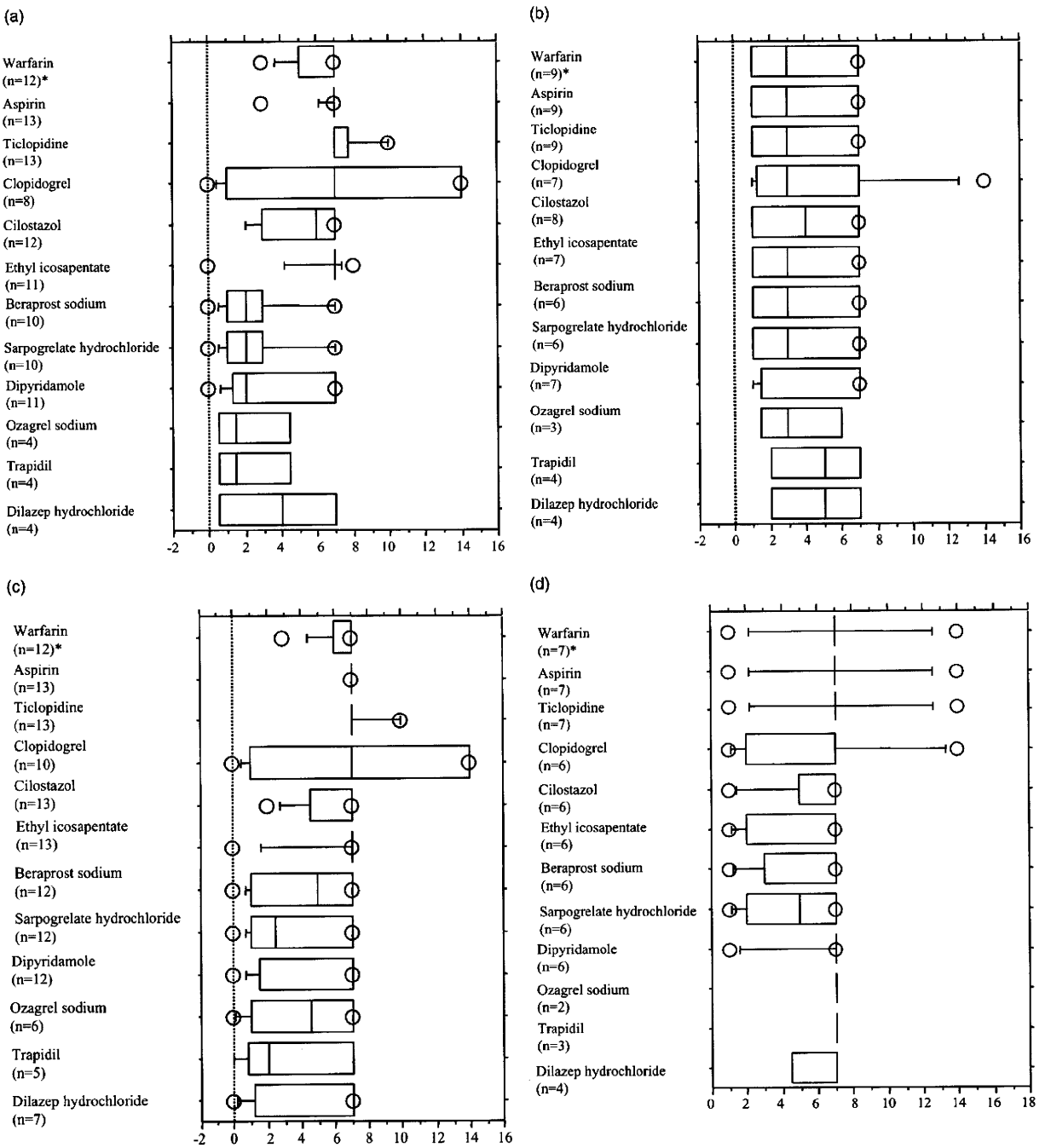

Figure 1 Duration of discontinuation for endoscopic procedures in anticoagulation and antiplatelet therapy. (a) Duration of discontinuation before endoscopic biopsy. (b) Duration of discontinuation after endoscopic biopsy. (c) Duration of discontinuation before endoscopic mucosal resection. (d) Duration of discontinuation after endoscopic mucosal resection. *The number is that of endoscopists who described the exact days. The others of 13 endoscopists did not have their standard policy in their hospitals.

information, it may not be necessary to discontinue the other agents nominated in the present study as antiplatelet agents before endoscopic procedures; however, the legitimacy to neglect these agents because of a risk of hemorrhage cannot be judged from the guidelines. Furthermore, regarding all the antiplatelet agents, a recommended duration of discontinuation after the procedures is very obscure.

Ido *et al.* reported their own efforts to establish provisional rules concerning management of anticoagulation and antiplatelet therapy for endoscopic procedures in cooperation with related departments in their hospital.[17] The description about the duration of discontinuation before and after endoscopic procedures for each disease is very concrete for each agent, although a recent agent, clopidogrel, and a few other antiplatelet agents are not described. We are convinced that most endoscopists prefer more practical guidelines authorized by our endoscopy society similar to the report by Ido *et al.* Further innovation of the guidelines on antiplatelet agents in more detail is warranted.

Limitations of the present study may be the small number of participating hospitals and questionnaire surveys, which results in the possibility of recall bias. However, even with these limitations, the tendency for different management for patients with anticoagulation and antiplatelet therapy among different hospitals was clearly recognized and the problems that should be solved on this topic were clarified. Additionally, we recognize the importance of this type of survey, because, in the past, these surveys drove the proposal for the establishment and refinement of guidelines for managing patients taking these agents in Western countries.[18–20]

In summary, the present study revealed that there was little consensus in our daily practice regarding the management of anticoagulation and antiplatelet therapy for endoscopic procedures even after a few years have passed since the establishment of the Japanese guideline. A reference guideline with clearer descriptions based on solid evidence that can be updated at least every few years should be established in the near future to prevent unnecessary anxiety of endoscopists and inappropriate management of patients.

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Digestive Endoscopy

Feasibility of electrocautery snaring as the final step of endoscopic submucosal dissection for stomach epithelial neoplasms

O. Goto, M. Fujishiro*, S. Kodashima, N. Kakushima, S. Ono, N. Yahagi, M. Omata

Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan

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Abstract

Background. Endoscopic submucosal dissection (ESD) is a novel endoluminal technique that permits the resection of gastric neoplasms.
Aim. To analyse the feasibility of snaring as the final step of ESD.
Patients and methods. One hundred and ninety-nine consecutive gastric neoplasms resected by four ESD experts from January 2004 to May 2007 were investigated. Forty-five (22.6%) were finally resected finally using a snare. Rates of *en bloc* resection, complete (R0 plus *en bloc*) resection, mean operation time, and complications were assessed between the snaring and the non-snaring groups.
Results. *En bloc* resection rate was significantly lower and delayed bleeding rate was significantly higher in the snaring group than in the non-snaring group (91.1% [41/45] vs. 100% [154/154], 11.1% [5/45] vs. 1.9% [3/154], respectively), although complete resection rate (86.7% [39/45] vs. 92.9% [143/154]) and mean operation time (70.2 min vs. 75.8 min) were not significantly different between the two groups. Six perforation cases (3 [6.7%] in the snaring group, 3 [1.9%] in the non-snaring group) were observed, but snaring did not lead to perforation in any case. When the subjects were divided into small (≤2 cm) and large (>2 cm) tumours, *en bloc* resection rate in large tumours was still significantly different between the groups (76.9% [10/13] vs. 100% [67/67]), whereas in small tumours it was no longer significantly different (96.9% [31/32] vs. 100% [87/87]).
Conclusions. Snaring may facilitate successful ESD for smaller tumours, but multiple-piece resection should be taken into account especially for larger tumours.

Keywords: Endoscopic submucosal dissection; Piecemeal resection; Snaring resection; Stomach neoplasm

1. Introduction

Endoscopic submucosal dissection (ESD) is a recently developed endoscopic treatment used mainly for intramucosal neoplasms of the gastrointestinal tract. It is characterised by a circumferential mucosal incision and submucosal dissection beneath the lesion. ESD has an invaluable advantage over the other treatment modalities, because the target lesion can be endoluminally resected in one piece without organ resection, even if the lesion is large or associated with submucosal fibrosis [1,2]. However, ESD is a highly advanced technique among the various endoscopic treatments, and special expertise in addition to training under the supervision of experienced hands is preferable.

Among the several steps involved in ESD, the most difficult step is considered to be submucosal dissection, that, if it does not go as planned, may result in active bleeding or perforation. In the clinical scene of ESD, snaring in a half way, where *en bloc* resection may be possible after circumferential mucosal incision and an appropriate amount of submucosal dissection, can be performed as a substitute for completion of submucosal dissection. Otherwise, in the case of difficulty in continuing the submucosal dissection due to technical problems, we are unwilling to use a snare after proceeding with submucosal dissection as much as possible. It is thought that using a snare may shorten operation time spent on submucosal dissection or rescue the patient from a difficult situation, but it may lead to uncertainty for *en bloc* resection or to the possibility of an increased tumour-positive margin. So, we retrospectively analysed differences in the outcomes of ESD for stomach epithelial neoplasms between the groups with

* Corresponding author. Tel.: +81 3 3815 5411; fax: +81 3 5800 8806.
E-mail address: mtfujish-kkr@umin.ac.jp (M. Fujishiro).

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Table 1
Clinicopathological features of 199 gastric lesions in the snaring and the non-snaring groups

	Snaring ($N=45$)	Non-snaring ($N=154$)	p-Value
Age (y.o.) (mean ± S.D.)	68.5 ± 9.0	68.1 ± 8.5	0.825
Sex (male/female)	37/8	118/36	0.541
Location (U/M/L)[a]	15/20/10	43/47/64	0.054
Circumference (LC/GC/AW/PW)[b]	13/9/9/14	66/19/29/40	0.317
Gross type (elevated/depressed/combined)	19/25/1	58/87/9	0.577
Histology type (intestinal/diffuse/adenoma)	38/3/4	123/11/20	0.746
Submucosal fibrosis (presence/absence)	8/37	25/129	0.821
Depth of invasion (mucosa/submucosa)	35/10	122/32	0.837
Tumour size (mm) (mean ± S.D.)	17.8 ± 10.2	22.6 ± 15.7	0.056

[a] U, upper third; M, middle third; L, lower third of the stomach.
[b] LC, lesser curve; GC, greater curve; AW, anterior wall; PW, posterior wall of the stomach.

and without electrocautery snaring as the final step of ESD, in order to find out whether snaring has been beneficial or not in our case series.

2. Patients and methods

One hundred and ninety-nine consecutive lesions with a diagnosis of early gastric cancer (EGC) or gastric adenoma (GA) between January 2004 and May 2007 were retrospectively investigated. These cases had all been resected by four, very experienced ESD experts, each of whom had performed ESD on more than 30 cases of EGC or GA. Lesions in a remnant stomach after gastrectomy or in a gastric tube after oesophagectomy were excluded, because the number was small and the specific conditions might affect subsequent analysis. Cases whose medical records were insufficient for retrospective analysis were also excluded. Clinicopathological features of 199 lesions were divided into two groups, the snaring group ($n=45$) and the non-snaring group ($n=154$), according to the requirement of an electrocautery snare for tumour resection (Table 1). Age, sex, location and circumference of the tumour, gross type, histology type, coexistence of submucosal fibrosis, depth of invasion, and mean tumour size (the greatest diameter of the lesion on actual measurement) were not significantly different between the two groups, although the cases in the non-snaring group tended to be located predominantly in the lower third and be larger in comparison with those in the snaring group.

The indication for ESD was determined by preoperative prediction of GA or EGC without nodal metastases described by the previous study [3] from thorough endoscopic examinations with endoscopic biopsy. The ESD technique has been described elsewhere [4,5]. In brief, a flex-knife (KD-630L; Olympus, Tokyo, Japan) [6,7] was used as the main electrosurgical knife, and other knives, such as an insulation-tipped diathermic knife (IT knife) [8] and a hook-knife [9], were used when required by the lesion. These knives were used for mucosal cutting to isolate the lesion from surrounding non-neoplastic mucosa, and for submucosal dissection to detach the lesion from the muscle layer. A mixture of 10% glycerin plus 5% fructose and 0.9% saline preparation (Glyceol, Chugai Pharmaceutical Co., Tokyo, Japan) containing 0.005% indigo carmine and 0.0005% epinephrine was used to make a submucosal fluid cushion [10]. Hyaluronic acid was added to the injection solution when the lesion had ulcerative findings or when it was in a difficult location, such as the cardia [11]. Haemostatic forceps (HDB2422W; Pentax, Tokyo, Japan) were used for reduction in bleeding during the procedure or for the treatment of visible vessels on the mucosal defect after resection. During submucosal dissection, an electrosurgical snare 15 mm (SD-210L-15, Olympus) or 25 mm (SD-210L-25, Olympus) in diameter was used when the operator required. The decision as to whether a snare was used or not was completely left to the judgment of the operator in consideration of several factors, e.g., operation time, technical difficulty, encountering complications, patient's comorbidity (Table 2).

We investigated the differences between the snaring and the non-snaring groups in rates of *en bloc* resection and complete resection (the rate of cases revealing no tumour on the edge of the one-piece resected specimen), mean operation time and complications in an overall analysis. Moreover, as shown in Tables 3 and 4, all the subjects were divided into two groups according to tumour size (small [≤2 cm] or large

Table 2
The main indications for snaring

	$N=45$
Positive indication of snaring for faster resection[a]	20
Passive indication of snaring to end up resection	25
Because of poor conditions during the operation[b]	11
Massive bleeding during the operation	5
Perforation during the operation	3
Poor condition of the patient	3
Because of difficulties in the lesion itself[c]	14
The location of the tumour made ESD difficult	11
Submucosal fibrosis made ESD difficult	3

[a] Submucosal dissection would be easy to continue, but snaring was done only for the reason of saving time.
[b] Submucosal dissection would be unfavourable to continue, so snaring was done.
[c] Submucosal dissection would be difficult to continue, so snaring was done.

Table 3
Clinicopathological features of 80 gastric lesions >2 cm in size in the snaring and the non-snaring groups

	Snaring ($N = 13$)	Non-snaring ($N = 67$)	p-Value
Age (y.o.) (mean ± S.D.)	70.6 ± 8.1	68.5 ± 8.2	0.403
Sex (male/female)	13/0	52/15	0.113
Location (U/M/L)[a]	6/5/2	23/19/25	0.310
Circumference (LC/GC/AW/PW)[b]	2/2/3/6	32/6/14/15	0.139
Gross type (elevated/depressed/combined)	9/4/0	30/33/4	0.233
Histology type (intestinal/diffuse/adenoma)	12/1/0	52/8/7	0.401
Submucosal fibrosis (presence/absence)	2/11	11/56	>0.999
Depth of invasion (mucosa/submucosa)	8/5	47/20	0.531

[a] U, upper third; M, middle third; L, lower third of the stomach.
[b] LC, lesser curve; GC, greater curve; AW, anterior wall; PW, posterior wall of the stomach.

Table 4
Clinicopathological features of 119 gastric lesions ≤2 cm in size in the snaring and the non-snaring groups

	Snaring ($N = 32$)	Non-snaring ($N = 87$)	p-Value
Age (y.o.) (mean ± S.D.)	67.6 ± 9.3	67.9 ± 8.8	0.889
Sex (male/female)	24/8	66/21	>0.999
Location (U/M/L)[a]	9/15/8	20/28/39	0.137
Circumference (LC/GC/AW/PW)[b]	11/7/6/8	34/13/15/25	0.812
Gross type (elevated/depressed/combined)	10/21/1	28/54/5	0.830
Histology type (intestinal/diffuse/adenoma)	26/2/4	71/3/13	0.765
Submucosal fibrosis (presence/absence)	6/26	14/73	0.784
Depth of invasion (mucosa/submucosa)	27/5	75/12	0.774

[a] U, upper third; M, middle third; L, lower third of the stomach.
[b] LC, lesser curve; GC, greater curve; AW, anterior wall; PW, posterior wall of the stomach.

[>2 cm]), to compare the outcomes of the two groups for a sub-analysis, because the tumour size might affect the results. This was of particular concern in the snaring group because a snare might limit the ability to resect in terms of its size.

For the statistical analyses, Student's t-test for age, operation time and tumour size, Chi-square test for location, circumference, gross type and histology type and Fisher's exact probability test for the other variables were used. A p-value < 0.05 in each analysis was considered to be statistically significant.

3. Results

The overall results for using a snare in the ESD procedure are summarised in Table 5. *En bloc* resection rate was significantly lower in the snaring group than in the non-snaring group (91.1% vs. 100%). There were no significant differences in complete resection rate and mean operation time between the two groups. One of the major complications, delayed bleeding rate, was significantly higher in the snaring group than in the non-snaring group (11.1% vs. 1.9%). In all six cases, perforation was occurred during direct submucosal dissection and were not due to snaring.

Table 6 shows the results of the sub-analysis between the snaring and the non-snaring groups according to tumour size. In large tumours, *en bloc* resection rate in the snaring group was still significantly lower (76.9%) than in the non-snaring group (100%), whereas in small tumours, it was not significantly different between the two groups. In terms of operation time, there was no significant difference between the snaring and the non-snaring groups regardless of small or large tumour, although large tumours needed significantly longer time than small tumours in both groups. It was no longer significantly different in major two complications between the snaring and the non-snaring groups in this sub-analysis.

Table 5
Overall outcomes between the snaring and the non-snaring groups

	Snaring ($N = 45$)	Non-snaring ($N = 154$)	p-Value
En bloc resection rate (%)	**91.1**	**100**	**0.002***
Complete resection rate[a] (%)	86.7	92.9	0.225
Operation time (min) (mean ± S.D.)	70.2 ± 58.4	75.8 ± 58.6	0.574
Delayed bleeding rate[b] (%)	**11.1**	**1.9**	**0.016***
Perforation rate[c] (%)	6.7	1.9	0.130

[a] The rate of cases revealing no tumour on the edge of the resected specimen.
[b] The rate of cases needing emergency endoscopy due to hematemesis or melena.
[c] All cases were snared after perforation during submucosal dissection (perforation is not directly due to snaring).
* Statistically significant.

Table 6
The outcomes between the snaring and the non-snaring groups according to tumour size

	Tumour >2 cm			Tumour ≤2 cm		
	Snaring ($N=13$)	Non-snaring ($N=67$)	p-Value	Snaring ($N=32$)	Non-snaring ($N=87$)	p-Value
En bloc resection rate (%)	**76.9**	**100**	**0.004***	96.9	100	0.269
Complete resection rate[a] (%)	69.2	88.1	0.099	93.8	96.6	0.610
Mean operation time (min) (mean ± S.D.)	103.1 ± 67.1	103.7 ± 67.1	0.976	56.9 ± 49.6	54.3 ± 39.7	0.773
Delayed bleeding rate[b] (%)	15.4	1.5	0.067	9.4	2.3	0.120
Perforation rate[c] (%)	0	3.1	>0.999	9.4	1.1	0.059

[a] The rate of cases revealing no tumour on the edge of the resected specimen.
[b] The rate of cases needing emergency endoscopy due to hematemesis, melena.
[c] All cases were snared after perforation during submucosal dissection (perforation is not directly due to snaring).
* Statistically significant.

4. Discussion

As tips and tricks for ESD have accumulated, performing ESD has gradually permeated into the repertoire of surgical techniques, especially in Japan [12]. The technique has significant advantages over other endoscopic treatments in the areas of controlling the shape and size of the resected specimen and the high probability of *en bloc* resection of an entire lesion. However, ESD is sometimes very difficult to perform and the operator must overcome many hurdles during the procedure.

The snaring technique is a basic one used in polypectomy or endoscopic mucosal resection (EMR) for pedunculated or small flat lesions [13]. Although snaring is easily performed and considered to save time, the disadvantage is uncertainty of *en bloc* resection [1,14]. When snaring is applied during ESD procedure, we should always consider this disadvantage; if snaring causes multiple-piece resection, the original advantage of ESD, the precise histopathological evaluation of an entire lesion, will be lost.

Why, then, is a snare used as the final step in ESD? There are various reasons. The most frequent one may be its time-saving advantage. However, Table 5 shows that snaring did not shorten operation time in our case series. Then, considering these results, does snaring actually shorten the operation time? The answer should be absolutely "yes". In some cases, a snare was used because it took an unexpectedly long time to dissect the submucosal layer beneath the tumour. In other cases, it was used because continuation of the submucosal dissection until complete detachment of the lesion from the muscle layer was impossible. So, when the results obtained from the overall analysis are considered in their clinical context, we are convinced that snaring absolutely shortens the procedure. If snaring had been planned beforehand for time-saving purposes, then normal operations that had not involved these extenuating circumstances would have been finished in much shorter time.

Although the overall analysis shows that *en bloc* resection rate was significantly lower in the snaring group than in the non-snaring group, the sub-analysis according to tumour size yielded quite important evidence for us. The results indicated that almost all small tumours (2 cm as a cut-off line between small and large tumours) could be removed in *en bloc* fashion. On the other hand, the rate of *en bloc* resection of large tumours in the snaring group was significantly lower than that in the non-snaring group. Furthermore, the larger the tumour size is, the longer operation time is. A dilemma emerges in the tradeoff between operation time and possibility of *en bloc* resection, especially for large tumours. It is reported that piecemeal resection leads to non-curative treatment, causing non-evaluable histopathology of the tumour and potentially local recurrence [1]. Even though it takes longer time to resect, the first priority should be curative resection of the tumour for the cases of no complications. So, in conditions where long operation time does not become a disadvantage for patients, such as under general anaesthesia, we recommend to accomplish complete submucosal dissection by an electrosurgical knife instead of using a snare. However, this consideration should be withdrawn when poor conditions during the operation occurred, and snaring should be used as a salvage technique, considering risks and benefits of the patient. Another possible point at issue of increasing the number of incomplete resections due to a burning effect of snaring for margins of resected specimens was not observed in our case series, which may surely encourage us to use a snare when *en bloc* resection is undoubtedly possible, in order to shorten operation time.

In terms of complications, delayed bleeding frequently occurred when a snare was used. In the "snaring" series, a snare was used in some cases of declared technical difficulties, presence of comorbidity or occurrence of complications. These variables might have determined the higher rate of delayed bleeding observed in this subgroup of patients, although the reason for this is still unclear. Another explanation is that snaring might cause insufficient coagulation of submucosal vessels, which implies that non-bleeding visible vessels should be more intensively treated after resection of the tumour with a snare, especially in the area detached by a snare. Although perforation occurred in six cases, the causes for perforation were not related to snaring, but were

due to inappropriate submucosal dissection by electrosurgical knives in all cases. On the contrary, an appropriate use of a snare may decrease perforation rate owing to reduction in time spent on direct submucosal dissection.

In summary, we suggest that lesions >2 cm in size should be resected in complete submucosal dissection style without snaring, in order to avoid multiple-piece resection, and that lesions ≤2 cm in size may be treatable through snaring in order to reduce operation time. However, the shortcoming of this report was a retrospective, non-controlled setting, even though the results were obtained from the consecutive data. A prospective, randomised controlled study about the feasibility of "planned" snaring is desirable to confirm our results, especially for smaller tumours.

Practice points

- Snaring resection may be acceptable for small lesions (≤2 cm) as the final step of endoscopic submucosal dissection (ESD) for stomach epithelial neoplasms to save operation time.
- Complete submucosal dissection by an electrosurgical knife is recommendable for large tumours (>2 cm) to avoid multiple-piece resection.

Research agenda

- A prospective, randomised trial is favourable to elucidate the feasibility of snaring as the final step of ESD, especially for small lesions.

Conflict of interest

None declared.

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DNA methylation: a marker for carcinogen exposure and cancer risk

Takeshi Nakajima · Shotaro Enomoto · Toshikazu Ushijima

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Abstract Cancers arise as a consequence of multiple genetic and epigenetic alterations. Many genes aberrantly methylated in cancers have been identified in recent years, and their use in cancer diagnosis and therapy is currently under investigation. During our genome-wide screening for a novel tumor-suppressor gene in gastric cancers, we found that only a small amount of aberrant methylation was present, even in non-cancerous gastric mucosae. A subsequent large-scale analysis of the gastric mucosae of healthy individuals and gastric cancer patients using quantitative methylation-specific PCR (qMSP) revealed that *Helicobacter pylori* infection potently induced aberrant DNA methylation in non-cancerous gastric mucosae and that these high methylation levels can decrease following cessation of the *H. pylori* infection. *Helicobacter pylori* infection induced the methylation of specific genes among 48 genes that can be methylated in gastric cancer cell lines. Most importantly, the methylation levels in the gastric mucosae of individuals without *H. pylori* infection correlated with their risk of gastric cancer. These findings show that a field for cancerization is formed by *H. pylori* infection and that this field can be measured using DNA methylation as a marker. The concept of an "epigenetic field for cancerization" has been also demonstrated for colon and breast cancers, and it is possibly present for other cancers and other diseases. Applied knowledge of epigenetic changes in human diseases has now started to make an impact on the prevention, diagnostics, and therapeutics of these diseases.

Keywords Cancer · DNA methylation · Epigenetic · Field cancerization · Field defect · Gastric cancer · *Helicobacter pylori*

T. Nakajima · S. Enomoto · T. Ushijima (✉)
Carcinogenesis Division,
National Cancer Center Research Institute,
5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan
e-mail: tushijim@ncc.go.jp

T. Nakajima
Endoscopy Division, National Cancer Center Hospital,
5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

Introduction

Epigenetic modifications are defined as DNA-associated modifications that are faithfully inherited upon somatic cell division, such as DNA methylation at CpG sites, histone modifications, and polycomb complex formation [1]. DNA methylation, in particular, is faithfully replicated upon cell division [2, 3], and is known to serve as a machinery for cellular memory [4]. At the same time, epigenetic modifications show plasticity during development, adaptation, and diseases. Epigenetic modifications are reprogrammed during the formation of germ cells, and dynamic and coordinated changes take place during development and differentiation [5]. Epigenetic changes are also physiologically induced in somatic cells to maintain the memory of exposure to environmental stimuli [6, 7].

Our increasing knowledge of epigenetic changes in human diseases has now started to make an impact on the prevention, diagnostics, and therapeutics of these diseases. From a viewpoint of environmental health and preventive medicine, epigenetic alterations in non-disease tissues are becoming important because it is becoming clear that they can be used as markers for disease risk and past exposure to some disease-inducing factors. In this review, we provide a brief introduction to aberrant DNA methylation in cancers, describe our experimental findings on the presence of aberrant DNA methylation in non-cancerous gastric

mucosae, including a description of its use as a marker for both the risk of gastric cancers and past exposure to *Helicobacter pylori*, an established gastric carcinogen and, finally, discuss the concept of field cancerization and its usefulness an a diagnostic marker in other cancers.

Aberrant DNA methylation in cancers

Aberrant DNA methylation in carcinogenesis

The existence of aberrant DNA methylation in cancer tissues has been known since the early 1980s, but it was not until the early 1990s that it was shown to have a causal involvement in human cancers [1, 8]. Aberrant DNA methylation in cancers is often summarized as (1) genome-overall hypomethylation and (2) regional hypermethylation. Genome-overall hypomethylation is mainly due to hypomethylation of repetitive DNA sequences, such as LINE and Alu (SINE), that constitute a major part of the genome and are normally methylated [9]. Such hypomethylation can cause chromosomal instability and, consequently, tumors [10] as well as aberrant expression of normally methylated genes, such as melanoma antigen genes (MAGEs) [11]. The aberrant activation of oncogenes due to promoter demethylation (hypomethylation) has as yet not been established.

Regional hypermethylation refers to the aberrant methylation of normally unmethylated sequences, most of which are clusters of CpG sites, denoted CpG islands (CGIs). Importantly, when a CGI is located in a gene promoter region, its methylation consistently leads to transcriptional silencing of its downstream gene (Fig. 1). This also applies to many tumor-suppressor genes, such as *CDKN2A* (various cancers), *CDH1* (gastric cancers), *APC* (colorectal cancers), and *BRCA1* (breast cancer). Methylation-silencing of tumor-suppressor genes is now known to be involved in various human cancers [1]. In addition to aberrant DNA methylation being causally involved in carcinogenesis (driver methylation), recent genome-wide studies have revealed the presence of many genes whose methylation is considered to be a consequence of carcinogenesis (passenger methylation) [12–14]. This fact clearly demonstrates the need to carefully analyze the role of any newly detected gene in terms of its role in carcinogenesis.

Clinical use of aberrant DNA methylation

Both diagnostic and therapeutic applications of aberrant DNA methylation in cancers are being developed [15]. One diagnostic application is the use of cancer-specific patterns of aberrant DNA methylation to detect cancer cells and cancer-derived DNA. Different from mutations, aberrant DNA methylation can be detected with high sensitivity – for example, at a sensitivity of one aberrantly methylated DNA molecule among 1000 molecules. The chemical stability of DNA is also an advantage in this application. A second diagnostic application is the association of patterns of aberrant DNA methylation in cancer tissues with tumor characteristics, such as histological type, risk of disease progression, sensitivity to chemotherapy, and molecular alterations [15, 16]. An example of this can be found in neuroblastomas, where the methylation pattern is very closely associated with survival risk [17]. Thirdly, methylation in non-cancerous tissues is now recognized as a marker for cancer risk and exposure to carcinogenic factors, which will be the main topic of this review.

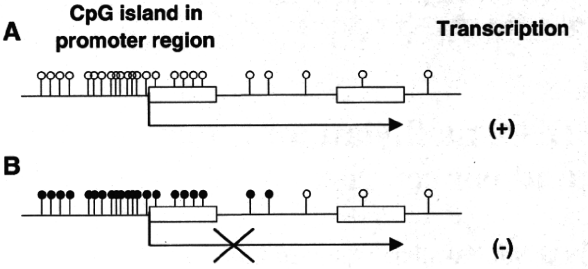

Fig. 1 Methylation of a promoter CpG island (*CGI*) and transcription of its downstream gene. *Open* and *closed circles* Unmethylated and methylated CpG sites, respectively. **a** In a normal cell, most CpG sites within a promoter CGI are unmethylated. **b** Methylation of most CpG sites (dense methylation) of the promoter CGI completely blocks transcription. If such methylation occurs in a tumor-suppressor gene, it leads to inactivation of the tumor-suppressor gene

In terms of therapeutic purposes, epigenetic abnormalities are now used as promising targets. The Federal Drug Agency has approved two demethylating agents, 5-aza-cytidine (5-aza; Vidaza) and 5-aza-2′-deoxycytidine (5-aza-dC; Decitabine), for hematological malignancies [18]. In addition, preclinical trials are ongoing for solid tumors. Demethylating agents currently seem to have therapeutic windows, being active in tumor cells but having few side-effects in normal cells. The concept of "maximum tolerance dose" is not valid for demethylating agents, and an optimal dose for maximum demethylating activity should be achieved [18]. Further investigations are necessary on the most suitable dosing, including the identification of appropriate marker genes and tissue, and on the target specificity in cancer and normal cells.

Aberrant methylation in non-cancerous gastric mucosae

Presence of "aberrant" DNA methylation in non-cancerous gastric mucosae

In human gastric cancers, *CDKN2A* (*p16*), *CDH1* (*E-cadherin*), and *hMLH1* are inactivated more frequently by the

aberrant methylation of promoter CGI than by mutations or chromosomal losses [19]. During previous study carried out in our laboratory in which we identified a novel tumor-suppressor gene, *LOX* [20], we observed that aberrant DNA methylation was present even in non-cancerous gastric mucosae of gastric cancer patients, although at a very low level [21, 9]. It is unlikely that these cancer cells contaminated non-cancerous samples because aberrant methylation in non-cancerous gastric mucosae was observed too often to be contamination.

Methylation in non-cancerous gastric mucosae and *H. pylori* infection

To clarify the meaning of "a small amount" of aberrant methylation in non-cancerous gastric mucosae, we quantified the methylation levels in gastric mucosae of healthy volunteers and in non-cancerous gastric mucosae of gastric cancer patients [22]. Healthy volunteers (individuals without gastric cancer) and gastric cancer patients were classified according to their status of *H. pylori* infection, a major etiological factor for gastric cancers [23, 24], at the time of sampling. The numbers of methylated and unmethylated DNA molecules were counted, using quantitative methylation-specific PCR (qMSP), for eight regions of seven genes, all of which can be methylated in gastric cancers. Methylation levels were calculated as the number of methylated molecules present among the total number of DNA molecules. This value was considered to represent the fraction of cells with methylation in a gastric mucosa.

All of the eight regions showed a similar tendency in terms of methylation levels. Among healthy volunteers, methylation levels were 5.4- to 303-fold higher in *H. pylori*-positive individuals than *H. pylori*-negative individuals [22]. This finding strongly indicates that *H. pylori* infection can potently induce aberrant DNA methylation in non-cancerous gastric mucosae. In addition, *H. pylori*-positive individuals had higher methylation levels than *H. pylori*-negative gastric cancer cases, most of whom were considered to have had prior exposure to *H. pylori* infection (Fig. 2a).

Temporary and permanent components of methylation level induced by *H. pylori*

The above results indicate that high methylation levels will decrease to certain levels after cessation of the *H. pylori* infection. Since endogenous DNA demethylase has not been established and active demethylation in a cell was unlikely to take place, this decrease is considered to be passive demethylation due to cell turnover. A gastric gland consists of one stem cell, multiple progenitor cells, and many differentiated cells [25], and it is expected that methylation in stem cells will persist forever (permanent component) while methylation in progenitor and differentiated cells will disappear (temporary component). It is therefore likely that *H. pylori* infection induced both permanent and temporary components of methylation, and that

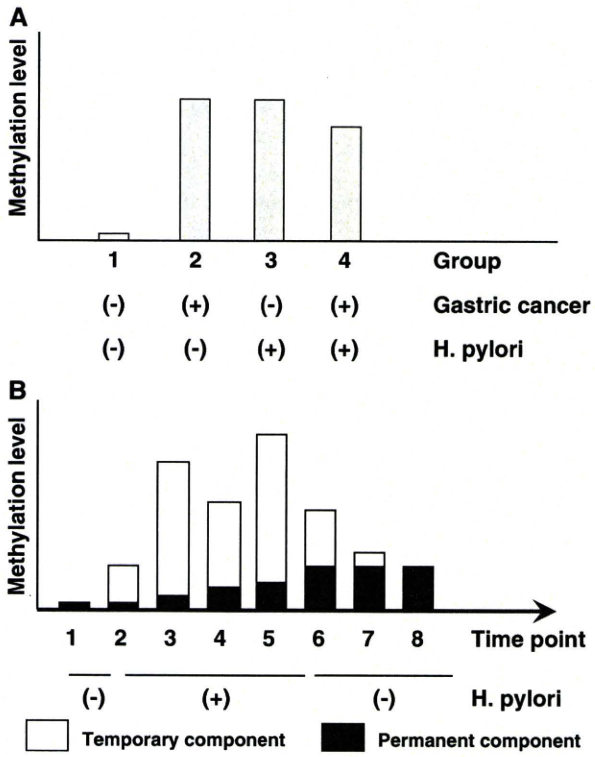

Fig. 2 Methylation induction by *Helicobacter pylori* infection and gastric cancer risk. **a** Schematic representation of methylation levels in the gastric mucosae of individuals with and without gastric cancer, and with and without *H. pylori* infection. Methylation levels were measured in DNA extracted from gastric biopsy specimens. Without *H. pylori* infection, there is a significant difference between healthy volunteers (*group 1*) and gastric cancer cases (*group 2*). With *H. pylori* infection, the methylation level is high in both healthy volunteers (*group 3*) and gastric cancer cases (*group 4*). *H. pylori*-positive individuals had higher methylation levels than *H. pylori*-negative gastric cancer cases, most of whom were considered to have had prior exposure to *H. pylori* infection. Modified from Maekita et al. [22]. **b** A hypothetical temporal profile of gastric methylation levels during the course of *H. pylori* infection in years to decades. Time point 1: Without *H. pylori* infection, the methylation is initially. Time points: 2–5, *H. pylori* infection induces both permanent (*closed box*) and temporary (*open box*) components of methylation, and the total methylation level fluctuates due to fluctuation of the temporary component. Time points 6–8: after *H. pylori* infection discontinues, the temporary component disappears, and the increase in the permanent component stops. It is speculated that the permanent component is due to methylation in stem cells and that the temporary component is due to methylation in progenitor and differentiated cells. The permanent component is correlated with damage in stem cells, and thus with gastric cancer risk

the temporary component disappeared after cessation of the *H. pylori* infection (Fig. 2b).

To support this hypothesis, we eradicated *H. pylori* and measured methylation levels 6 weeks after the eradication. When the eradication was successful, *FLNc* (filamin C) methylation levels decreased to certain levels. When eradication failed, methylation levels fluctuated, depending upon the individual (Nakajima, in preparation). The decreased methylation levels in individuals following successful eradication of *H. pylori* was considered to be due to disappearance of the temporary component, leaving only the permanent component in place.

Methylation levels in gastric mucosae as a marker for gastric cancer risk

In the study described above [22], individuals without *H. pylori* infection, whose methylation levels were considered to reflect the fraction of stem cells with methylation, had methylation levels that were 2.2- to 4.9-fold higher in cases of gastric cancer than in healthy volunteers. We also newly collected non-cancerous gastric mucosae of patients with a single gastric cancer and those with multiple gastric cancers. Patients in the latter group, who were considered to have a higher risk of gastric cancers [26], had a significantly higher *FLNc* methylation level than patients with a single gastric cancer ($P < 0.01$, t test) [27]. These results strongly indicate that methylation levels in non-cancerous gastric mucosae are a good candidate biomarker for gastric cancer risk. In order to confirm their clinical usefulness, a prospective study is currently being planned.

Methylation of specific genes in gastric mucosae by *H. pylori* infection, and its promising potential

In the initial study where eight regions of seven genes were analyzed [22], all eight regions were methylated in the presence of *H. pylori* infection. There are two possibilities explaining this result: (1) these eight regions are regions that can be methylated in gastric cancers, or (2) *H. pylori* infection induces genome-wide, non-specific methylation of CGIs. To distinguish between these two possibilities, we analyzed the methylation of 48 genes in gastric mucosae of individuals with and without *H. pylori* infection. These 48 genes were selected because they can be methylation silenced in gastric cancer cell lines [14]. Some genes were resistant to methylation induction, and some were consistently methylated in individuals with *H. pylori* infection (unpublished data; for review, see [28]. Since low transcription levels are known to trigger promoter methylation [12, 29], it was concluded that *H. pylori* infection can induce decreased transcription of specific genes and that some of these can be methylated. This concept can be expanded to one in which some carcinogenic factors have the potential to induce methylation of specific genes in non-cancerous tissues and that the specific methylation profile of an individual can be used as a marker for past exposure to specific carcinogenic factors.

Epigenetic field for cancerization

The finding that methylation levels in non-cancerous gastric mucosae correlate with gastric cancer risk has the potential to be generalized to cancers of other tissues. Since this finding is closely related with the concept of field defect, or field for cancerization, which has a long history, we first provide a short review of the concept of field for cancerization and then discuss "epigenetic field for cancerization".

The concept of field for cancerization

The concept of "field for cancerization" was first used by Slaughter et al. in 1953 for describing oral cavity cancer [30] and was based on the phenomenon that, even after curative resection of a primary cancer, metachronous (secondary multiple) primary cancers developed further. This occurrence indicated that the background mucosae of a cancer patient were already predisposed to cancer development, providing a field for cancerization. In recent decades, the concept has been applied to cancers of many other organs, especially squamous cell carcinomas of the head and neck (HNSCC) [31, 32], squamous cell carcinomas of the esophagus [33, 34], adenocarcinomas from the Barrett's esophagus [35], stomach cancers [36], breast cancers [37], and skin cancers [38]. The presence of cells with mutations of tumor-related genes, such as *p53*, in the field for cancerization has been shown for head and neck cancers (21–52%) [31, 32] and skin cancers (5/8) [38].

Epigenetic field for cancerization

As the deep involvement of aberrant DNA methylation in human cancers became clear, the occasional presence of aberrant DNA methylation in non-cancerous tissues was recognized in the colon [39, 40], liver [41], Barrett's esophagus [42], and stomach [43]. The presence of aberrant methylation in non-cancerous tissues suggested the involvement of the former in the field for cancerization. However, since DNA methylation can show non-significant fluctuation, analysis of control non-predisposed tissues

from healthy individuals (or patients with cancers at other sites) is essential to demonstrate the association between DNA methylation and the field for cancerization. This was first achieved in the liver [41] and subsequently in the colon [44], stomach [22], and breasts ([45]; Table 1). Our study of the stomach is characterized by a marked difference achieved by quantitative methylation analysis and by the clear presence of an inducing factor, *H. pylori*, in addition to the systemic collection of non-predisposed tissues. These reports from multiple institutions strongly support the existence of an "epigenetic field for cancerization" in addition to a genetic field for cancerization.

Advantages of DNA methylation as a marker for a field for cancerization

There are several advantages to using DNA methylation as a marker of a field of cancerization. First, for some cancers, such as gastric cancers, aberrant DNA methylation of tumor-suppressor genes is more commonly observed than mutations [19]. In such cancers, an epigenetic field for cancerization is likely to be present and can be detected using appropriate marker genes. Second, the fractions of cells with aberrant methylation of marker genes can be much larger than those with mutations. In the case of non-cancerous gastric mucosae of human gastric cancers, the former were in the range of 10^{-3}–10^{-1} [22, 27]. In contrast, the fractions of cells with mutations of a *LacI* marker gene were in the range of 10^{-4}–10^{-3} in the colon and liver of mice heavily exposed to a carcinogen [46]. Third, novel marker genes can be easily isolated because techniques for genome-wide screening for changes in DNA methylation are now available [12]. An ideal marker gene should be methylated in association with methylation of tumor-suppressor genes, but at much higher frequencies (Fig. 3). Finally, methylated DNA molecules can be precisely quantified, even when present at a frequency of 1×10^{-3} [22]. For an assessment of the cancer risk in an individual, plus–minus judgment has limited meaning, and quantitative analysis is essential. Using qMSP of biopsy materials, we were able to predict the gastric cancer risk of individuals.

Inducing factors of aberrant DNA methylation and their detection

Helicobacter pylori was involved in the induction of the field for gastric cancers. Infection by *H. pylori* is known to induce severe chronic inflammation. Chronic inflammation is also present in ulcerative colitis for colon cancers, chronic hepatitis for liver cancers, and Barrett's esophagus for esophagus adenocarcinomas. Therefore, chronic inflammation, possibly specific types, is likely to induce aberrant DNA methylation in normal tissues and thus form a field for cancerization. Interestingly, interleukin 6, whose polymorphisms are involved in the susceptibility of various cancers, is known to induce expression and activity of DNA methyltransferase [18]. Further investigations are necessary to clarify which cytokines are really involved.

Epilogue

The presence of an epigenetic field for cancerization, induced by *H. pylori* infection, is now evident for human gastric cancers. For gastric cancer patients with *H. pylori* infection, we fortunately have a realistic choice: eradication therapy for *H. pylori*. This will prevent further

Table 1 Reports on epigenetic field for cancerization (modified from [53]

Cancer	Inducing factor	Analyzed gene	References
Liver	HBV and/or HCV	*CDKN2A,hMLH1,THBS-1* and five MINT loci	Kondo et al. [41]
Colorectal cancer			
Sporadic	Unknown	*MGMT*	Shen et al. [44]
UC associated	UC	*CDKN2A*	Hsieh et al. [40]
UC associated	UC	*ER, MYOD, CDKN2A*, and *CSPG2*	Issa et al. [52]
Barrett's cancer	Barrett's esophagus	*APC,CDKN2A*, and *ESR1*	Eads et al. [48]
Lung cancer	Smoking?	*CDKN2A, MGMT, DAPK, SOCS1, RASSF1A, COX2*, and *RARβ*	Guo et al. [49]
Gastric cancer	*H. pylori*	*CDKN2A,LOX,THBD, HRASLS,FLNc,HAND1*, and *p41ARC*	Maekita et al. [22]
Breast cancer	Unknown	*CYP26A1*	Yan et al. [45]
Renal cancer	Unknown	*CDKN2A, hMLH1,THBS-1*, and five MINT loci	Arai et al. [47]

UC, Ulcerative colitis; HBV, hepatitis B virus; HCV, hepatitis C virus

exacerbation of an epigenetic field for cancerization and also enable us to measure the risk of metachronous gastric cancers accurately. Follow-up procedures will be modified depending upon the risk. Even if a high risk has already accumulated, there is a possibility that demethylating therapy could reduce the risk. If this scenario is eventually realized, clinical management of gastric cancers will experience a great change.

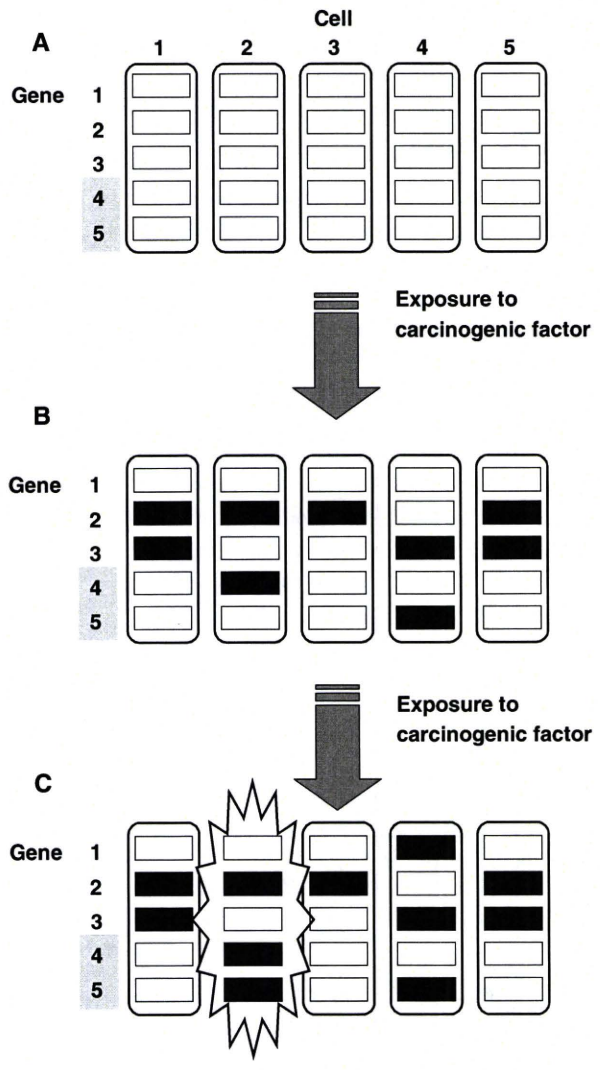

Fig. 3 Gene function and a good marker gene. Genes 4 and 5 are tumor-suppressor genes and, when both are methylated, cell transformation takes place. **a** In a normal tissue, no or little methylation is present. **b** After exposure to a carcinogenic factor, such as chronic inflammation, specific genes become methylated. Good marker genes (genes 2 and 3) are those readily methylated upon exposure to the carcinogenic factor in association with the methylation of tumor-suppressor genes, which are generally resistant to methylation. Specific methylation of genes 2 and 3, not gene 1, is a candidate marker for exposure to the carcinogenic factor. **c** After repeated exposure to the carcinogenic factor, the fractions of cells with methylated marker genes increase, and some cells (cell no. 2) can acquire methylation of multiple tumor-suppressor genes and transform

The concept of epigenetic field cancerization also seems to be valid for colon and breast cancers [44, 45] and, possibly, for liver cancers, esophageal adenocarcinomas, lung cancers, and renal cancers [41, 47–49]. Since the involvement of epigenetic alterations seems not to be limited to cancers [50, 51], there is even a possibility that an epigenetic field defect could be identified for various diseases. It is clear that if disease risk at a time point can be measured by a DNA methylation marker, it will help people to change their lifestyles for more intensive disease prevention.

The clarification of just how much individual carcinogenic factors can contribute to human cancers is an important issue in public health. It is known that some carcinogenic factors leave their fingerprint in the tissues damaged by them – even if they themselves are no longer present – as specific DNA methylation patterns. If more fingerprints could be identified that are as distinct as that left by *H. pylori*, these could be used to identify carcinogenic factors involved in individual cancers. This would enable the appropriate efforts and resources to be focused on the elimination of carcinogenic factors at both individual and public levels.

Research in epigenetics is now very active world-wide. Sweeping changes in the clinical management of cancer patients and elimination procedures of carcinogenic factors are in sight.

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DISEASES OF THE ESOPHAGUS

Case report

Detailed comparison between endocytoscopy and horizontal histology of an esophageal intraepithelial squamous cell carcinoma

M. Fujishiro,[1] S. Kodashima,[1] K. Takubo,[2] N. Kakushima[1] and M. Omata[1]

[1]*Department of Gastroenterology, University of Tokyo, Tokyo, Japan,* [2]*Department of Clinical Pathology, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan*

SUMMARY. **Endocytoscopy allows the real-time microscopic observation of living cells. Unlike the cross-sectional images obtained by conventional histology, endocytoscopy provides cellular images in a plane parallel to the surface of the mucosa. However, there is little knowledge about the endocytoscopic diagnosis of carcinomas. Using a specimen obtained by the endoscopic submucosal dissection of an intraepithelial esophageal squamous cell carcinoma, a detailed comparison between endocytoscopic and horizontal histological images was made, revealing the similarity between the images. Sharp lateral borders between atypical and normal epithelium and differences in cellularity and the sizes and shapes of the nuclei were clearly identified by endocytoscopy. Further horizontal histological investigations of this case also showed the variety of endocytoscopic images in non-cancerous and cancerous epithelia.**

KEY WORDS: **endocytoscope, esophageal neoplasm, histology, ultra-high magnification.**

INTRODUCTION

Endocytoscopy enables a real-time microscopic observation of living cells[1–4] and has the potential to guide endoscopic biopsy. However, it is not known what the endocytoscopic images represent in relation to standard cross-sectional histopathological images. A more appropriate evaluation would correlate the endocytoscopic images to that of histological images obtained in a plane parallel to the tissue surface. Increased cellularity, uneven sizes and shapes of nuclei and a distinct border interface between atypical and normal epithelium are likely to be the minimum essential findings to make an endocytoscopic diagnosis of malignancy. We here describe a case of an esophageal intraepithelial squamous cell carcinoma analyzed *in vivo* with endocytoscopy and correlate it with a corresponding horizontal histological examination in order to confirm the feasibility of an endocytoscopic diagnosis of carcinoma or a normal epithelium in the esophagus.

CASE REPORT

A 82-year-old man with three esophageal squamous cell carcinomas (two intraepithelial and one invasive) underwent endoscopic submucosal dissection (ESD) of these lesions, as described elsewhere.[5] All the lesions were completely resected *en bloc*, and a conventional histopathological assessment was performed for the invasive carcinoma and the larger intraepithelial carcinoma to make a final histological decision of curability. Because the remaining smaller intraepithelial carcinoma (7 mm in size), resected with a tumor-free margin confirmed by iodine staining (Fig. 1) had little influence on further management, the lesion was investigated for a detailed comparison between the endocytoscopy and horizontal histology after informed consent was obtained from the patient.

Immediately after ESD, the resected specimen was evaluated by endocytoscopy to assess the cancerous region, normal epithelium and the interface between carcinoma and normal epithelium following staining

Address correspondence to: Dr Mitsuhiro Fujishiro, MD, PhD, Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo, Japan.
Email: mtfujish-kkr@umin.ac.jp
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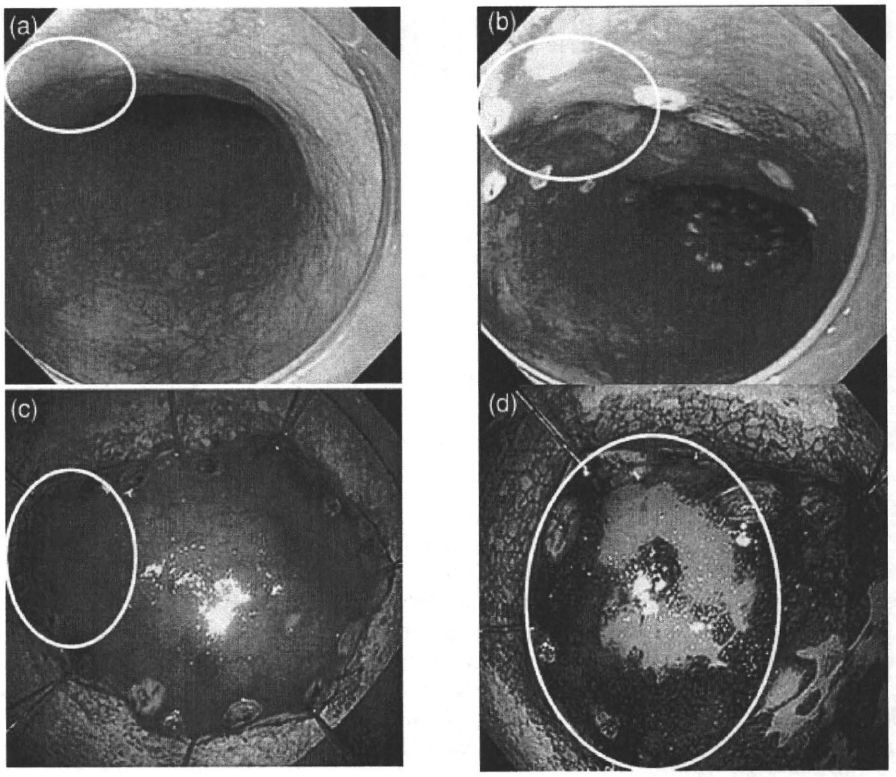

Fig. 1 Intra-epithelial esophageal carcinoma with endocytoscopic and horizontal histological observations, (a) ordinal endoscopic view, (b) iodine-staining endoscopic view with marking around it (the marking is to encircle a coexisting intra-epithelial carcinoma), (c) resected specimen by endoscopic submucosal dissection (a single resection is made with a coexisting intra-epithelial carcinoma), (d) close-up image of the resected specimen with iodine staining. *The circle in each figure shows the same area in both endocytoscopic and horizontal histological observations.

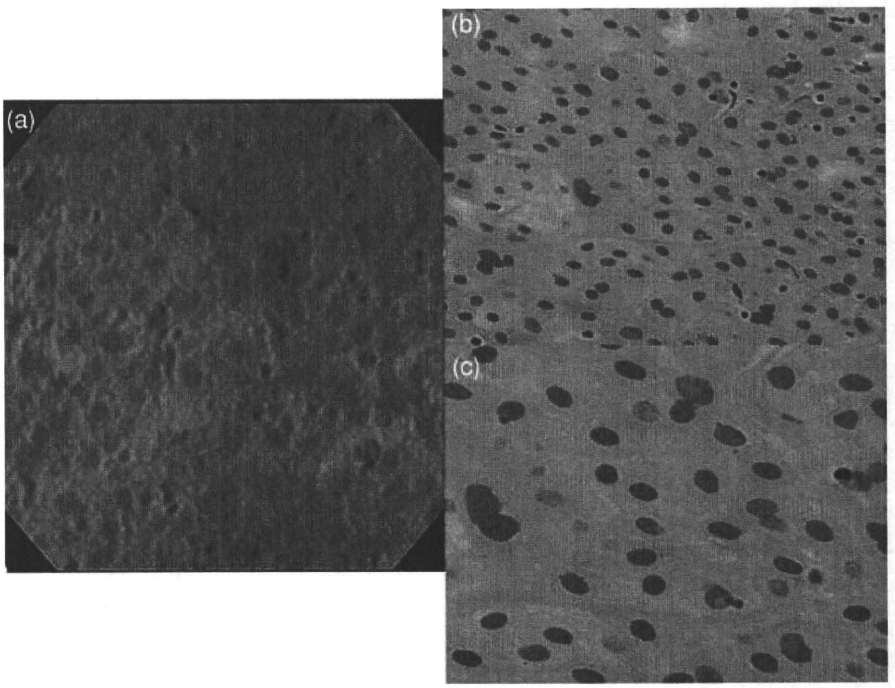

Fig. 2 Endocytoscopic and horizontal histological images of the intra-epithelial carcinoma showing the increased cellular density and the irregular arrangement and uneven sizes and shapes of the nuclei, (a) endocytoscopic image (1% methylene blue staining, original magnification 450× on a 35-cm monitor), (b) histological image (hematoxylin and eosin staining, original magnification 200×), (c) histological image (hematoxylin and eosin staining, original magnification 400×).

with 1% methylene blue.[6] A prototype endocytoscopy system, which consisted of a 3.2-mm diameter soft-catheter-type endocytoscope (XEC-300-2, Olympus Medical Systems Co., Tokyo, Japan), VISEA video system center (OTV-S7V, Olympus Medical Systems Co., Tokyo, Japan), and high-brightness light source (CLH-SC, Olympus Medical Systems Co. Tokyo, Japan), was used. The endocytoscope provides a fixed magnification of 450× (on a 36-cm monitor), which covers a 300 × 300-μm area of tissue. After observation, the specimen containing the entire lesion with surrounding normal epithelium was fixed with formalin and embedded in paraffin. Horizontal histological sections of the carcinoma, normal epithelium and normal–cancer border were made from each block and stained with hematoxylin and eosin.

A comparison of the endocytoscopic and histological images was performed. The endocytoscopic images bore a close resemblance to the surface horizontal histological images. The carcinoma showed increased cellular density with a completely irregular arrangement and uneven-sized and -shaped nuclei (Fig. 2), whereas the normal epithelium showed a regular cellular arrangement and even-sized and -shaped nuclei (Fig. 3). The tissue interface between the carcinoma and the normal epithelium was clearly identified in the endocytoscopic image as well as in the histological image (Fig. 4).

DISCUSSION

Endocytoscopy enables cells at the luminal surface to be viewed in their natural living state. In the attempt to obtain a real-time histological analysis at endoscopy (an optical biopsy), several methods, including endocytoscopy, have proved promising for potential clinical use.[7] Natural images can be obtained using endocytoscopy alone, because this system uses white light and is similar to an optical microscope, conferring a great advantage over other techniques in the application of histopathological knowledge to interpret the images. As shown in this case, it was possible to distinguish between the carcinoma and the normal epithelium endocytoscopically and to correlate these findings with a histologic diagnosis obtained at a plane similar to that of endocytoscopy. Key findings such as the degree of cellularity, nuclear size and shape, and a distinct normal–neoplastic interface could be clearly identified by endocytoscopy in order to identify the presence of a neoplastic lesion.

Inoue *et al.*[8] recently reported on their excellent progress in establishing a preliminary classification with five grades of endocytoscopic atypia. The classification corresponded well with the Vienna histological classification[9] and the overall accuracy was 82%. However, there are still limited data of the characteristics of endocytoscopic images of various

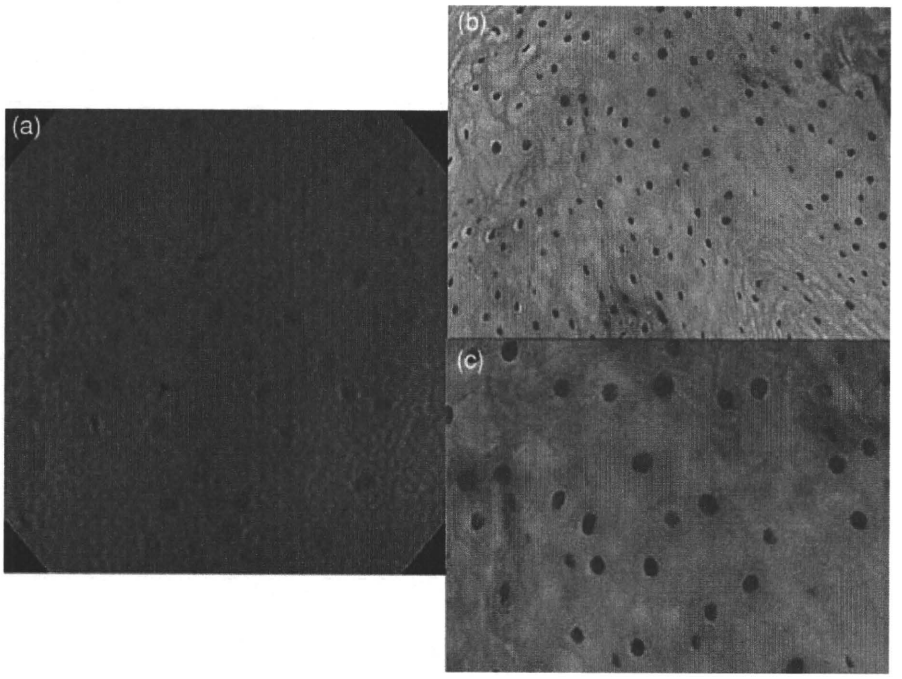

Fig. 3 Endocytoscopic and horizontal histological images of the normal epithelium showing the regular cellular arrangement and even sizes and shapes of nuclei, (a) endocytoscopic image (1% methylene blue staining, original magnification 450× on a 35-mm monitor), (b) histological image (hematoxylin and eosin staining, original magnification 200×) and (c) histological image (hematoxylin and eosin staining, original magnification 400×).

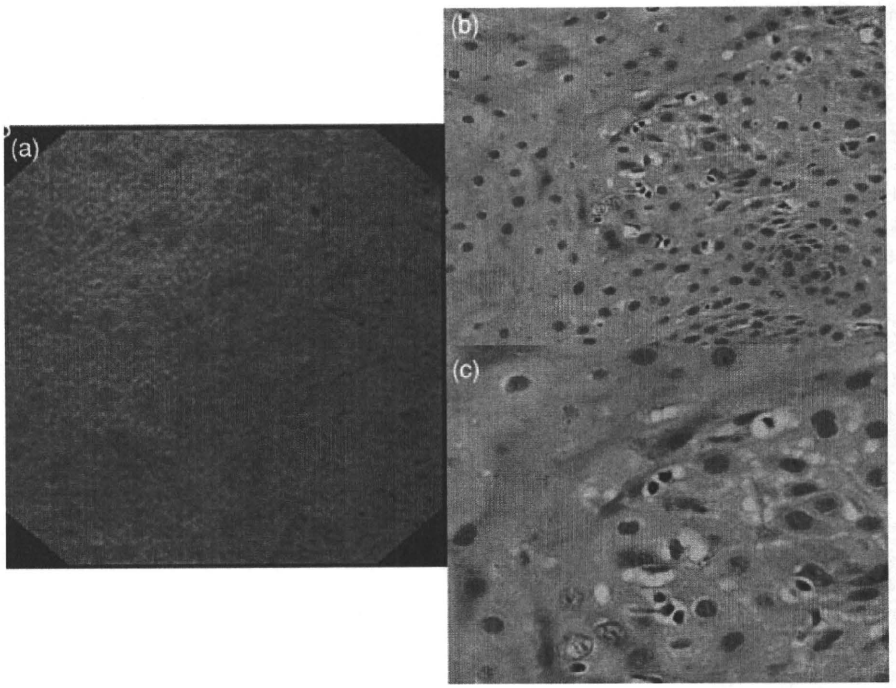

Fig. 4 Endocytoscopic and horizontal histological images of the border of intraepithelial carcinoma and normal epithelium. The lateral border is clearly identified by both the (a) endocytoscopic image (1% methylene blue staining, original magnification 450× on the 35-mm monitor), and (b) the horizontal histological images (hematoxylin and eosin staining, original magnification 200×) and (c) histological image (hematoxylin and eosin, original magnification 400×).

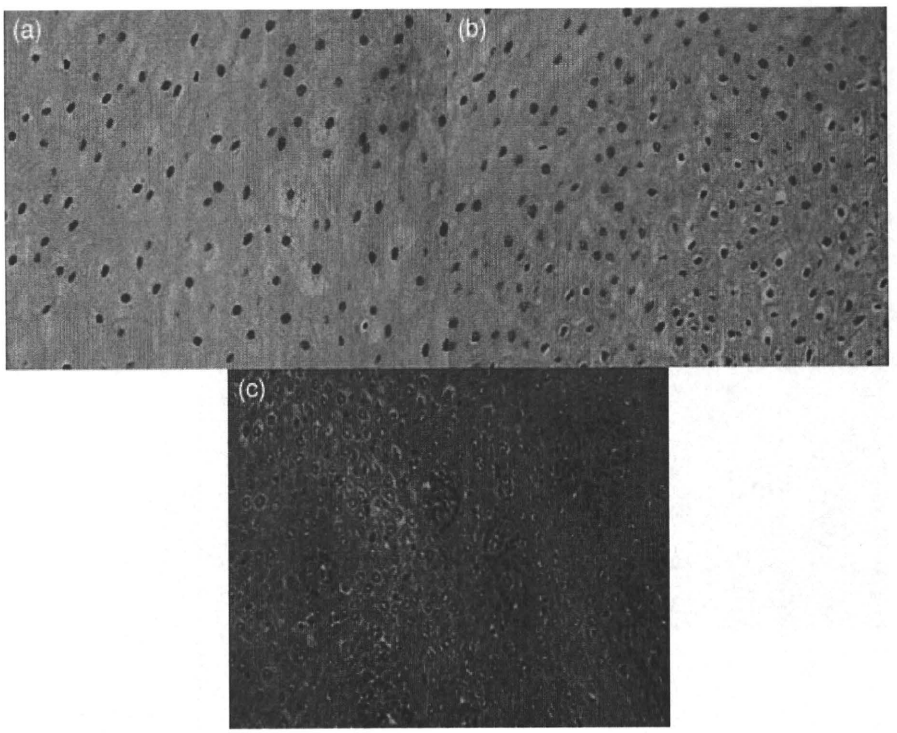

Fig. 5 Horizontal histological images from (a) the surface, (b) the middle and (c) the deepest image in the normal epithelium (hematoxylin and eosin staining, original magnification 200×). The cellular density and size of nuclei gradually increase from the surface to the deepest in a normal stratified squamous epithelium. Intra-papillary capillary vessels and light nuclei can be observed in the deepest part.

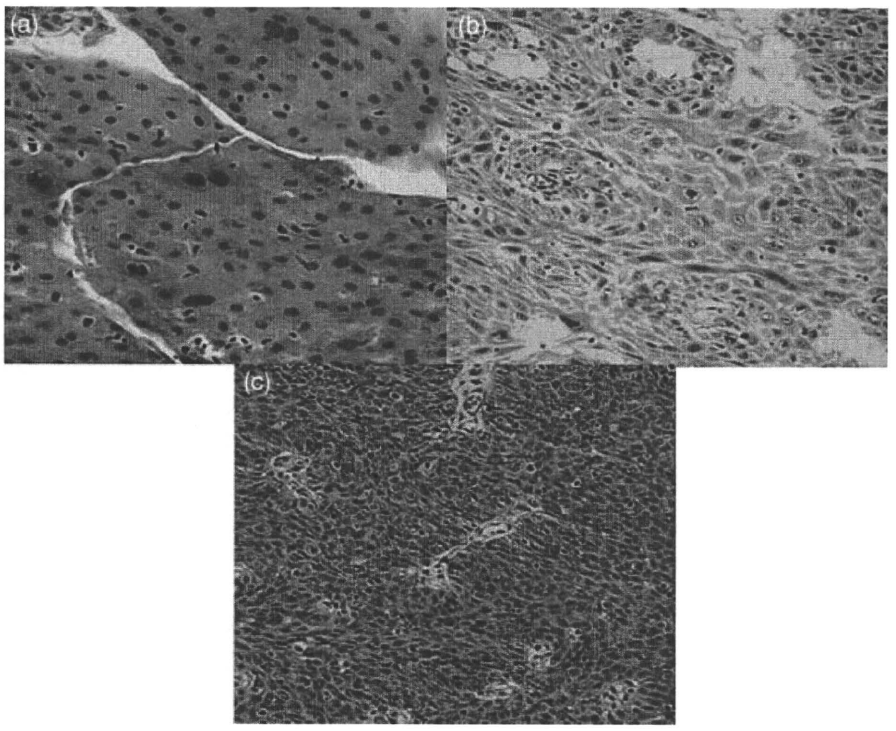

Fig. 6 Horizontal histological images from (a) the surface, (b) the middle, (c) the deepest surface image (hematoxylin and eosin, original magnification 200×) in the intraepithelial carcinoma. The cellular density and size of the nuclei are high, even on the surface and the cellular arrangement and shape of the nuclei are irregular in each part of the intraepithelial carcinoma. The images look quite different according to their depth.

conditions. Some observations can be made from this case study, which may lead to further progress in the knowledge of endocytoscopic images. Cellular density and the size of nuclei gradually decrease from the stratum basale epidermis to the surface in the normal stratified squamous epithelium. Thus, when erosion occurs, one may expect that the endocytoscopic images will show increased cellular density without showing the irregularity of size and shape of the nuclei (Fig. 5). On the other hand, the cellular density and size of nuclei are high even at the surface and the cellular arrangement and shape of the nuclei are irregular in intraepithelial carcinoma (Fig. 6).

From this case study, we also recognized the disadvantages of endocytoscopy. A major limitation is that critical factors such as the depth of the invasion cannot be assessed by endocytoscopy. This requires another imaging modality, such as confocal endoscopy or optical coherence tomography.[10,11] However, there is no doubt that endocytoscopy represents a powerful tool that enables the observation of living cells *in vivo* at the mucosal surface and the diagnosis of neoplastic lesion based on differences in cellular characteristics. The establishment of endocytosocopic diagnosis is promising, but needs further study in a larger cohort, including comparisons of *in vivo* endocytoscopy with biopsy or a histology of endoscopically or surgically resected specimens.

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