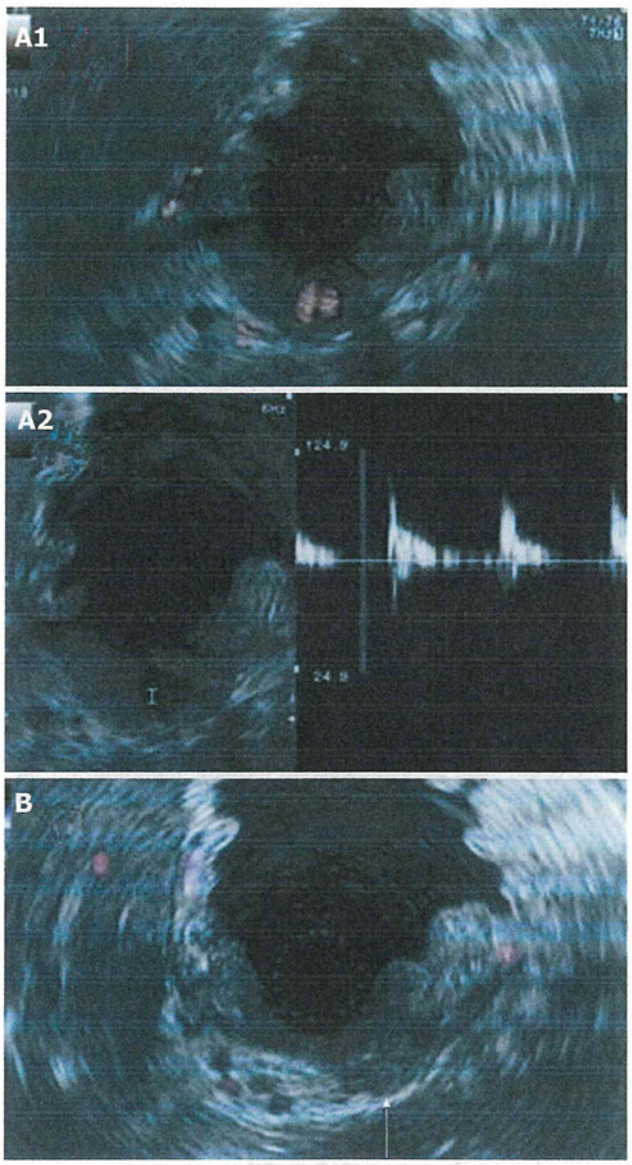

Figure 4 Endoscopic ultrasonography findings. A1: Endoscopic ultrasonography showed an anechoic region whose entire periphery was hypoechoic beneath the gastric mucosa. Power Doppler showed blood flow in the anechoic region. Upper gastrointestinal endoscopy showed a 2 cm, submucosal tumor-like protrusion with a red, eroded upper region located in the lesser curvature of middle of the body of the stomach (arrow); A2: Pulsed wave Doppler showed pulsatile blood flow in the anechoic region. This finding led to the diagnosis of an aneurysm; B: The cessation of blood flow to the pseudoaneurysm was confirmed with endoscopic ultrasonography which was performed 1 wk after treatment (arrow).

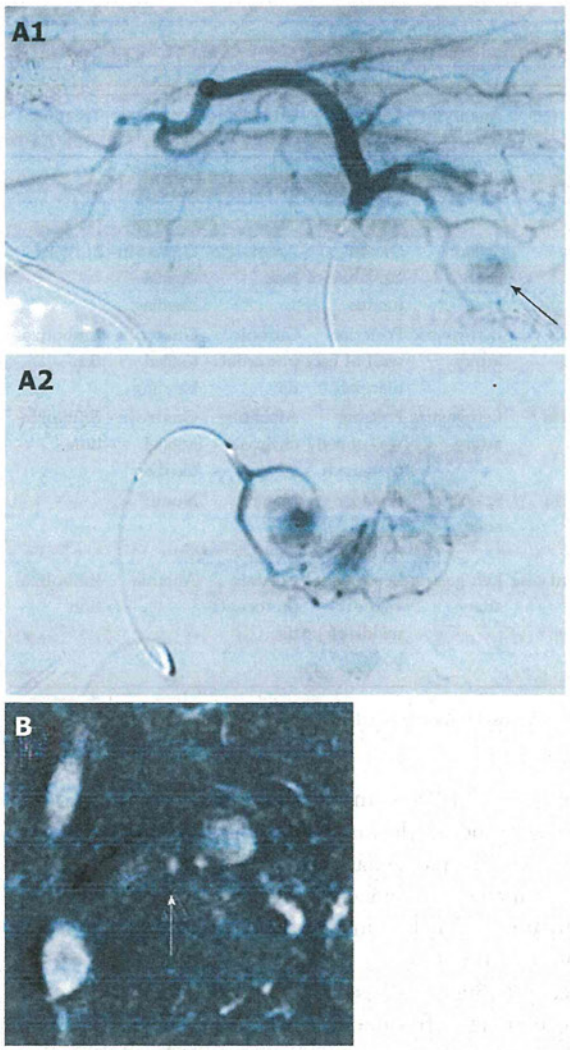

Figure 5 Angiography findings. A1: The pseudoaneurysm of the left gastric artery was diagnosed on angiography (arrow). The left hepatic artery diverged from the left gastric artery; A2: The microcatheter was advanced in the region of the pseudoaneurysm, and the pseudoaneurysm was embolized with histoacryl and lipiodol; B: A small pseudoaneurysm was observed in the splenic artery (arrow), and the splenic artery was embolized by coils.

mission revealed a communication between the tail duct and the pseudocyst. It is thought that the splenic pseudoaneurysm was bleeding into the pseudocyst because the splenic artery was adjacent to the pseudocyst on MDCT. No bleeding from Vater's papilla was observed when carrying out ERCP, but it was presumed that hemosuccus was the cause of this bleeding as the patient had black stool in the week preceding admission and was markedly anemic upon admission. The resulting progress of anemia triggered the discovery of a pseudoaneurysm in the left gastric artery which was on the verge of rupturing.

Although a pseudoaneurysm complicating chronic pancreatitis occurs relatively infrequently and affects only 6% to 9% of patients[1], 40% to 60% of ruptured pseudoaneurysms result in a fatal outcome[2]. Pseudoaneurysms are primarily attributed to the digestion and lysis of the arterial wall near the pancreas by errant activated pancreatic enzymes[3]. The splenic artery is the most commonly affected site. Pseudoaneurysms also frequently form in the gastroduodenal, pancreaticoduodenal, and hepatic arteries, but rarely in the left gastric artery[4,5]. Aneurysms of the left gastric artery mimicking a gastric submucosal tumor are also extremely rare[2,4].

The MDCT examination performed on admission may have missed the aneurysm because the lesion was small or because collateral circulation attributable to pancreatitis-induced pancreatic arteriovenous occlusion resulted in the imaging of many winding blood vessels which, in turn, complicated the identification and diagnosis of the aneurysm. EUS, which can show the gastric

Table 1 Cases of pseudoaneurysm diagnosed on endoscopic ultrasonography

Reported by	Aneurysm site	SMT-like lesion site	Underlying disease	Symptoms	Treatment
Mosler *et al*[7]	Splenic artery	Posterior wall of cardiac part	None	Anemia	
Chaya *et al*[8]	Splenic artery	Greater curvature of fundus	Arteriosclerosis	Gastrointestinal bleeding	Surgery
Falodia *et al*[2]	Left gastric artery	Posterior wall of cardiac part	Chronic pancreatitis	Gastrointestinal bleeding	Embolization
Jani *et al*[9]	Left gastric artery	Posterior wall of body of stomach	Alcoholic cirrhosis	Gastrointestinal bleeding	Embolization
Higuchi *et al*[10]	Splenic artery × 4	Posterior wall of fundus	None	None	-
Present case 2011	Left gastric artery	Lesser curvature of middle of body	Chronic pancreatitis	Anemia	Embolization

SMT: Submucosal tumor; -: No description.

wall in fine detail, is an excellent tool for diagnosing gastric submucosal lesions[6]. The added Doppler functionality of the particular EUS device used in the present case made the device better suited than MDCT for diagnosing and following small aneurysms resembling submucosal tumors.

Recently, higher rates of detection have been related to the increased frequency of imaging studies such as EUS[5]. A search of the literature revealed only this case and 8 other cases of submucosal tumor-like protrusions diagnosed as pseudoaneurysms on EUS[2,7-10]. The responsible vessel was the splenic artery in 6 cases and the left gastric artery in 3. The submucosal tumor-like lesion was often located in the fundus or cardiac area (7 of 9) and posterior wall (7 of 9). Two of the patients had chronic pancreatitis, one had alcoholic cirrhosis, one had arteriosclerosis, and five had no underlying disease. The lesions were coincidentally discovered during upper gastrointestinal endoscopic screening in four of these patients. Three of the patients had gastrointestinal bleeding that was treatable with either embolization or surgery (Table 1).

The danger of re-bleeding after embolization increases if pancreatitis continues even following treatment, but we believe that we were able to successfully control bleeding by avoiding stent implantation in the pancreatic duct and by avoiding bleeding. A pseudoaneurysm should be suspected when a gastric submucosal tumor-like protrusion is seen in a patient with chronic pancreatitis. We recommend that EUS be carried out, and if a pseudoaneurysm is diagnosed, then interventional radiology should be performed as soon as possible. In addition, the successful control of pancreatitis was believed to be the key to successful bleeding control.

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Identification of a DNA methylation marker that detects the presence of lymph node metastases of gastric cancers

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Abstract. The accurate detection of the presence of lymph node metastases (LNM) of gastric cancers (GCs) is useful for the implementation of necessary and sufficient treatment, but current methods of detection are unsatisfactory. In the present study, we focused on DNA methylation markers since they have several advantages, including biological and chemical stability and informativeness even in the presence of contaminating cells. Using three metastatic lymph nodes and three primary GCs without LNM, methylation bead array analyses were performed, which enabled the interrogation of 485,577 CpG sites. A total of 31 CpG sites that were hypermethylated in the metastatic lymph nodes, compared with the GCs without LNM, were isolated. Using primary GCs with and without LNM (28 GCs with LNM and 10 without), their methylation levels were measured using quantitative PCR following treatment with sodium bisulfite or a methylation-sensitive restriction enzyme. Of the genomic regions around the 31 CpG sites, 10 regions demonstrated higher methylation levels in the GCs with LNM compared with the GCs without LNM ($P<0.05$). Finally, the hypermethylation of the 10 regions was validated using another set of samples (129 GCs with LNM and 20 without). Hypermethylation of the region around the cg06436185 CpG site predicted the presence of LNM at a sensitivity of 43% and specificity of 85%. Additionally, the hypermethylation of the region was associated with a poor survival rate among GC patients with LNM. The results of the present study indicated that the methylation status of the region was a promising candidate marker to detect the presence of LNM of GCs and may reflect the malignant potential of GCs.

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Key words: DNA methylation, gastric cancer, lymph node, metastasis

Introduction

Gastric cancer (GC) is one of the most prevalent malignancies worldwide and remains a leading cause of cancer-related mortality (1,2). Since the presence of lymph node metastases (LNM) is associated with a significantly poorer prognosis of GC patients (3-5), radical resection with free-margin gastrectomy and extended lymphadenectomy are performed for patients with advanced GC to eradicate LNM (6). Such an aggressive resection of the lymph nodes is associated with higher patient morbidity and/or mortality rates (7-9). Alternatively, the absence of LNM allows for minimally invasive surgery, which provides an improved quality of life following treatment. Therefore, the accurate detection of LNM is useful for the implementation of necessary and sufficient treatment.

To detect the presence of LNM, much effort has been made in the fields of imaging and molecular markers. Imaging modalities, including computed tomography (CT), endoscopic ultrasonography (EUS) and ^{18}F-fluorodeoxyglucose positron emission tomography (FDG-PET) are used in clinical practice. However, the sensitivities of these modalities are 77.2, 82.8 and 71%, respectively, and the specificities are 78.3, 74.2 and 74%, respectively (10-13). Moreover, these imaging modalities are almost powerless to detect micrometastases (14,15). With regard to molecular markers, analyses that targeted specific RNA and protein expression have been made. Although a number of these markers were associated with the presence of LNM of GCs (16-19), their utility has not been confirmed by independent studies. Therefore, genome-wide or comprehensive analysis of molecular markers for LNM of GCs is required and validation of the utility of the markers is essential for clinical application.

As a molecular marker, DNA methylation is advantageous, as its status is stable even if a cell is placed in different environments (biologically stable) and DNA is chemically stable, even in clinical materials. In addition, DNA methylation profiles are not disturbed by the presence of a small population of contaminating cells. As a strategy, we used metastatic lymph nodes and primary GCs without LNM for genome-wide analysis as cells with the abililty of LNM may constitute only a small population of the cells in primary GCs with LNM. Differences in methylation levels may be extremely small and may not be detected by the analysis between primary GCs with and without LNM. Alternatively, in metastatic lymph nodes, cancer cells are expected to possess the aberrant DNA methylation following clonal selection. Moreover, the methylation levels of appropriate marker CpG sites in the metastatic lymph nodes are expected to be relatively high compared with those in primary GCs with LNM.

In the present study, we aimed to identify CpG sites with a methylation status associated with the presence of LNM of GCs via a genome-wide methylation analysis using metastatic lymph nodes and primary GCs without LNM and to validate the isolated candidate markers.

Materials and methods

Patients, tissue samples and DNA extraction. A total of 187 GC surgical samples were obtained from patients who underwent gastrectomy with extended lymph node dissection (D2) at the National Cancer Center Hospital (Tokyo, Japan) and Aichi Cancer Center Hospital (Aichi, Japan) between 1994 and 2011 with informed consent. A total of three metastatic lymph nodes were obtained from 3 of the 187 patients. No patients had undergone prior chemotherapy or radiotherapy. Prognostic information of 55 GC patients with LNM was available and the mean follow-up period after surgery was 3,024 days. Disease grades were classified according to the 6th edition of the TNM classification by the UICC. Samples were stored at -80˚C and a high molecular weight DNA was extracted using the phenol/chloroform method. The 187 samples were divided into screening (28 GCs with LNM and 10 without) and validation (129 GCs with LNM and 20 without) sets in advance, between which no significant differences in clinicopathlogical data were observed (Table I). This study was conducted with the approval of the Aichi Cancer Center and National Cancer Center.

Genome-wide methylation analysis. Genome-wide screening of differentially methylated CpG sites was performed using an Infinium HumanMethylation450 BeadChip array, which covers 485,577 CpG sites (Illumina, San Diego, CA, USA) (20). Genomic DNA (1 μg) was treated with sodium bisulfite using a Zymo EZ DNA Methylation kit (Zymo Research, Irvine, CA, USA) and the bisulfite-modified DNA was amplified prior to hybridization to the array. The array was scanned with an iScan System (Illumina) and the data were analyzed using GenomeStudio Methylation Module Software (Illumina). A CpG site was considered to be informative if the sum of the signals for methylated and unmethylated sequences at the CpG site was significantly higher (at $P<0.05$) than signals of the negative control probes on the same array. Methylation levels were represented by β values, with a β value of 0 corresponding to no methylation and 1 corresponding to full methylation.

Quantitative methylation-specific PCR (qMSP). Sample DNA was treated with sodium bisulfite and purified as described previously (21). qMSP was performed using real-time PCR with bisulfite-modified DNA and specific primers (Table II, Fig. 1A). A methylation level was expressed as a percentage of the value of methylated DNA reference (PMR) calculated as the [(number of fragments methylated at a target locus in sample/number of the *Alu* sequences in sample)/(number of fragments methylated at a target locus in *Sss*I-treated DNA/ number of the *Alu* sequences in *Sss*I-treated DNA)]x100 (22).

Quantitative PCR following treatment with a methylation-dependent restriction enzyme (qPTMR). A fully unmethylated control was prepared by amplifying human blood genomic DNA with phi29 DNA polymerase (Illustra GenomiPhi HY kit, GE Healthcare, Buckinghamshire, UK) (23). DNA (1 μg) was treated with *Msp*JI (New England Biolabs, Beverly, MA, USA), which cleaves DNA 9 bp downstream from the ^{m}CNNR sequence (24,25), in a 30 μl reaction [4 U of *Msp*JI, 1X NEB buffer 4 (New England Biolabs) and 0.1 mg/ml BSA] at 37˚C for 20 h. Following purification, the DNA was treated with *Msp*JI again and dissolved in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) at a concentration of 5 ng/μl without purification. Using 1 μl of the solution, quantitative PCR (qPCR) was performed by real-time PCR with primers that encompassed a target *Msp*JI site (Fig. 1B). To normalize the quantity of input DNA, the number of copies of a standard sequence, which may be amplified with a primer pair (5'-TTGCTTGAAGTTTTGTTGCTGTAGT-3' and 5'-AATAAACTCAGTTGTGACATGGACA-3') and contains no *Msp*JI site, was measured by qPCR. A percentage of the value of unmethylated reference (PUR) was calculated as the [(number of fragments at target locus in sample/number of the standard sequence in sample)/(number of fragments at target locus in GenomiPhi-amplified DNA/number of the standard sequences in GenomiPhi-amplified DNA)]x100. For convenience, the methylation level was expressed as 100-PUR.

Statistical analysis. Statistical analyses were conducted using PASW statistics version 18.0.0 (SPSS Japan Inc., Tokyo, Japan). The difference between the mean values of the two groups of samples was evaluated using Welch's t-test. The Fisher's exact test was used to evaluate the significant difference in relative frequency of the phenomena between two independent groups. Survival curves were computed according to the Kaplan-Meier method and the log-rank test was employed to evaluate the level of significant difference. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Genome-wide screening using metastatic lymph nodes and GCs without LNM. To isolate the CpG sites that are hypermethylated specifically in GCs with LNM, genome-wide methylation analysis was performed using metastatic lymph nodes (n=3) and GCs without LNM (n=3) using an Infinium HumanMethylation450 BeadChip array. The samples used for this analysis were prepared from 6 patients in the screening

Table I. Clinicopathological data of sample sets.

	N	Age (years)	P-value	Gender	N	P-value	T stage	N	P-value
Genome-wide analysis set[a]									
Meta (-)	3	72±4	0.17	Male	2	1.0	T1	0	0.51
							T2	1	
				Female	1		T3	1	
							T4	1	
Meta (+)	3	59±13		Male	2		T1	0	
							T2	0	
				Female	1		T3	1	
							T4	2	
Screening set									
Meta (-)	10	69±6	0.13	Male	7	0.53	T1	0	0.17
							T2	1	
				Female	3		T3	6	
							T4	3	
Meta (+)	28	63±11		Male	18		T1	0	
							T2	0	
				Female	10		T3	14	
							T4	14	
Validation set									
Meta (-)	20	63±11	0.71	Male	13	0.6	T1	0	0.14
							T2	3	
				Female	7		T3	8	
							T4	9	
Meta (+)	129	62±10		Male	91		T1	0	
							T2	4	
				Female	38		T3	55	
							T4	70	

[a]This set comprised samples from the screening set.

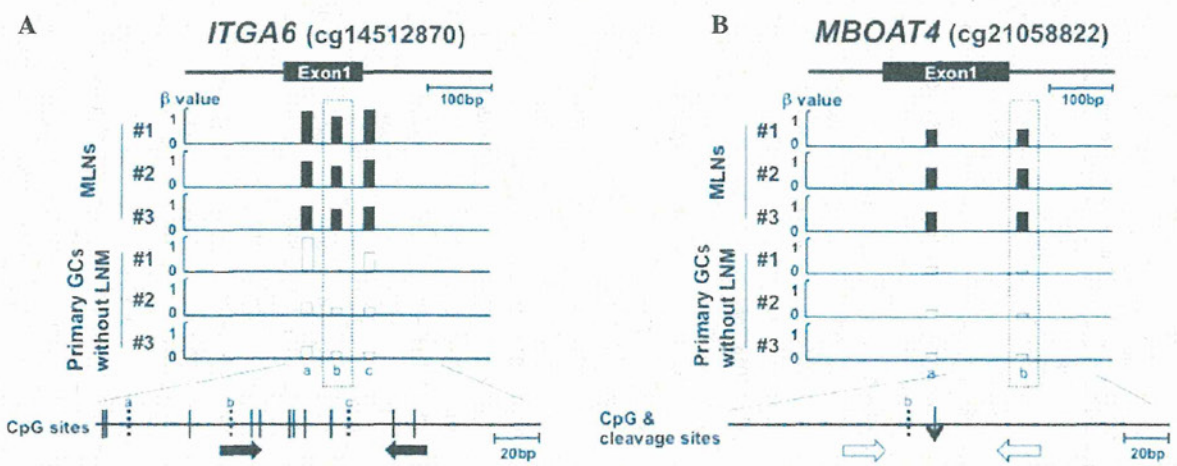

Figure 1. Representative genomic regions around the CpG sites differentially methylated between metastatic lymph nodes and GCs without LNM and primer design in the regions. Below the genomic structure of a region, β values (methylation levels) of the CpG sites carried by Infinium bead array are shown. The differentially methylated CpG site is marked by a rectangle with dotted line. A CpG map is drawn at the bottom, vertical lines (solid and broken lines) indicate CpG sites and broken lines indicate CpG sites whose β values were measured. (A) A region whose methylation level was assessed by qMSP. Primers specific to the methylated sequence (closed arrows) were designed on CpG sites around the differentially methylated sites based on the bisulfite-modified sequence. (B) A region whose methylation level was assessed by qPTMR. Primers (open arrows) were designed to amplify the region encompassing the *Msp*JI-cleaved site (thin vertical arrow) based on the unmodified sequence. GC, gastric cancer; MLN, metastatic lymph nodes; LNM, lymph node metastases; qMSP, quantitative methylation-specific PCR; qPTMR, quantitative PCR following treatment with a methylation-dependent restriction enzyme.

Table II. CpG sites identified by bead-chip array analysis.

No.	Probe name (IlmnID)[a]	Gene symbol	Location (Chr: base)	Relation to CpG island	Position to gene	P-value[b]		Cut-off (YI)	Primer sequences (5'-3')		Annealing temp.	PCR type	Mg^{2+c} (μM)
						Screening	Validation		Forward	Reverse			
1	cg23218354	-	Chr1: 2885244	Island	-	0.05	0.17	10.1 (0.48)	TGGTTTTTATACGGGGGATTTAC	ACTAAACCAAAACGACGATTACG	60	qMSP	1.5
2	cg13239126	*KIAA1026*	Chr1:15256136	-	Body	0.24	-	-	CTCCAGAGAGACAGGCATGGTT	CAAGCCTGACCTTCCCTCTCC	60	qPTMR	1.5
3	cg16112880	*TMEM9*	Chr1:201123745	Island	TSS200	0.41	-	-	CCCGCCCTCTCCTAGCTTCTAT	GGCTGACGTTCCCTTTTCTGGT	63	qPTMR	1.5
4	cg14512870	*ITGA6*	Chr2:173330342	-	Body	0.01	0.07	32.5 (0.44)	TATAGTTGCGATATTATCGTTC	AAACTACCGAAATAAACGCT	51	qMSP	2.5
5	cg09866366	*ABCF3*	Chr3:183903315	Shore	TSS1500	0.34	-	-	TCGTTAGATTACGGGTGTTTC	CAAAACGCATATATAACGATAACG	58	qMSP	2.5
6	cg08812108	-	Chr6:2515318	-	-	0.03	0.24	56.3 (0.44)	AGCGTTGGCGTTAGGTAGGGTAGTTC	CCAAATAACCACCTACGTCTTTACG	63	qMSP	1.5
7	cg06728252	*ABT1*	Chr6:26598149	Island	Body	0.24	-	-	CGCGTAGATCGGTTCGTGAGAC	GCCACGCGCTTAACTATACG	63	qMSP	1.5
8	cg08972588	*TNXB*	Chr6:32014674	-	Body	0.64	-	-	CCTGAGCAAGAATGAGGCCAGA	GGGGACAAGGGGGAGATCACA	65	qPTMR	2.5
9	cg22126965	*COX19*	Chr7:1015501	Shore	TSS1500	0.50	-	-	GGTTTAGAAAGGTTTAGCGAATTGTTC	AACAACCGCAAACAACG	62	qMSP	2.5
10	cg18450582	*DYNC1I1*	Chr7:95546539	-	Body	0.32	-	-	ACCTTGGCCTCTGGATTGTGGA	GCACTGCCTGCCTGAAAGGAGA	64	qPTMR	1.5
11	cg02005782	-	Chr7:105857664	-	-	0.59	-	-	GAAGTCAGCCAGGCATTGGAAG	CCCAGCTGCCTTTCTGATCTCT	65	qPTMR	1.5
12	**cg06436185**	*PRKAG2*	Chr7:151442351	-	Body	0.04	0.03	28.8 (0.24)	ATTTAGTTTTTTGTACGGTTGC	CCCAATAAAACGACGTAACG	55	qMSP	2.5
13	cg21058822	*MBOAT4*	Chr8:30002223	-	TSS200	0.38	-	-	GGCTGTCTCTGGTCTTTTTATC	AGAAAGCCAGTTTTTATTCTGC	61	qPTMR	1.5
14	cg12089032	-	Chr8:72881203	-	-	0.03	0.09	40.6 (0.41)	GCAAGTTAAGGCATCGTAGGAAAGC	GGCAGAGAGGAACAGCTCCTAAG	66	qPTMR	1.5
15	cg23170346	-	Chr8:134863880	-	-	0.95	-	-	CTAGCCACATCCATAGCAGACAGG	CACTCAGCAATGCAAACAGTCTTG	66	qPTMR	1.5
16	cg19878482	*C8orf73*	Chr8:144655026	Shore	TSS200	0.10	-	-	GGAGTTTTTCGGGTTCGGTTTC	CAAAAACCCATTATAAACACGTCCGT	65	qMSP	2.5
17	cg01263942	*DIP2C*	Chr10:695859	-	Body	0.01	0.12	23.2 (0.38)	GTTCGTTATTTGCGTTTTCGTGC	CAACGAAAAAACTCCATAAACCG	59	qMSP	2.5
18	cg03015672	*ARHGAP12*	Chr10:32216066	Shore	5'UTR	0.88	-	-	AGAACAGTGGAGCCGCATGCAA	CCAAAGCAGGCAGTGAAAGCGT	66	qPTMR	1.5
19	cg10326726	*MSMB*	Chr10:51549505	-	TSS200	0.16	-	-	CAACCCTCTGTAAACACTCAAT	TATAGACAGGTACATCCAGGCA	57	qPTMR	2.5
20	cg19864370	-	Chr10:80354592	-	-	0.00	0.29	70.6 (0.69)	GAATAGCTTAGGCCCCTGTCAT	GATAGTGCTAGCCCTTGGGAAT	60	qPTMR	1.5
21	cg03850986	*ABLIM1*	Chr10:116408382	-	Body	0.38	-	-	TGATAAAAATGCTCTGGAATTAG	TGGAGATGTAATGTAGTACACCATA	51	qPTMR	1.5
22	cg25885280	*SHANK2*	Chr11:70760166	-	Body	0.34	-	-	GCGGTGGGGGATTTCTGTAAGGA	GAGCAGGGTGTGCCTTCTCAGGG	68	qPTMR	1.5
23	cg26894278	*CRYL1*	Chr13:21016241	-	Body	0.22	-	-	GTTAAGTTTAAATGGAGCCTTG	TGACAGGATTACAATAAGGCTA	56	qPTMR	1.5
24	cg04339360	*KLF5*	Chr13:73635568	Shore	Body	0.04	0.31	25.4 (0.43)	TAGTCAAGAAAAGAAACCTGTGCAA	TGCCAAACTACCTCAATTCTGTTTA	61	qPTMR	1.5
25	cg16206504	-	Chr13:114917223	Shelf	-	0.02	0.35	35.2 (0.41)	CGAGATTGTAGGCGGTTGTTC	CCTAACTATTACAACAATACCGAACG	63	qMSP	1.5
26	cg14851578	-	Chr14:106187192	Shore	-	0.08	-	-	GGAGTGTGGGTTACGTGTGATTAC	CAATCTCGCCCACTCACG	66	qMSP	1.5
27	cg02990302	*C16orf80*	Chr16:58155189	-	Body	0.04	0.45	56.2 (0.65)	TCCTTTCCTTAGCTCCTTCCAG	AAAAACAGTCGGCTCTTTGTGA	63	qPTMR	1.5
28	cg08292959	*MGAT5B*	Chr17:74878420	Island	Body	0.97	-	-	GGCACCTGCCACTCCATCCG	TGCACTCTGGGCTGTACCACAGTG	63	qPTMR	1.5
29	cg15645685	*PBX4*	Chr19:19730175	Shore	TSS1500	0.26	-	-	CTAATGCTCCCTGCATCCTCAG	TAAACAAGCGAGGTCACTCTTCAGC	64	qPTMR	1.5
30	cg14571622	*NLRP8*	Chr19:56499348	-	3'UTR	0.01	-	-	TGGGGCTTGATTGATCAGTTCC	CCAGGGTTCAAAGCTGAGGTTC	62	qPTMR	1.5
31	cg27050343	*OTC*	ChrX:38211596	-	TSS200	0.15	-	-	AATTTTTGGGTTTAAGTGATTCGTTC	AAAAAAATAATTACTAACCGAACACG	62	qMSP	1.5

[a]IlmnID is a unique CpG site identifier from the Illumina CG database; underlined IlmnID indicates P<0.05 in the screening set; bold IlmnID, P<0.05 in the validation set. [b]Difference was evaluated by Welch's t-test. [c]Final concentration in a PCR analysis. Island, CpG island; Shore, within 2,000 bp of end of CpG island; Shelf, within 2,000 bp of end of Shore; TSS200, within 200 bp upstream of the transcription start site; TSS1500, 200-1,500 bp upstream of the TSS; 5'UTR, in the 5' untranslated region of the gene; Body, within exons or introns of the gene; 3'UTR, in the 3' untranslated region of the gene; YI, maximized Yoden index.

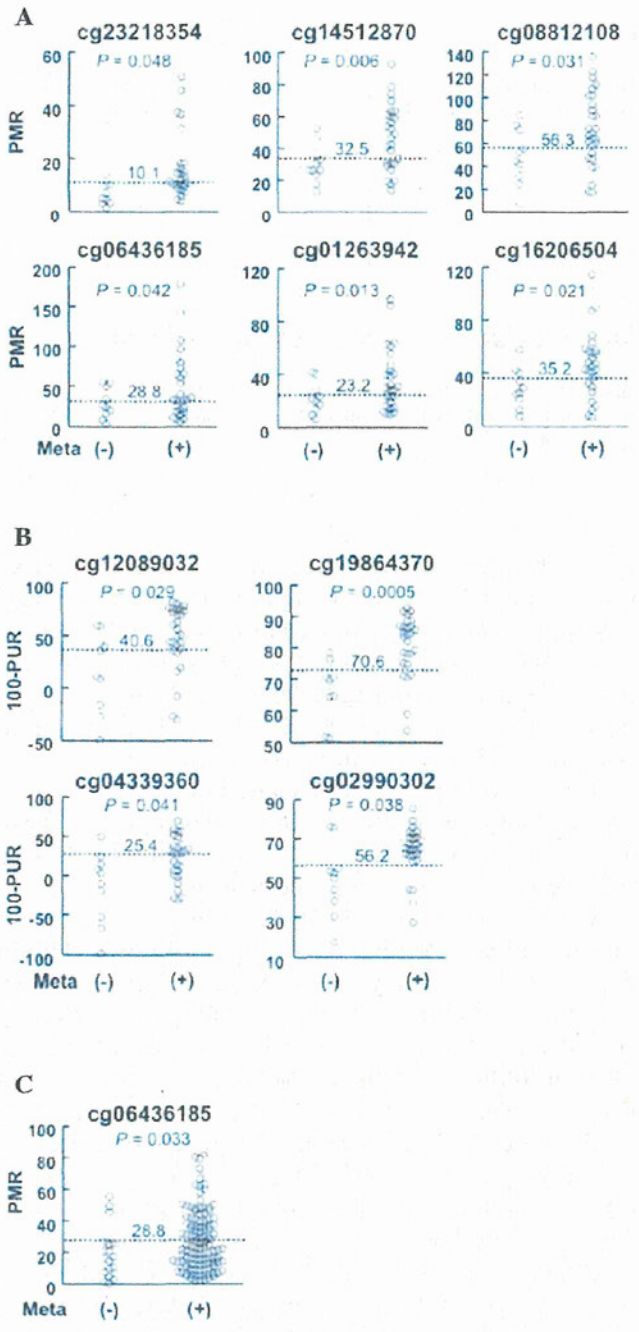

Figure 2. Methylation levels of the candidate genomic regions in primary GCs with and without LNM. Methylation levels were measured by (A) qMSP and (B) qPTMR in the screening sets. The screening set consisted of 10 GCs without LNM and 28 with LNM. (C) Methylation level of the region around cg06436185 in the validation sets was measured by qMSP. The validation set consisted of 20 GCs without LNM and 129 with LNM. Meta (-), GCs without LNM; Meta (+), those with LNM. Horizontal dotted lines are the cut-off methylation levels and the number on the line indicates the value of the level. GC, gastric cancer; LNM, lymph node metastases; qMSP, quantitative methylation-specific PCR; qPTMR, quantitative PCR following treatment with a methylation-dependent restriction enzyme; PUR, percentage of the value of unmethylated reference; PMR, percentage of the value of methylated DNA reference.

set (Table I). The mean number of informative CpG sites was 485,170 (SD 209) in the metastatic lymph nodes and 485,001 (SD 514) in the GCs without LNM (P=0.63). We searched for CpG sites that were highly methylated in the three metastatic lymph nodes [β value > a) 0.6, b) 0.5 and c) 0.4] and hardly

Table III. Association between methylation levels of the genomic region around cg0643618 and clinical characteristics.

		Methylation level		
Parameters	N	Mean	SD	P-value
Age				
≤60	77	32.2	24.0	0.26
>60	110	28.1	25.2	
Gender				
Female	58	34.9	26.4	0.07
Male	129	27.6	23.6	
T category				
T3	83	26.8	27.1	0.07
T4	96	33.6	22.5	

methylated in the three primary GCs without LNM (β value <0.2) and the number of hypermethylated CpG sites was a) 1, b) 31 and c) 209, respectively. To obtain a practicable number of candidate CpG sites, we adopted a cut-off β value of 0.5 and the 31 CpG sites were selected for further analysis (Table II).

Selection of informative candidate genomic regions among primary GCs. Using primary GCs with and without LNM (screening set, Table I), the methylation levels of genomic regions around the 31 CpG sites were measured by qMSP or qPTMR, which are accurate and sensitive enough to detect aberrant DNA methylation in a small population of cells. Of the 31 regions, 10 regions exhibited higher methylation levels in GCs with LNM (1.4- to 1.9-fold) than in those without LNM (Table II and Fig. 2A and B). For each of the 10 genomic regions, a cut-off methylation level was established in order that the Youden index (sensitivity + specificity - 1) would be maximized (Table II and Fig. 2).

Validation of the candidate genomic regions in a different set of samples. To validate the hypermethylation of the 10 candidate genomic regions in GCs with LNM, the methylation levels were analyzed in an independent sample set (validation set, Table I). A region around the cg06436185 CpG site revealed significantly higher methylation levels in GCs with LNM (1.5-fold) than those without (P=0.033, Fig. 2C), whereas the other nine regions were not validated (Table II). The region was located in the gene body of the *PRKAG2* gene and did not belong to a CpG island (Table II). Therefore, it was unlikely that the methylation status of the region around cg06436185 affected the transcription of a gene. Using a cut-off level established in the analysis of the screening set (28.8%), the presence of LNM was detected at a sensitivity of 43% and a specificity of 85%. This result indicated that a methylation level of this region is a candidate marker for the detection of the presence of LNM.

Association between the methylation level of the genomic region around the cg06436185 CpG site and clinicopathological characteristics. Associations between the methylation level of the genomic region around cg06436185