Clinical Study

Oncology

Oncology 2012;82:298–304
DOI: 10.1159/000336792

Received: December 2, 2011
Accepted after revision: January 23, 2012
Published online: April 28, 2012

Clinical Benefit of High-Sensitivity KRAS Mutation Testing in Metastatic Colorectal Cancer Treated with Anti-EGFR Antibody Therapy

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Key Words
Colorectal cancer · Cetuximab · KRAS mutation testing

Abstract
Objective: We compared high-sensitivity KRAS mutation testing with direct sequencing for predicting the efficacy of antiepidermal growth factor receptor antibodies in patients with metastatic colorectal cancer (mCRC). ***Methods:*** We analyzed the KRAS status in 61 tumors from cetuximab-treated mCRC patients by both direct sequencing and a high-sensitivity method: 2-step PCR restriction fragmentation length polymorphism (RFLP). Therapeutic effects in each mutational status were evaluated. ***Results:*** The incidences of KRAS mutations determined by direct sequencing and 2-step PCR RFLP were 34.4 and 52.5%, respectively ($p = 0.02$). Patients were categorized into 3 groups [W/W, wild-type by both methods (n = 29); W/M, wild-type by direct sequencing, detected mutation by 2-step PCR RFLP (n = 11); M/M, mutant-type by both methods (n = 21)]. The response rate for cetuximab in the W/M group (0%) was the same as that in the M/M group, and was significantly lower than in the W/W group (41.4%) ($p < 0.001$). Progression-free survival in the W/M group (11.0 weeks) was similar to that in the M/M group (8.0 weeks), and was significantly shorter than in the W/W group (18.0 weeks) ($p < 0.002$). ***Conclusion:*** High-sensitivity KRAS mutation testing is useful for selecting true responders to cetuximab.

Introduction

The recent development of antibody drugs that target molecules such as vascular endothelial growth factor (VEGF) or epidermal growth factor receptor (EGFR) has remarkably improved treatment outcomes of metastatic colorectal cancer (mCRC). They have been widely used as first- to third-line therapy for mCRC [1–3]. However, retrospective subset analyses and prospective randomized phase III trials have revealed that an anti-EGFR antibody agent was effective only for mCRC with wild-type KRAS but not for that with a KRAS mutation [3–7]. Inappropriate characterization of mCRC patients leads to a delay of the administration of appropriate therapy resulting in a poor outcome for patients and unnecessarily high medi-

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Table 1. Patient characteristics

Total number of patients		61
Median age (range)		66.5 (33–84)
Sex	Male	39
	Female	22
Performance status	0	23
	1	28
	2	10
Primary tumor	colon	23
	rectum	38
Differentiation	well	24
	moderately	32
	poorly	5
Metastatic site	liver only	15
	lung only	8
	peritoneum only	5
	≥2 sites	33
Number of previous chemotherapies	1	10
	2	34
	≥3	17
Chemotherapy regimen	cetuximab + FOLFIRI	5
	cetuximab + CPT11	24
	cetuximab alone	22
	others	10

cal expenses which affect the health care system. Ideally, cost-effective and evidence-based treatment should be developed in the field of chemotherapy. When we treat patients with mCRC, it is very important to accurately determine the presence or absence of a KRAS mutation, and anti-EGFR antibodies should not be administered in those cases with a KRAS mutation.

KRAS protein encoded by the KRAS gene is a small G protein and is located downstream of the EGFR-induced cell-signaling pathway [8]. Binding of a ligand, such as EGF or transforming growth factor-α (TGF-α), to EGFR, activates KRAS protein, and the cell proliferation signal is transmitted to the downstream RAF/MEK/ERK pathway. In colorectal cancer with a KRAS mutation, despite blockage of EGFR with an anti-EGFR antibody agent upstream of KRAS, cell proliferation signals from the mutated KRAS protein are constitutively transmitted downstream, resulting in treatment failure. Mutation in the KRAS gene occurs as a point mutation in codon 12 or 13 in 90% or more cases, and it is detected in 30–40% of cases of colorectal cancer [9]. Currently, direct sequencing is widely used for KRAS mutation analysis; however, several critical disadvantages of direct sequencing for diagnosis have been indicated. One is its low sensitivity; it cannot detect mutations accurately unless there are at least 20–50% cells with a KRAS mutation in the tissue sample. On the other hand, restriction fragment length polymorphism (RFLP) and the amplification refractory mutation system (ARMS) have been developed as high-sensitivity methods to detect KRAS mutations [10]. With these methods, the KRAS mutation is detected when cells with the mutation account for at least 0.1 and 1% of cells in the sample, respectively. In particular, 2-step PCR RFLP (mutant-enriched PCR RFLP) has made it possible to analyze the KRAS mutation at high sensitivity with a formalin-fixed paraffin-embedded block sample. Although differences in sensitivity and specificity between tests for the KRAS mutation have been noted, few studies have investigated the clinical impact of the accuracy of KRAS mutation testing on treatment with an anti-EGFR agent. In this study, we assessed the KRAS status by both 2-step PCR RFLP and direct sequencing in mCRC treated with cetuximab and evaluated the relationship between the mutation status and the efficacy of cetuximab.

Patients and Methods

Patient Characteristics

We retrospectively assessed 68 patients with mCRC who were treated by cetuximab monotherapy or by combination therapy using cetuximab plus a cytotoxic agent as second- or third-line treatments in three hospitals (Tokushima University Hospital, Sapporo Medical Center Tonan Hospital, and Kochi Health Sciences Center) from September 2008 to August 2009. Among these patients, 61 had measurable lesions detected by computed tomography (CT) scan. All of these patients had histologically proven colorectal adenocarcinoma. All tumor samples were obtained from the primary colorectal tumor by biopsy (n = 5) or by surgery (n = 56). The patients' clinical and pathologic characteristics are listed in table 1. Almost all patients (92%, 56/61) had been refractory to oxaliplatin and irinotecan administration. Tumor response was evaluated by CT scan according to the Response Evaluation Criteria in Solid Tumors (RECIST) [11]. Objective tumor response was classified as complete response, partial response, stable disease or progressive disease. Patients with complete response or partial response were defined as responders.

This retrospective study was conducted with the approval of the institutional review board.

DNA Extraction

Ten slices of a 10-μm tissue section were cut from each of the formalin-fixed paraffin-embedded tumor blocks. Serial sections were stained with hematoxylin and eosin to confirm the presence of carcinoma tissue. Genomic DNA was extracted using a QIAmp DNA FFPE tissue kit (Qiagen GmbH, Germany) and quantified by spectrophotometry.

Table 2. Correlation between 2-step PCR RFLP and direct sequencing for the detection of KRAS mutations

2-Step PCR RFLP	Direct sequencing		
	wild-type	mutant-type	
Wild-type	29 (47.5%)	0	29 (47.5%)
Mutant-type	11 (18.0%)	21 (34.4%)	32 (52.5%)
Total	40 (65.6%)	21 (34.4%)	

Direct Sequencing of KRAS Gene

Reactions were set up in 50-μl volumes as follows: 1.25 unit of Taq polymerase (Takara, Japan), 0.5 μmol/l of each primer (forward primer 5′-ACTGAATATAAACTTGTGGTAGTTGGAGCT-3′ and reverse primer 5′-TCAAAGAATGGTCCTGCACC-3′), 0.2 mmol/l of deoxyribonucleoside triphosphates, 10 × PCR buffer and 2 μg genomic DNA. PCR conditions comprised an initial denaturation step at 94°C for 3 min, followed by 35 cycles at 95°C for 30 s, 54°C for 30 s, 72°C for 30 s and a final extension at 72°C for 5 min. The PCR products were sequenced after purification using an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems-HITACHI, Japan).

2-Step PCR RFLP for KRAS Mutations

To detect KRAS codon 12 and 13 mutations, the PCR products were amplified using mismatch primers, which were designed to introduce an *MvaI* restriction site for codon 12 and a *BglI* restriction site for codon 13 of the wild allele, and analyzed by the RFLP method, as described by Kahn et al. [12]. In brief, the mismatched nucleotide primers used for the enzymatic amplification of KRAS sequences were as follows (nucleotide substitutions are underlined): 12&13F: 5′-ACTGAATATAAACTTGTGGTAGTTGGACCT-3′, wtR: 5′-TCAAAGAATGGTCCTGCACC-3′, 12mtR: 5′-TCAAAGAATGGTCCTGGACC-3′, and 13mtR: 5′-AACAAGATTTGCCTCTATGGCTGGATCA-3′. The 1st step in PCR amplification was performed on 2 μg of genomic DNA in a final reaction volume of 50 μl, which contained 0.2 mmol/l of deoxyribonucleoside triphosphate, 10 × PCR buffer, 0.5 μmol/l of 12 and 13F primer and wtR primer, and 1.25 units of Taq polymerase. The PCR condition was as follows: 94°C for 3 min and 30 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 45 s and finally 5 min at 72°C. Aliquots (2 μl) of the first PCR product were then digested with 20 units of *MvaI* (Takara) and 10 units of *BglI* (Takara) in a final volume of 20 μl at 37°C for 2 h under conditions recommended by the supplier. Ten-microliter aliquots of these *MvaI* and *BglI* digests were used in a 2nd-step PCR for mutations in codons 12 and 13, respectively. These aliquots were diluted to a final volume of 50 μl as described above. The PCR for codon 12 was performed with the 12&13F and 12mtR primers, whereas the PCR for codon 13 was performed with the 12&13F and 13mtR primers. The 2nd-step PCR conditions were as described above. Aliquots (25 μl) of KRAS codon 12 and 13 products obtained after 2nd-step PCR were then digested with either *MvaI* (for codon 12) or *BglI* (for codon 13) at 37°C for more than 2 h and were then electrophoresed through a polyacrylamide gel. This RFLP analysis by *MvaI* digestion for KRAS codon 12 mutations generated 114-bp, 29-bp, and 14-bp fragments if there was no mutation and 143-bp and 14-bp fragments if there was a mutation. Similarly, the RFLP analysis by *BglI* digestion for codon 13 mutations generated 74-bp, 32-bp, and 14-bp fragments if there was no mutation and 106-bp and 14-bp fragments if there was a mutation.

Statistical Analysis

All data were analyzed by using the Statistical Package for the Social Sciences (SPSS) statistics 18 package software (SPSS Inc., Japan). The positive rates of KRAS mutations with the 2-step PCR RFLP and direct sequencing were compared by the χ^2 test. Progression free survival (PFS) was estimated by the Kaplan-Meier method. Comparison between each group was performed by the log-rank test. $p < 0.05$ was considered statistically significant.

Results

Prevalence of KRAS Mutations by Direct Sequencing and 2-Step PCR RFLP

A total of 61 specimens of colorectal cancer tissues were examined for KRAS codon 12 and 13 mutations by both direct sequencing and 2-step PCR RFLP, and positive rates by the 2 methods were compared. KRAS mutations were detected in 34.4% (21/61) of specimens by direct sequencing and in 52.5% (32/61) of specimens by 2-step PCR RFLP. The positive rate of KRAS mutations by 2-step PCR RFLP was significantly higher than that by direct sequencing ($p = 0.02$). Table 2 shows the correlation between KRAS wild-type and mutant-types by direct sequencing and 2-step PCR RFLP. All of the 21 mutant-type patients identified by direct sequencing were also determined to have the mutant-type by 2-step PCR RFLP. Of the 40 wild-type patients identified by direct sequencing, 11 (18.0%) were determined to be the mutant-type by 2-step PCR RFLP. Figure 1 shows results of the KRAS analysis in all 11 patients with discordant findings. In the analysis of direct sequencing, no abnormal signals were observed in the nucleotide sequence of codon 12 or 13 in any of those 11 patients (fig. 1a). In the analysis of 2-step PCR RFLP, however, 143-bp bands (which represent a mutation of codon 12) or 106-bp bands (fig. 1b) (which represent a mutation of codon 13) were observed in all 11 patients.

Response Rate according to KRAS Mutation Status

In order to examine the relationship between KRAS mutation status and the response to cetuximab, we investigated the response rate for each KRAS mutation status by the 2 different methods. The response rate in KRAS wild-type patients determined by direct sequencing was 30.0% (12/40), whereas that determined by 2-step PCR RFLP was 41.4% (12/29) (table 3), with the latter being higher. None of

those patients with a KRAS mutation determined by either direct sequencing or 2-step PCR RFLP were responders.

We then categorized the patients into 3 groups as follows: W/W – wild-type by both methods, W/M – wild-type by direct sequencing but mutant-type by 2-step PCR-RFLP and M/M – mutant-type by both methods. When we compared baseline characteristics among the 3 groups, we found no statistically significant differences (suppl. table, www.karger.com/doi/10.1159/000336792). The response rate was compared among the 3 groups. The response rate in the W/M group was 0% (0/11), which was the same as in the M/M group (0/21); these rates were significantly lower than that in the W/W group (41.4%, 12/29) ($p < 0.001$). This indicates that patients with the mutant-type identified by 2-step PCR RFLP, irrespective of being positive or negative by direct sequencing, would have no response to cetuximab.

PFS in Patients with KRAS Wild- and Mutant-Types

The relationship between PFS and KRAS mutation status by the 2 methods was analyzed. Kaplan-Meyer curves for PFS in KRAS wild- and mutant-types by direct sequencing and 2-step PCR RFLP are shown in figure 2a, b. The PFS in the KRAS wild group determined by direct sequencing was significantly longer than in the KRAS mutant group with a p value of 0.01. The PFS in the KRAS wild group determined by 2-step PCR RFLP was even longer with greater significance in the KRAS mutant group with a p value of less than 0.001. The PFS in the KRAS wild group identified by 2-step PCR RFLP was longer than that by direct sequencing although there was no statistical significance. Figure 2c shows Kaplan-Meyer curves for the W/W, W/M and M/M groups. The median PFSs were 8.0 weeks (95% CI 6.9–9.1) for the M/M group, 11.0 weeks (95% CI 6.5–15.5) for the W/M group and 18.0 weeks (95% CI 13.5–22.5) for the W/W group. The PFS in the W/M group was almost the same as that in the M/M group, and was significantly shorter than that in the W/W group ($p = 0.002$). Thus, the detection of KRAS mutations by 2-step PCR RFLP reflected a poor prognosis regardless of the results by direct sequencing, and this method could be a significant predictor of appropriate candidates for cetuximab therapy.

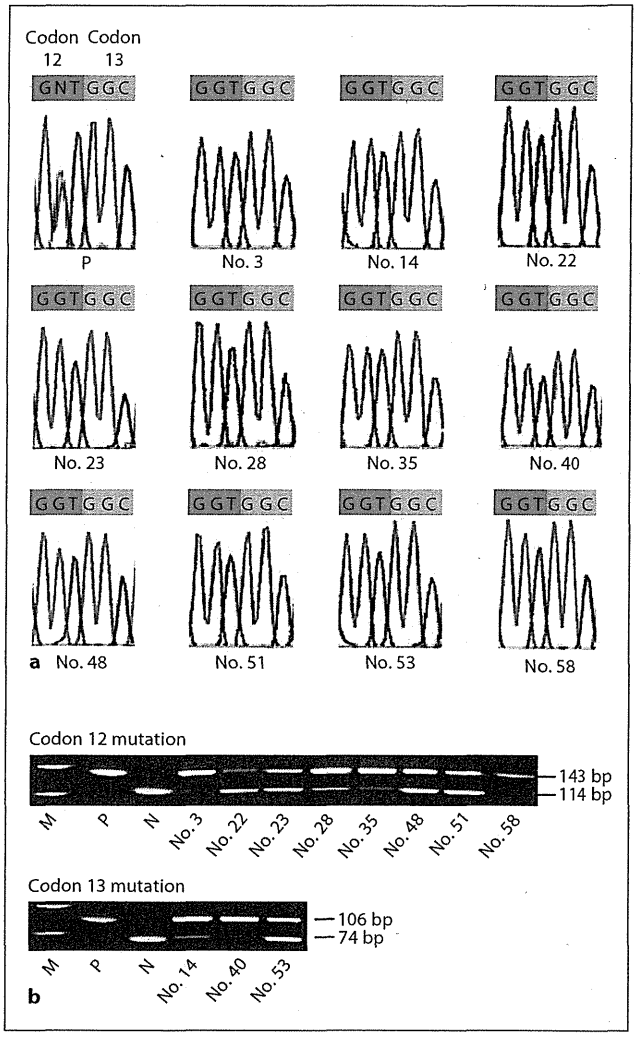

Fig. 1. Data on 11 patients with discordant KRAS mutation status between direct sequencing and 2-step PCR RFLP. **a** In the analysis by direct sequencing, no abnormal signals were found in the nucleotide sequence of codons 12 and 13 in any of the 11 patients. **b** In the analysis by 2-step PCR RFLP, however, 143-bp bands (which represent a mutation of codon 12) or 106-bp bands (which represent a mutation of codon 13) were observed in all 11 patients. ASPC-1, which is known to have a KRAS codon 12-point mutation, and HCT116, which has a KRAS codon 13 mutation, were used as positive controls. P = Positive control; M = marker; N = normal mucosa.

Discussion

In this study, the incidences of KRAS codon 12 or 13 mutations in mCRC by direct sequencing and 2-step PCR RFLP were 34.4 and 52.5%, respectively, with the latter being significantly higher than the former ($p = 0.02$). The response rate (41.4%) in patients with wild-type by 2-step PCR RFLP was higher than that by direct sequencing (30.0%), suggesting that 2-step PCR RFLP is useful for selecting true responders to cetuximab, excluding the pa-

Table 3. Correlation between KRAS status and response to cetuximab

	Number	CR	PR	SD	PD	RR	p value
Direct sequencing							
KRAS wild	40	1	11	17	11	30% (12/40)	<0.001
KRAS mutant	21	2	0	10	11	0% (0/21)	
2-step RFLP							
KRAS wild	29	1	11	12	5	41.4% (12/29)	<0.001
KRAS mutant	32	0	0	15	17	0% (0/32)	
Direct sequencing/2-step PCR RFLP							
W/W	29	1	11	12	5	41.4% (12/29)	<0.001
W/M	11	0	0	5	6	0% (0/11)	
M/M	21	0	0	10	11	0% (0/21)	

CR = Complete response; PD = progressive disease; PR = partial response; RR = response rate; SD = stable disease.

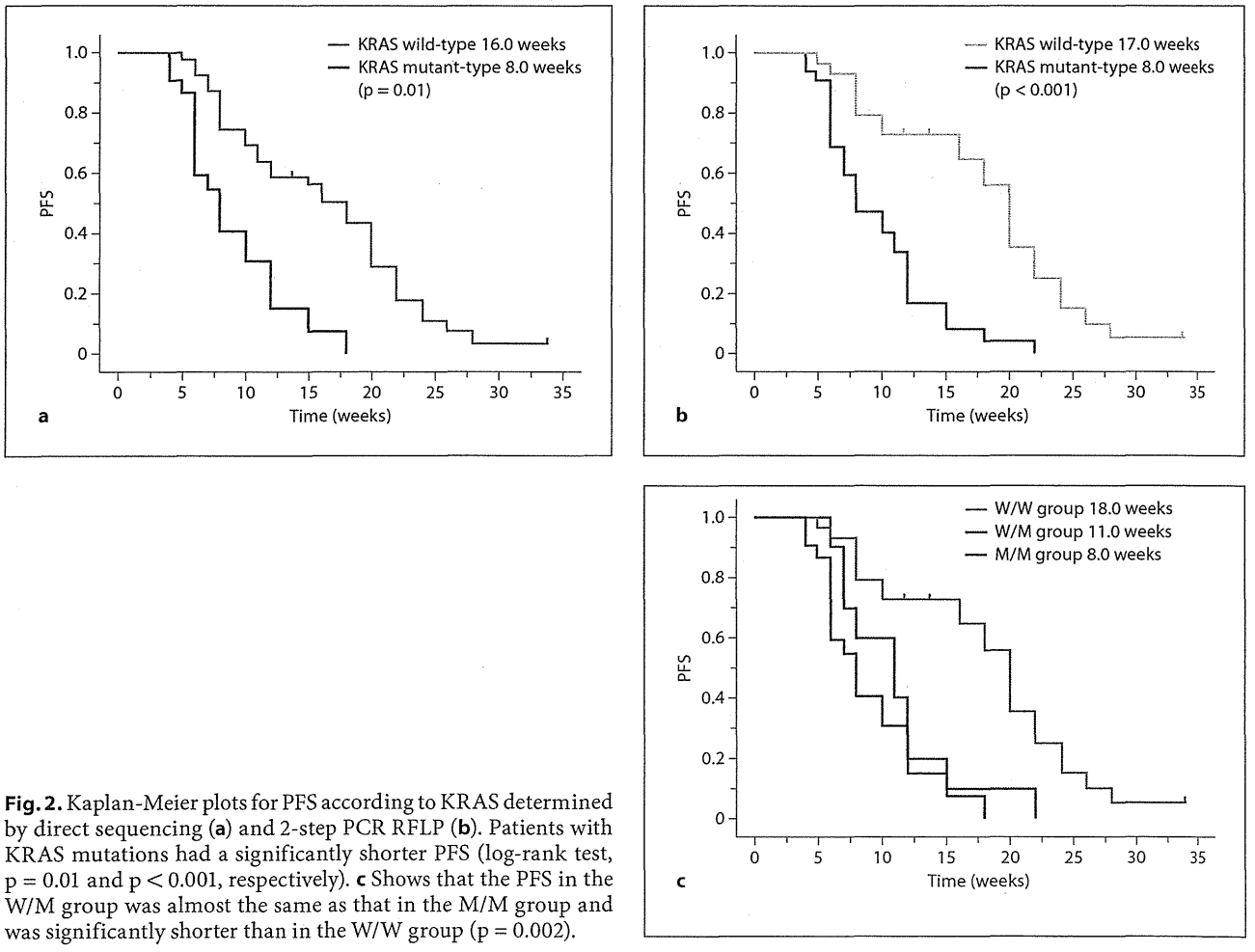

Fig. 2. Kaplan-Meier plots for PFS according to KRAS determined by direct sequencing (**a**) and 2-step PCR RFLP (**b**). Patients with KRAS mutations had a significantly shorter PFS (log-rank test, $p = 0.01$ and $p < 0.001$, respectively). **c** Shows that the PFS in the W/M group was almost the same as that in the M/M group and was significantly shorter than in the W/W group ($p = 0.002$).

tients with a false negative. Moreover, the response rate and PFS in the 11 cases in the W/M group were almost the same as those in the M/M group and were significantly lower and shorter, respectively, than those in the W/W group. These results indicated that the KRAS mutation status determined by 2-step PCR RFLP is more precisely predictive of the response to cetuximab than that by direct sequencing.

According to The Catalogue of Somatic Mutations in Cancer (COSMIC), a public mutation database [13], the incidence of a KRAS mutation in codons 12 and 13 in colorectal cancer was 31.3% (5,111/16,345) based on the 368 papers reviewed between 1992 and 2008. The mutation rate by direct sequencing in the current study was compatible with that in the COSMIC database. This is reasonable because the direct sequencing method has been employed as a gold standard in most previous studies on the KRAS mutation. However, it does not provide satisfactory sensitivity, as DNA with the KRAS mutation must be present in 20–50% of the sample. On the other hand, many sensitive methods have been reported previously. Bando et al. [14] reported that KRAS mutations detected by ARMS assay improved prediction of cetuximab efficacy. Two-step PCR RFLP, employed in this study, has the highest sensitivity and requires only 0.1% of mutated DNA at codon 12 or 13 [10, 12]. This high sensitivity is achieved by selective PCR amplification of mutant KRAS gene sequences employing a 2-step PCR in combination with restriction enzyme digestion.

The significant advantage of a high-sensitivity method might be explained by the following potential scenarios. First, a higher sensitivity method can detect fewer numbers of KRAS mutations in a sample that is contaminated with other noncancerous cells. In general, formalin-fixed paraffin-embedded specimens include normal colorectal cells, inflammatory cells such as lymphocytes, neutrophils and macrophages and other noncancerous cells such as fibroblasts. When a tissue sample is contaminated with more than a certain amount of these noncancerous cells, failure to detect the KRAS mutation in DNA from cancer cells is possible. Second, intratumoral heterogeneity of the KRAS mutation in colorectal cancer tissue is plausible. To date, several studies have reported heterogeneity of the KRAS mutation in colorectal cancer tissue [15–17]. Losi et al. [16] reported that heterogeneity of the KRAS mutation was observed within the tumor tissue in about 20% of advanced colorectal cancer cases. Therefore, unless a high-sensitivity method is employed, a KRAS mutation that only partly exists in colorectal cancer tissue may not be detectable.

Although involvement of BRAF mutations, PI3KCA mutations and PTEN loss of expression have been suggested in resistance to cetuximab [18–20], KRAS is the only target gene that is clinically used to date. In our study, it was possible to select cetuximab responders efficiently by employing a high-sensitivity method to analyze the KRAS mutation. Although macro- or micro-dissection and preparation of fresh-frozen tissue samples have been used to try to improve the sensitivity of direct sequencing, these procedures are hard to introduce to clinical practice due to high cost and inconvenience. In this respect, a high-sensitivity method using paraffin-embedded tissues provides a simple, accurate and clinically useful way for analysis of the KRAS mutation. The 2-step PCR RFLP method may take somewhat longer in comparison with the conventional direct sequencing method. However, the examination times needed are at most 3–5 days, which seems to be an acceptable range. Moreover, the method does not require any special and expensive equipment, and therefore is very economical. In view of these points, it appears that the 2-step PCR RFLP method can be easily applied to clinical use.

Analyses of the cell proliferation signaling for the KRAS wild and mutant samples could provide additional valuable information on how the mutant KRAS escapes cetuximab the therapy. Further investigations using pre- and post-therapeutic samples are warranted in the future.

In conclusion, 2-step PCR RFLP is very useful for selecting true responders to cetuximab, in comparison with direct sequencing. This highly sensitive KRAS mutation detection system should be applied in the clinical setting for efficient chemotherapy.

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J Gastroenterol
DOI 10.1007/s00535-012-0575-2

ORIGINAL ARTICLE—ALIMENTARY TRACT

The impact of narrow band imaging for colon polyp detection: a multicenter randomized controlled trial by tandem colonoscopy

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Received: 8 December 2011 / Accepted: 24 February 2012

Abstract
Background Previous studies have yielded conflicting results on the adenoma detection rate with narrow band imaging (NBI) compared with white light imaging (WLI). To overcome the confounding factors of these studies, we aimed to evaluate the colonic adenoma detection rate with primary NBI versus that with primary WLI by using consistent NBI system, endoscope, and imaging settings, and experienced colonoscopists.
Methods In this multicenter prospective trial, 813 patients were randomized to undergo high-definition, tandem colonoscopy in the right colon with either NBI followed by WLI (NBI–WLI group) or WLI followed by NBI (WLI–NBI group). The NBI settings were fixed at surface structure enhancement level A-5 and adaptive index of hemoglobin color enhancement level 3. All detected polyps were resected or biopsied for histopathological analysis. The primary and secondary outcome measures were the adenoma detection rates and miss rates, respectively, with primary imaging.
Results The NBI–WLI and WLI–NBI groups comprised 389 and 393 patients, respectively, who met the inclusion criteria. The groups did not differ significantly in age, gender, institution, indication for colonoscopy, bowel preparation, or observation time. The adenoma detection rates of primary NBI and WLI were 42.3 and 42.5 %, respectively [difference not significant (NS)]. The adenoma miss rate was significantly less with primary NBI than with primary WLI (21.3 vs. 27.8 %; $p = 0.03$).
Conclusions NBI does not improve the adenoma detection rate during primary colonoscopy; however, it has a lower miss rate for adenoma lesions in the proximal colon than WLI.

Keywords Adenoma detection rate · Colonoscopy · Screening

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Introduction

Early detection and removal of colorectal adenoma lesions by screening colonoscopy are the most effective means of colorectal cancer prevention [1–3]. The adenoma detection rate is an important quality indicator for colonoscopy; moreover, this detection rate is an independent predictor of the risk of colorectal cancer after screening colonoscopy

Published online: 24 March 2012

[4]. Colonoscopy is considered the gold standard for the detection and treatment of colorectal polyps; however, white light imaging (WLI) has an adenoma miss rate of 10–30 % during colonoscopy [5–7]. Various methods, such as pan-colonic dye-spraying [8, 9], wide-angle colonoscopy [10, 11], Third Eye Retroscope colonoscopy [12, 13], and cap-fitted colonoscopy [14] reportedly reduce the adenoma miss rate. Similarly, some researchers have indicated improvements in the adenoma detection rate by performing colonoscopy with narrow band imaging (NBI) [15–21].

NBI is an innovative imaging technology that uses narrow band width filters [22, 23]. The center wavelengths of the dedicated trichromatic optical filters are 540 and 415 nm, with bandwidths of 30 nm. NBI enables endoscopic imaging with a one-touch electrical button and without indigo carmine dye-spraying. It also helps in clearly visualizing the microvascular structure of the organ surface, because the 415-nm light is well absorbed by hemoglobin. Given that the microvascular surface of an adenoma lesion is thicker and more irregular than that of normal mucosa, surface microvascular irregularities are useful landmarks for identifying an early neoplasm in the gastrointestinal tract; such lesions appear brownish during NBI. In addition, lesion detection and diagnosis can be performed simultaneously with NBI.

Muto et al. [24] reported the efficiency of NBI for the early detection of superficial cancers in the head and neck region and the esophagus. In the colorectal region, this modality was expected to enable the early detection of adenoma lesions; however, both positive [15–17] and negative [18–21] results have been reported, and some researchers have concluded that there was no improvement in the adenoma detection rate of NBI compared with that of WLI. One reason for these conflicting findings could be a difference between the optical-electronic technologies employed in the video endoscopes in the different NBI systems used [sequential system (LUCERA; Olympus Optical, Tokyo, Japan) vs. non-sequential system (EXERA II; Olympus Optical)]. Further, differences in the endoscope (low-resolution vs. high-resolution) and imaging (surface structure enhancement and index of hemoglobin color enhancement) settings can lead to different findings in the detection of the same lesion [25, 26]. Moreover, the colonoscopist's experience may have a considerable impact on the detection rate: if the colonoscopist does not have sufficient training in the chromoendoscopy of flat and depressed lesions with an NBI system, the usefulness of NBI for adenoma detection may not be evident. Finally, we note that most of the previous studies of NBI used a single-center design.

To overcome the aforementioned confounding factors, we aimed to evaluate the colonic adenoma detection rate achieved with NBI versus that achieved with WLI by using consistent NBI system, endoscope, and imaging settings, and experienced colonoscopists.

Patients and methods

Study population

Consecutive patients who were scheduled to undergo total colonoscopy with NBI at six institutions were considered eligible for inclusion in the study. The study was performed in university settings/academic centers. Patients with a history of surgical colorectal resection or those with inflammatory bowel disease, familial adenomatous polyposis, or hereditary non-polyposis colorectal cancer were excluded.

The institutional medical ethics committees approved the study protocol, which adhered to the tenets of the Declaration of Helsinki, and all patients gave written informed consent for diagnosis and treatment before the procedures. This study was registered in the University Hospital Medical Network Clinical Trials Registry (UMIN 000002934).

This study was supported by the Ministry of Health, Labour and Welfare of Japan, and there are no conflicts of interest between the authors and this or any other organization or company.

Study design

To investigate whether a lesion detected by primary imaging could be identified subsequently by the other type of imaging, or whether a lesion missed by primary imaging could be identified subsequently by the other type of imaging, the enrolled patients were randomized to undergo tandem colonoscopy with either NBI followed by WLI (NBI–WLI group) or WLI followed by NBI (WLI–NBI group).

After the endoscopists had achieved complete colonoscope insertion into the cecum with WLI, they were informed of the patient's allocation. Patients with poor bowel preparation, those with melanosis coli, those with multiple polyps unresectable in a single endoscopic examination, and those with advanced cancer were withdrawn.

We examined only the right colon, including the cecum, ascending colon, and transverse colon, because of a previous report of positive adenoma detection with NBI in this region, and to reduce the patient's discomfort during insertion and withdrawal.

Randomization

Random assignment was performed in each case by an investigator using a computer-aided system on the Medical Research Support website (Kyoto, Japan). A minimization algorithm was used to balance the selection of the primary

examination, according to the following 4 stratification variables: institution, age (<60 and ≥60 years), gender, and indication for colonoscopy.

Endoscopic equipment and setting

All procedures were performed up to the cecum by using a high-definition colonoscope (CF-H260AZI; Olympus Optical). A video endoscope system (EVIS LUCERA SPECTRUM; Olympus Optical) was used without a magnifying system. The NBI settings were fixed at surface structure enhancement level A-5 and adaptive index of hemoglobin color enhancement level 3. Twenty-seven endoscopists, each of whom had performed more than 5000 colonoscopies and more than 500 NBI colonoscopies, participated in this study.

Endoscopic procedure

For bowel preparation, 2–3 L of polyethylene glycol solution was administered in the morning on the day before the procedure. Scopolamine butylbromide (10 mg) was administered in the absence of contraindications, and midazolam (0.03 mg/kg) and/or pethidine hydrochloride (35 mg) was used for conscious sedation only when a patient complained of discomfort or pain. An examiner assessed the quality of bowel preparation according to the extent of mucosal visualization after suction of the fluid residue, as follows: excellent (approximately 100 % mucosal visualization following suction of fluid residue); good (approximately 90 % mucosal visualization); fair (less than 90 % mucosal visualization); poor (large amounts of solid fecal matter were found) [27]. The endoscopists who participated in the study were blinded to the indication for the procedure and and to the findings of previous colonoscopy.

In the NBI–WLI group, the colonoscope was withdrawn from the cecum to the splenic flexure with NBI, reinserted into the cecum, and then withdrawn again to the splenic flexure with WLI; in the WLI–NBI group, the same steps were performed with WLI first and then with NBI. The same endoscopist performed the primary and secondary examinations for the same patient. Patients were maintained in a supine position during NBI–WLI and WLI–NBI examinations, because changing the position did not influence the detection and miss rate [28].

In the primary examination, the endoscopists diagnosed lesions using the image obtained upon the detection of the lesion. At the same time, lesions less than 20 mm in diameter that were diagnosed as adenomas were removed endoscopically, and all lesions that were diagnosed as hyperplastic polyps were biopsied. All endoscopic treatments were performed using WLI. The same procedure was followed for the secondary examination. Adenoma lesions more than 20 mm in diameter were observed with both NBI and WLI and were removed by endoscopic mucosal resection or endoscopic submucosal dissection on another day in the hospital.

We did not use chromoendoscopy during the NBI or WLI because it elevates the adenoma detection rate; however, when observation with chromoendoscopy was diagnostically required, it was performed after the secondary examination.

In the primary examination, all lesions diagnosed as adenomas were removed by hot biopsy, snare polypectomy, endoscopic mucosal resection on the same day, or endoscopic submucosal dissection on another day, and all lesions diagnosed as hyperplastic polyps were biopsied. The location of each lesion was defined according to landmarks such as the hepatic flexure and splenic flexure. The lesion size was estimated by using open endoscopic biopsy forceps and/or a snare. Macroscopically, the lesions were classified according to the Paris classification of superficial gastrointestinal lesions [29]. We measured the total observation time, excluding mucosal washing, the diagnostic time, and the therapeutic time using a stopwatch. A doctor who was not the examiner, or a nurse, operated the stopwatch.

Histologic examination

All resected and biopsy specimens were retrieved, immediately fixed in 10 % buffered formalin solution, and examined histologically by hematoxylin and eosin staining. Experienced gastrointestinal pathologists blinded to the endoscopic diagnosis determined the histopathological diagnosis according to the World Health Organization (WHO) criteria [30]. Only traditional serrated adenoma (TSA) was included in the category of serrated adenoma.

Statistical analysis

The primary outcome measure was the detection rate of non-advanced adenoma lesions [adenoma with low-grade dysplasia (LGD)] and advanced adenoma lesions [adenoma of ≥10 mm or with villous histology in 25 % of polyps or with high-grade dysplasia (HGD) and submucosal invasive cancer] in the primary examination. Assuming an adenoma detection rate of 61 % in the right colon with WLI, from the pilot study at the National Cancer Center Hospital East and an increase of 16 % in the detection rate with NBI [17], the necessary sample number was calculated to be 369 patients in each group, 738 patients in total. Hence, 400 patients were required in each group for the probability of an α error to be 0.05 with a power of 0.80 (reflecting a β error of 0.2). The secondary outcome measure was the

adenoma miss rate in the primary examination; we defined a missed adenoma lesion as one detected only during the secondary examination.

Nominal and ordinal variables are expressed as frequencies and percentages. Continuous variables are expressed as means and standard deviations (age, adenoma lesions per patient) or medians and ranges (withdrawal time). Continuous data were compared by using the Mann–Whitney *U*-test. Pearson's χ^2test or Fisher's exact test was used to analyze categorical data and compare proportions. SPSS version 11 (SPSS, Chicago, IL, USA) was used for the statistical analyses. All statistical tests were two-tailed and significance was defined as $p < 0.05$.

Results

Group characteristics

Between October 2008 and March 2010, 813 patients were enrolled in this study. Of the 813 enrolled patients, 406 and 407 patients were randomly assigned to the NBI–WLI and WLI–NBI groups, respectively (Fig. 1). Three patients were withdrawn just before the primary examination, because of refusal to participate in the study ($n = 1$) and cardiac arrhythmia ($n = 2$). The colonoscope reached the cecum in 809 (99.9 %) of the remaining 810 study patients. Then 27 patients were withdrawn because of poor bowel preparation ($n = 8$), melanosis coli ($n = 6$), multiple polyps unresectable in a single endoscopic examination ($n = 5$), advanced cancer ($n = 4$), duplicated registration ($n = 1$), and other factors ($n = 3$). Finally, we analyzed 389 and 393 patients in the NBI–WLI and WLI–NBI groups, respectively.

The characteristics of the groups are listed in Table 1. The 782 analyzed patients included 553 (70 %) men, and the mean patient age was 63.2 ± 10.1 years. The indications for colonoscopy were polyp surveillance ($n = 553$), screening ($n = 183$), any abdominal symptoms ($n = 45$).and family history of colorectal cancer ($n = 1$). The bowel preparation was described as excellent, good, and fair in 246, 439, and 97 patients, respectively. The groups did not differ significantly in gender, age, indication for colonoscopy, bowel preparation, or institution. No complications occurred with the endoscopic treatment.

Table 2 shows the total observation times of the examinations. The observation times did not differ significantly between the groups.

Detection rates

The numbers of patients with lesions detected by primary NBI and WLI, including adenoma and hyperplastic polyp lesions, were 191 and 187, respectively (Table 3). The detection rate of adenoma lesions did not differ significantly between primary NBI and primary WLI (42.4 vs. 42.5 %). When we compared the detection rates of primary NBI and WLI by adenoma characteristics, the percentages of patients were not significantly different in terms of the number of lesions, non-advanced or advanced adenoma, and polypoid or flat and depressed adenoma.

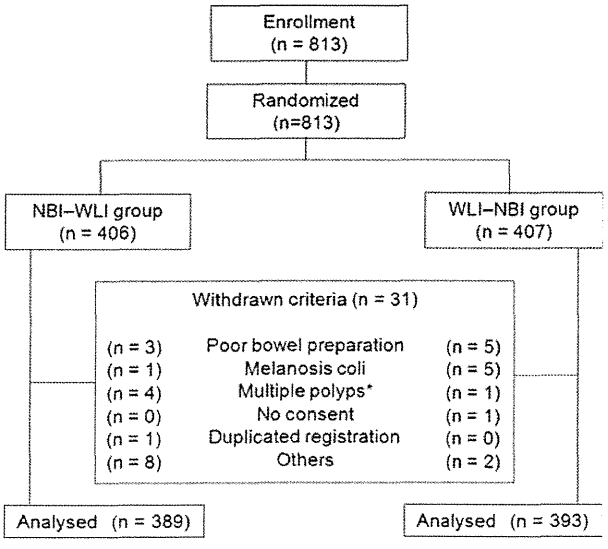

Fig. 1 CONSORT diagram. Overview of the study design. *Multiple polyps* (*asterisk*) many polyps unresectable in a single endoscopic examination, *NBI* narrow band imaging, *WLI* white light imaging

Table 1 Patient characteristics

Characteristic	NBI–WLI group ($n = 389$)	WLI–NBI group ($n = 393$)	p
Male gender	267 (69)	277 (70)	0.57
Mean (SD) age (years)	63.2 (10.2)	63.3 (9.9)	0.58
Indication for colonoscopy			0.67
Polyp surveillance	280	273	
Screening	88	95	
Any abdominal symptom	21	24	
Family history of CRC	0	1	
Bowel preparation			0.25
Excellent	115	131	
Good	219	220	
Fair	55	42	

Data represent the number of patients (%) unless indicated otherwise

NBI narrow band imaging, *WLI* white light imaging, *CRC* colorectal cancer

Table 2 Observation time

Time (s)	NBI–WLI group			WLI–NBI group			Primary imaging			Total		
	NBI	WLI	*p*	WLI	NBI	*p*	NBI	WLI	*p*	NBI	WLI	*p*
Median	210	164	0.67	180	180	0.98	210	180	0.76	190	180	0.78
Range	59–1112	52–1230		60–1200	20–1200		59–1112	60–1200		20–1200	52–1230	

NBI narrow band imaging, *WLI* white light imaging

Table 3 Detection rates of primary NBI and WLI

	Primary NBI ($n = 389$)	Primary WLI ($n = 393$)	*p*
Patients with any lesion	191 (49.1)	187 (47.6)	0.67
Patients with adenoma lesions	165 (42.4)	167 (42.5)	0.98
Mean (SD) no. of lesions per patient	0.79 (1.23)	0.79 (1.27)	0.98
Patients with 1–2 lesions	135 (34.7)	133 (33.8)	0.88
Patients with ≥3 lesions	30 (7.7)	34 (8.7)	
Patients with non-advanced adenoma[a]	106 (27.2)	112 (18.5)	0.59
Patients with advanced adenoma[a]	59 (15.2)	55 (14.0)	
Patients with polypoid adenoma only	30 (7.2)	45 (11.5)	0.06
Patients with flat and depressed adenoma	135 (34.1)	122 (31.0)	

Data represent the number of patients (%) unless indicated otherwise

[a] Advanced adenoma: adenomas ≥10 mm or with villous histology in 25 % of the polyps or with high-grade dysplasia or invasive cancer

Characteristics of the detected adenoma lesions

The total numbers of adenoma lesions detected by primary NBI and WLI were 306 and 310, respectively (Table 4), and those identified by secondary WLI and NBI were 83 and 119, respectively. The adenoma miss rates of primary NBI and WLI were significantly different (21.3 vs. 27.8 %; $p = 0.03$). In terms of location, there was no significant difference in the detection rate. Morphologically, polypoid lesions were detected significantly more often by primary NBI ($p = 0.006$). Further, 4-mm or smaller lesions ($p = 0.04$) and LGD ($p = 0.04$) were detected significantly more often by primary NBI. There was no significant difference in the detection rate for advanced adenoma. Figure 2 shows representative images of polyps detected by one imaging technique and missed by the other.

Discussion

The present study was the first randomized tandem colonoscopy trial in a multicenter setting for comparing the adenoma detection and miss rates of NBI and WLI. The results did not show any objective advantage of NBI over WLI in terms of improved detection of adenoma lesions in primary colonoscopy; however, NBI had a lower adenoma miss rate in the proximal colon than WLI by tandem colonoscopy.

The results of previous randomized trials comparing the adenoma detection rate of colonoscopy with NBI against that of colonoscopy without NBI are controversial. For example, Uraoka et al. [17] reported that the total number of adenoma lesions detected by colonoscopy with NBI was significantly higher than that detected by high-definition colonoscopy alone ($p = 0.02$) and adenomatous lesions in the right colon were identified more often by NBI ($p = 0.02$). Similarly, Inoue et al. [16] noted a significantly higher number of patients with detected diminutive (<5 mm) adenomas ($p = 0.011$) and lesions in the distal colon ($p = 0.02$) in their NBI group than in their control group. On the other hand, Rex and Helbig [18] reported no significant difference in the percentage of patients with adenomas detected by WLI versus NBI ($p = 0.68$). Further, Adler et al. [21] reported no significant difference between their NBI and their control groups in terms of the general adenoma detection rate (0.32 vs. 0.34 %). We attribute these varied results to differences in factors such as the NBI systems, endoscope and imaging settings, and the learning curves among the studies.

Differences in the NBI systems can be explained by differences between the optical-electronic technologies employed in video endoscopes in the previous studies: a sequential system (LUCERA) was used in the studies conducted in Japan and the United Kingdom, whereas a non-sequential system (EXERA II) was used in the other Western studies. Though we used only the LUCERA system in the present study, the present study was also a negative study of the rate of adenoma detection. Hence, we consider that the video endoscope system alone is not a reason for the negative study in the adenoma detection rate of NBI.

Darkness and noise of the viewing screen cause problems in NBI without high-definition colonoscopy, and

Table 4 Clinicopathologic characteristics of adenoma lesions detected during primary and secondary examinations

Characteristic	Primary		Secondary		*p*
	NBI	WLI	WLI	NBI	
Adenoma lesions	306 (78.7)	310 (72.2)	83 (21.3)	119 (27.8)	0.03
Location					
Cecum	57 (90.5)	49 (89.1)	6 (9.5)	6 (10.9)	0.80
Ascending colon	120 (78.9)	129 (71.7)	32 (21.1)	51 (28.3)	0.13
Transverse colon	129 (74.1)	132 (68.0)	45 (25.9)	62 (32.0)	0.20
Morphology					
Polypoid	136 (89.5)	139 (78.1)	16 (10.5)	39 (21.9)	0.006
Ip	8 (88.9)	12 (100)	1 (11.1)	0	0.24
Isp	13 (100)	15 (83.3)	0	3 (16.7)	0.12
Is	115 (88.5)	112 (75.7)	15 (11.5)	36 (24.3)	0.006
Flat and depressed	170 (72.0)	171 (68.6)	67 (28.0)	80 (31.4)	0.42
IIa	167 (71.4)	167 (68.2)	67 (28.6)	78 (31.8)	0.45
IIa + IIc	3 (100)	3 (75.0)	0	1 (25.0)	0.35
IIc	0	1 (50.0)	0	1 (50.0)	-
Size (mm)					
1–4	148 (74.4)	154 (65.3)	51 (25.6)	82 (34.7)	0.04
5–9	108 (79.4)	109 (76.8)	28 (20.6)	33 (23.2)	0.59
≥10	50 (92.6)	47 (92.2)	4 (7.4)	4 (7.8)	0.93
Histopathological findings					
TSA	7 (77.8)	12 (85.7)	2 (22.2)	2 (14.3)	0.62
LGD	277 (77.7)	283 (70.9)	80 (22.3)	116 (29.1)	0.04
LGD with villous	2 (100)	0	0	0	-
HGD	12 (93.3)	7 (92.9)	1 (6.7)	1 (7.1)	0.72
HGD with villous	2 (100)	6 (100)	0	0	-
Invasive cancer	6 (100)	2 (100)	0	0	-

Data represent the number of lesions (%)

NBI narrow band imaging, *WLI* white light imaging, *TSA* traditional serrated adenoma, *LGD* adenoma with low-grade dysplasia, *HGD* adenoma with high-grade dysplasia

these problems are usually solved by using high-definition colonoscopy. In addition, the wider colorectal lumen than the esophageal lumen in NBI is considered to be a reason for the screen darkness. An NBI setting different from that used in esophageal observation by high-definition colonoscopy is, therefore, indispensable for polyp detection in the colon and rectum. Uraoka et al. [25, 26] have reported that the A-5 image setting of the surface structure enhancement function, together with the level 3 adaptive index of hemoglobin color enhancement function, seem to be the most suitable settings for the detection of colorectal adenomas. In accordance with these findings, we used only high-definition colonoscopy to compare WLI with NBI and we applied surface structure enhancement level A-5 and adaptive index of hemoglobin color enhancement level 3 for NBI.

Our study results did not show a significant difference between NBI and WLI in the primary outcome measure but results were significantly different in the secondary outcome measure. Specifically, we found no significant difference in the adenoma detection rates by primary NBI versus primary WLI. We consider these results reliable because there was no significant difference in the bowel preparation results or total observation times between the groups. Only expert colonoscopists performed the procedures in this study; therefore, it is necessary to clarify the usefulness of NBI for adenoma detection by all colonoscopists, including novices, in the future. Further, the detection rates of adenoma lesions by primary NBI and WLI were 78.7 and 72.2 % when we considered the detection rate of adenoma lesions by both primary and secondary examinations to be 100 %. In other words, the adenoma miss rates by primary NBI and WLI were 21.3 and 27.8 % ($p = 0.03$). The higher miss rate of WLI is similar to that reported previously. [5–7] Furthermore, Kaltenbach et al. [20] reported that NBI did not improve the colorectal adenoma miss rate compared to WLI in a randomized controlled trial using tandem colonoscopy (NBI–WLI vs. WLI–WLI; 12.6 vs. 12.1 %, respectively). Their adenoma miss rate using WLI was lower than the adenoma miss rate of 10–30 % reported in other studies. However, when we compared our results with their findings, it was evident that our miss rate was high. The differences between the study of Kaltenbach et al. and our

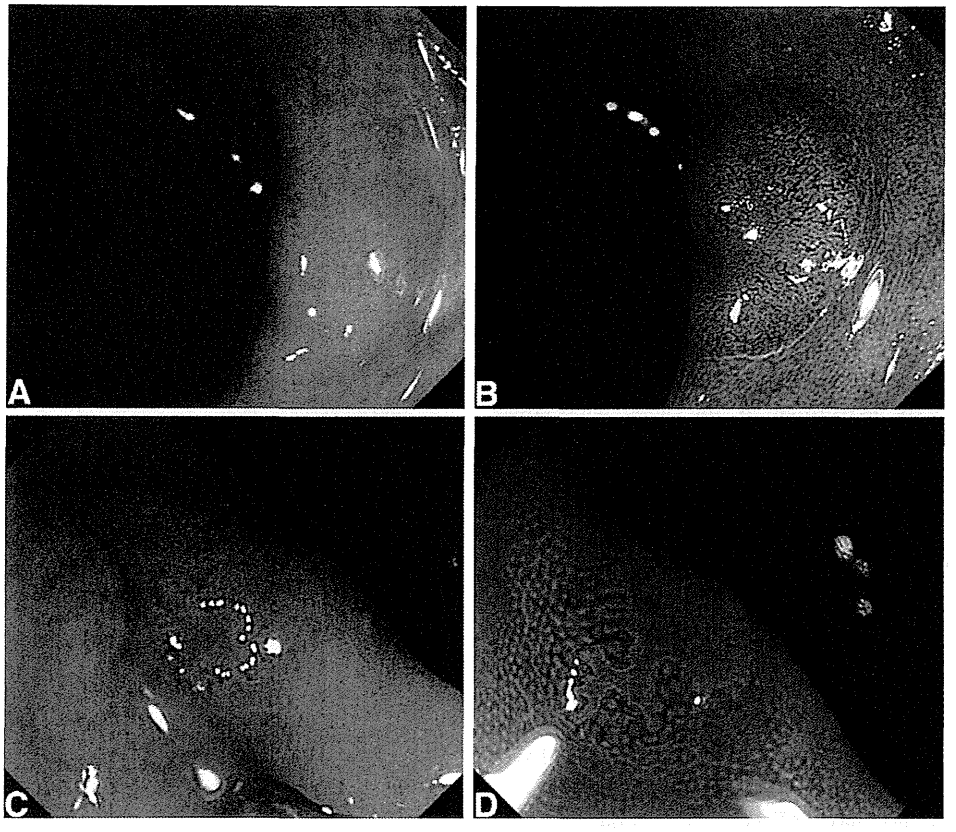

Fig. 2 A flat elevated lesion was detected in the transverse colon. The size of the lesion was 7 mm in diameter. The final histopathological diagnosis was adenoma with low-grade dysplasia (LGD). **a** The polyp was missed by WLI; **b** the same lesion was identified by subsequent NBI. **c, d** A depressed lesion was detected in the transverse colon. The size of the lesion was 5 mm in diameter. The final histopathological diagnosis was LGD. **c** The polyp was missed by WLI; **d** the same lesion was identified by subsequent NBI

study are the use of LUCERA versus EXERA II and the single-center versus multicenter design. Furthermore, we believe that a difference in the resolution of NBI and WLI may have influenced the results, because we were able to detect small lesions with NBI.

In line with previous reports [16, 17], we found that significantly higher numbers of small lesions (<5 mm) and/or LGD lesions were detected by NBI than by WLI16, 17. Further, nearly all the adenoma lesions we detected were flat elevated or polypoid in shape, and two were depressed. Depressed lesions are considered to have a higher malignant potential than polypoid ones of similar size [31–34]. The superiority of NBI over WLI in the detection of depressed lesions was not proven in the present study; however, we believe NBI is a promising modality for detecting small neoplastic lesions. The advantage of NBI endoscopy is simply to get the NBI view when we use a one-touch electrical button and to avoid indigo carmine dye-spraying. In addition, we can diagnose a lesion at the same time as it is detected. In the colorectal region, NBI is useful for differentiating non-neoplastic from neoplastic lesions, and magnifying NBI is effective for determining the depth of invasion in early neoplasms [35–37].

In the present study, we could not evaluate serrated lesions because the pathological diagnosis of serrated lesions (particularly, sessile serrated adenoma) is not yet unified among Japanese pathologists. The number of TSAs detected by primary NBI and WLI were 7 and 12, respectively, and those identified by secondary WLI and NBI were 2 and 2, respectively. The miss rates of primary NBI and WLI for these lesions were not significantly different (22.2 vs. 14.3 %; $p = 0.62$).

This study has several limitations. First, the procedures were conducted only in the right colon, because Uraoka et al. [17] reported higher adenoma detection rates with NBI in the right colon, and a higher adenoma miss rate has been reported in the right colon than in the left colon [5]. Further, because complete back-to-back colonoscopy is sometimes uncomfortable for patients without sedation, we defined the region from the cecum to the splenic flexure as the target area in our study. Another limitation is that WLI was used for colonoscope insertion in both the study groups, which could have

influenced the NBI results if some lesions were identified during insertion. However, we used the same imaging condition and study design for tandem colonoscopy in both the groups. Moreover, the detected lesions were removed endoscopically using WLI, because of the darkness of the screen with NBI. We cannot entirely exclude the possibility that some switches of endoscopic treatment influenced the detection rates in both the groups. Endoscopic treatment is, however, usually focused on the small area in which the polyp is located and the examiner likely concentrates on the endoscopic treatment rather than on the detection of additional lesions. Another limitation is that both the NBI and WLI examinations were performed by the same endoscopist. There may be investigator bias. However, we believe this does not substantially influence the results, because this was a multicenter trial and the endoscopists performed procedures for both the NBI–WLI and WLI–NBI groups. Another limitation is that, in the distinction between neoplasia and non-neoplasia, NBI may be expected to have a small advantage. In the present study, expert colonoscopists examined the lesions; such experts are able to distinguish between neoplasia and non-neoplasia using WLI as well as NBI. Furthermore, because all detected non-neoplastic lesions were removed or biopsied, the difference between NBI and WLI with respect to the distinction between neoplasia and non-neoplasia would not have affected the overall adenoma detection rate. Therefore, we believe that the primary endpoint of the adenoma detection rate was not affected by this discrepancy. Finally, the current NBI systems have problems such as darkness and noise. Even if these systems are used with high-definition colonoscopy, the brightness of the screen is still not sufficient. Further, NBI system-related improvement is necessary for enhanced adenoma detection.

In conclusion, NBI does not have a higher adenoma detection rate during primary colonoscopy than WLI, but it has a lower adenoma miss rate in the proximal colon by tandem colonoscopy. NBI can be expected to represent a suitable modality for screening colonoscopy, because the miss rate is low.

Acknowledgments This paper was presented at the 18th United European Gastroenterology Week (UEGW), October 23–27, 2010, in Barcelona, Spain. This study was supported in part by Grant No. H21-009 from the Ministry of Health, Labour and Welfare of Japan.

Conflict of interest The authors declare that they have no conflict of interest.

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Digestive Endoscopy (2012) **24**, 419–425 doi:10.1111/j.1443-1661.2012.01314.x

ORIGINAL ARTICLE

Nature of white opaque substance in gastric epithelial neoplasia as visualized by magnifying endoscopy with narrow-band imaging

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Background and Aims: Magnifying endoscopy (ME) with narrow-band imaging (NBI) revealed a white opaque substance (WOS) within the superficial part of the gastric neoplasia; however, its nature has remained obscure. A WOS noted within the duodenum was reported to comprise lipid droplets (LD) absorbed by the duodenal epithelium. We attempted to ascertain whether the WOS within gastric neoplasia could also comprise LD and whether the presence of this WOS could be correlated with a specific phenotype.

Methods: Forty-three patients with early gastric epithelial neoplasia underwent ME with NBI. The presence or absence of WOS in the neoplasias was recorded based on the findings of ME with NBI. One biopsy specimen was taken from each of the neoplasias. Cryostat sections underwent oil red O staining for LD. Serial sections were immunostained using the first antibody of CD10, MUC2, CDX2, human gastric mucin, MUC5AC and MUC6. The tissue phenotype was classified as intestinal (I), gastric (G) and gastrointestinal (GI) type based on the results of immunostaining. In total, 49 gastric neoplasias from 43 patients were investigated.

Results: Prevalence of LD in WOS-positive versus WOS-negative lesions was 96.2% (25/26) and 4.3% (1/23), respectively ($P < 0.001$, Fisher's exact test). WOS was present in GI- and I-type lesions, but not in G-type lesions.

Conclusions: WOS may be LD that have been accumulated in the superficial part of the gastric neoplasia of a certain intestinal phenotype.

Key words: fat droplet, gastric cancer, gastric epithelial neoplasia, lipid droplet, white opaque substance.

INTRODUCTION

The subepithelial microvascular architecture as visualized by high-resolution magnifying endoscopy (ME) is a reliable marker for making a precise diagnosis of gastrointestinal pathology.[1–4] When we incorporate narrow-band imaging (NBI)[5] with ME, we can clearly visualize both the subepithelial microvascular architecture and the microsurface structure.[6,7] However, we sometimes encounter difficulties when attempting to observe the subepithelial microvascular architecture in neoplastic lesions within the stomach, even by ME with NBI, because a white opaque substance (WOS) within the superficial part of the early gastric neoplasia obscures the microvessels that are just beneath the neoplastic epithelium.[8–11] We have already reported that the morphology of the WOS as visualized by ME is a new optical marker for differentiating between low-grade dysplasia and high-grade dysplasia/early carcinoma, making it an alternative to microvascular architecture.[8–11] However, the precise nature of this WOS is still unknown.

It has been reported that the endoscopic findings of a whitish duodenal non-neoplastic mucosa in both normal subjects[12] and patients with chylomicron retention disease[13,14] is due to lipid droplets (LD) that have substantially accumulated within the enterocytes. In addition, it has been reported that a WOS is also present in sporadic epithelial neoplasias within the duodenum and that the nature of this WOS could be LD that have accumulated within the duodenal neoplastic epithelium.[15] Accordingly, we assumed that the WOS within the gastric neoplasia could also be made up of LD, as is the case within the duodenum, if the gastric neoplasia acquired a certain phenotype similar to the small intestine. We thus attempted to ascertain first whether the WOS is made up of LD accumulated in gastric neoplasia and second whether the presence of this WOS could be correlated with a specific gastric neoplasia phenotype.

METHODS

Patients and endoscopic procedures

A total of 185 patients were referred to our endoscopic department for further endoscopic examination of known

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Meeting presentation: Part of this study was presented at the 18th United European Gastroenterology Week (*Endoscopy* 2010; **42**: A373) and at DDW 2011 (*Gastrointest. Endosc.* 2011; **4S**: AB229).

Received 23 December 2011; accepted 1 March 2012.

early gastric epithelial neoplasia between December 2009 and November 2010. Of these, we included consecutive patients who fulfilled the following inclusion criteria: (i) patients who gave written informed consent; (ii) patients who were assigned to the endoscopy lists of two experienced endoscopists, KY and TN; and (iii) patients who underwent ME with NBI. Patients who were receiving warfarin or any other anticoagulant treatment and patients who did not give informed consent were excluded from this study. Neoplasia of less than 10 mm in size was excluded in order to ensure that an adequate biopsy sample could be obtained from the neoplasia. This study was approved by the Medical Ethics Committee of Fukuoka University Chikushi Hospital.

Written informed consent was obtained from 43 patients. After fasting for 12 h before the endoscopic examination, they underwent ME with NBI which was carried out by two experienced endoscopists (KY and TN) who are familiar with this procedure and who understand the findings of ME with NBI, using a high-resolution magnifying upper GI endoscope (GIF-Q240Z; Olympus, Tokyo, Japan) or a high-definition magnifying upper GI endoscope (GIF-H260Z; Olympus), and an electronic endoscopy system (EVIS LUCERA SPECTRUM; Olympus), as described previously.[9]

When an epithelial neoplastic lesion was found during non-magnifying observation with white light imaging, visualization of the lesion was immediately zoomed up to maximal magnification with NBI. The presence or absence of a WOS was recorded for each of the neoplasias based on the findings of ME with NBI.

One targeted biopsy specimen was taken from each of the neoplasias and all the specimens were embedded in Tissue-Tek OCT compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and immediately frozen in liquid nitrogen-cooled isopentane (Sigma-Aldrich, Dorset, UK). Serial cryostat sections (5-μm thick) were cut at −20°C and the sections were mounted on glass slides for oil red O staining. The remainder of each specimen was fixed with 20% formalin overnight and embedded in paraffin. Seven serial sections (5-μm thick) were cut. One was stained with hematoxylin and eosin (HE) for standard histological investigations and the others were prepared for immunohistochemistry.

Oil red O staining

In order to investigate lipid accumulation in the gastric neoplasias, LD were observed using the oil red O staining method as described elsewhere.[16] First, the dried frozen sections were fixed with 20% formalin for 15 min and then the sections were washed with running tap water for 5 min. After the sections were rinsed with 60% isopropanol, they were stained with freshly prepared oil red O working solution for 15 min. Then the sections were again rinsed with 60% isopropanol. Counterstaining with Mayer's hematoxylin was carried out for 5 min and then the sections were rinsed with distilled water. Finally, they were mounted in glycergel mounting medium (Dako North America Inc., Carpinteria, CA, USA).

We prepared the reagent according to the following method.

1. Oil red O stock stain:

Dye (0.5 g oil red O (color index: 26125)) was dissolved in 100 mL isopropanol, using the very gentle heat of a water bath.

2. Oil red O working solution:

For use, the stock stain was diluted in 20 mL distilled water and was allowed to stand for 10 min. After the diluted solution was filtered into a Coplin staining jar, it was covered immediately. In each instance, the working solution was freshly prepared according to the above procedure, as the stain can easily become aggregated.

Immunohistochemistry and classification of phenotypes

After deparaffinization and rehydration, the sections were incubated with Super Block (ScyTek, Logan, UT, USA). To classify phenotypic expression, the presence of CD10, MUC2, CDX2, human gastric mucin (HGM), MUC5AC or MUC6 was investigated by immunohistochemical methods, according to previous studies.[17,18] We used monoclonal antibodies as the first antibodies as follows: CD10 (Leica Biosystems, Newcastle, UK), MUC2 (Leica Biosystems), CDX2 (BioGenex Laboratories, San Ramon, CA, USA), HGM (Leica Biosystems), MUC5AC (Leica Biosystems), and MUC6 (Leica Biosystems). Immunostaining was carried out using the labeled streptavidin biotin method that consists of a secondary antibody of biotinylated horse antimouse IgG (H + L) (Vector Laboratories, Burlingame, CA, USA) and alkaline phosphatase-conjugated avidin (Vector Laboratories).[19] When more than 5% of the neoplastic cells in the neoplastic areas were stained, it was classified as positive expression. When fewer than 5% of the neoplastic cells in the neoplastic areas were stained, it was classified as negative expression.

Histopathological assessment

Standard histological diagnosis was made by a single experienced pathologist (AI) who was blinded to the endoscopic findings. Diagnosis was based on either biopsied specimens or resected specimens according to the revised Vienna classification.[20]

Histological investigation of LD and the phenotypic classification of neoplasia were made by an experienced pathologist (HT) who was blinded to the endoscopic findings as follows. By histological investigation of the section stained by oil red O at 100× magnification rate, the presence or absence of LD was determined. If LD were evident, the histological distribution of the droplets was further recorded according to the localization of the droplets; that is: (i) they were present at a relatively elevated apical part between the crypts, at a cryptal part or at both an apical part and a cryptal part; and (ii) they showed intraepithelial, subepithelial or both intraepithelial and subepithelial distribution. The neoplastic phenotype was classified as intestinal (I) type if the neoplastic epithelium was positive for either CD10, MUC2 or CDX2; gastric (G) type if it was positive for either HGM, MUC5AC or MUC6; and gastrointestinal (GI) type if it showed both gastric and intestinal phenotypes.[17,18]

Statistical analysis

Comparison of the prevalence between the two groups was made by chi-squared test or Fisher's exact test. Statistical significance was taken as a P-value <0.05. SPSS 10.5J for Windows was used for statistical processing.

RESULTS

A total of 49 gastric epithelial neoplasias from 43 patients were included in this study. The average age (range) of the patients was 71 years (48–85 years). The male : female ratio was 30:13. Regarding the macroscopic findings of neoplasias according to the Paris classification,[21] the number of protruded (0-I), superficial-elevated (0-IIa), superficial-flat (0-IIb), and superficial-depressed (0-IIc) types was 1, 30, 3 and 15, respectively. When we divided the stomach into upper third (U), middle third (M) and lower third (L), four, 29 and 16 lesions were located in the U, M and L parts, respectively. Eleven neoplasias were followed up after a biopsy was taken. Twenty-seven lesions were completely resected by endoscopic submucosal dissection (ESD). The remaining 11 lesions were treated by surgical resection because the lesions were preoperatively diagnosed as carcinomas that had invaded the submucosa. The final histological diagnosis according to the revised Vienna classification was 19 low-grade neoplasias (Category 3), 13 high-grade neoplasias (Category 4) and 17 invasive carcinomas (Category 5).

Regarding the prevalence of the WOS based on the findings of ME with NBI, 26 (53.1%) of the 49 neoplasias demonstrated a WOS in the superficial part of the neoplasias. However, 23 (46.9%) of the 49 neoplasias did not show any WOS. Instead, the subepithelial microvascular architecture was clearly visualized in all of these 23 neoplasias.

The presence of the WOS as visualized by ME with NBI was dependent upon the presence of LD as histologically visualized by oil red O staining (Table 1, Figs 1–3). With regard to the 26 neoplasias that showed a WOS, 25 (96.2%) of 26 lesions were positive for LD, with only one (3.8%) lesion showing no LD. In contrast, with regard to the 23 neoplasias that did not demonstrate a WOS, only one (4.3%) of the 23 lesions was positive for LD, whereas 22 (95.7%) lesions did not show any LD. This strong correlation between the presence of a WOS and the presence of LD was statistically significant ($P < 0.001$, Fisher's exact test).

When we investigated the histological localization of LD for the 26 lesions that showed LD, the LD were not distributed within the crypt epithelium, but rather were found only at a relatively apical part between the crypts (Fig. 4). With regard to the vertical distribution of LD, in 10 (36.5%) of the 26 lesions, they were located within the surface epithelial cells (intraepithelial distribution) (Fig. 3) and in 16 (61.5%) of the lesions, they were located both in the epithelial cells (intraepithelial distribution) and in the superficial part of the lamina propria just beneath the surface epithelium (subepithelial distribution) (Table 2, Fig. 2).

Table 1. Histological prevalence of LD by oil red O staining according to the presence of WOS by ME with NBI

		WOS			
		Positive ($n = 26$)		Negative ($n = 23$)	
LD	Positive	25	(96.2%)	1	(4.3%)
	Negative	1	(3.8%)	22	(95.7%)

ME, magnifying endoscopy; LD, lipid droplets; NBI, narrow-band imaging; WOS, white opaque substance.

According to the results obtained by immunohistochemistry, 11, 17 and 21 of the 49 lesions were classified as G, GI and I type, respectively. The WOS was only present in either GI or I type; in contrast, the WOS was absent in G type (Table 3). The prevalence of the WOS was more evident in GI and I type than in G type ($P < 0.001$, Fisher's exact test).

DISCUSSION

In the present study, we demonstrated a strong correlation between the WOS as visualized by ME with NBI and lipid micro-droplets accumulated in the superficial (intraepithelial and subepithelial) part of gastric epithelial neoplasias (adenoma and cancer).

The reason why accumulated LD contribute to visualization of WOS was elucidated in bio-optical studies.[22,23] As LD have a higher reflective index than those of intracellular organelles and organic components of tissue,[22,23] and LD are categorized as Mie's scattering particles,[23] it follows that projected light is strongly scattered and reflected by LD. Accordingly, when LD accumulated in the epithelial and the subepithelial part of the mucosa strongly scatter and reflect projected light, the projected light cannot reach hemoglobin in microvessels located underneath the epithelium. Therefore, the opacity is increased. In addition, such strong backward scattering and reflection of light is recognized as white coloration by the human eye. Accordingly, accumulated LD may be the cause of WOS. In other words, this is the nature of the WOS that we previously reported in our clinical observation.[8–10] Prior to this study, this phenomenon had not been reported in gastric neoplasias.

The mechanism of accumulation of LD in the epithelium and subepithelium is still unknown. There are two possible mechanisms, one being that the LD are derived from external lipid absorbed by the epithelial surface (absorption hypothesis). The other is that neoplastic cells themselves synthesize LD from glucose or lipid supplied by capillaries (production hypothesis).[24] We are uncertain which hypothesis is correct.

In the case of intestinal epithelium and intestinal metaplasia, accumulated lipids are thought to be derived from digested micellar lipid.[25–28] In order to absorb lipid, lipolysis and micellar formation are essential. Up to 20% of dietary triglycerides can be hydrolyzed within the normal stomach.[26] Furthermore, bile and pancreatic juice that has been regurgitated into the atrophic stomach, whose luminal content is not acid, may have been able to hydrolyze triglycerides within the lumen and even to form micelles. If the amount of lipid accumulation increased after we loaded micellar lipids on the surface of the neoplasias, it would suggest that LD are resynthesized from external digested lipid (fatty acids and mono- or diglycerides). In fact, according to our preliminary observations, when we loaded patients with micellar lipid, WOS positivity and density increased (Yao K., unpubl. data, 2011). Accordingly, we speculate that resynthesis of triglycerides from absorbed external lipids may at least be one of the mechanisms for accumulation of LD. However, these observations need to be tested in further well-designed studies. In addition, further intensive laboratory work is needed to investigate absorbed lipid transfer and metabolism to clarify the actual mechanisms.

It has been proposed that the metabolism of cancer cells, and all proliferating cells, adapts to facilitate the uptake and

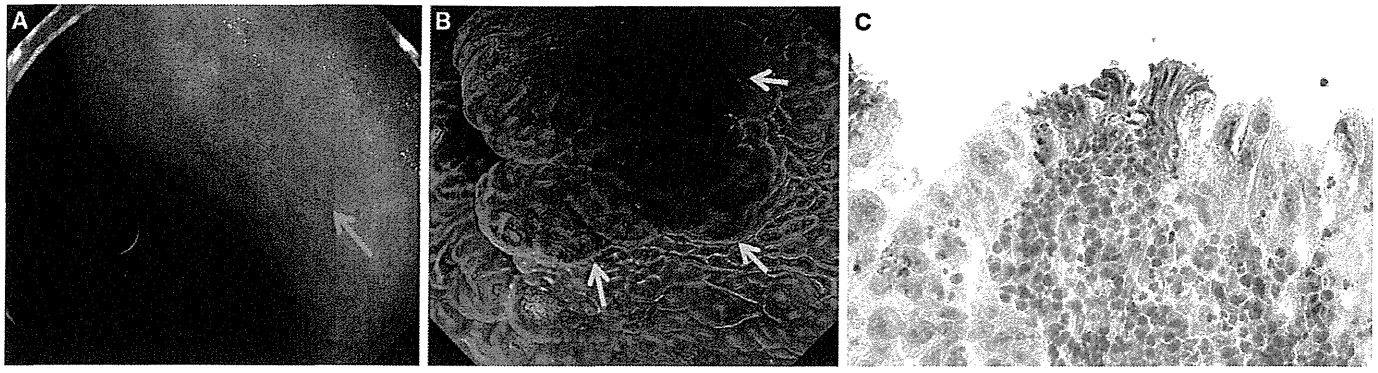

Fig. 1. (a) Standard endoscopic findings with white light of high-grade neoplasia of 0-IIa type. Slightly elevated reddened lesion (arrow) is present at the posterior wall of the gastric body. (b) Magnifying endoscopy (ME) with narrow-band imaging (NBI) findings. When we observed the margin of the lesion by ME with NBI, there was a clear demarcation line (arrow) between the background mucosa and the lesion. At that demarcation line, both the microvascular architecture and the microsurface structure in the background mucosa disappeared. Instead, dense brownish microvessels in the superficial part of the neoplasia became clearly visualized by ME with NBI. (c) Histopathological findings (oil red O staining, 100×). In the superficial part of the mucosa, no LD were detected by oil red O staining.

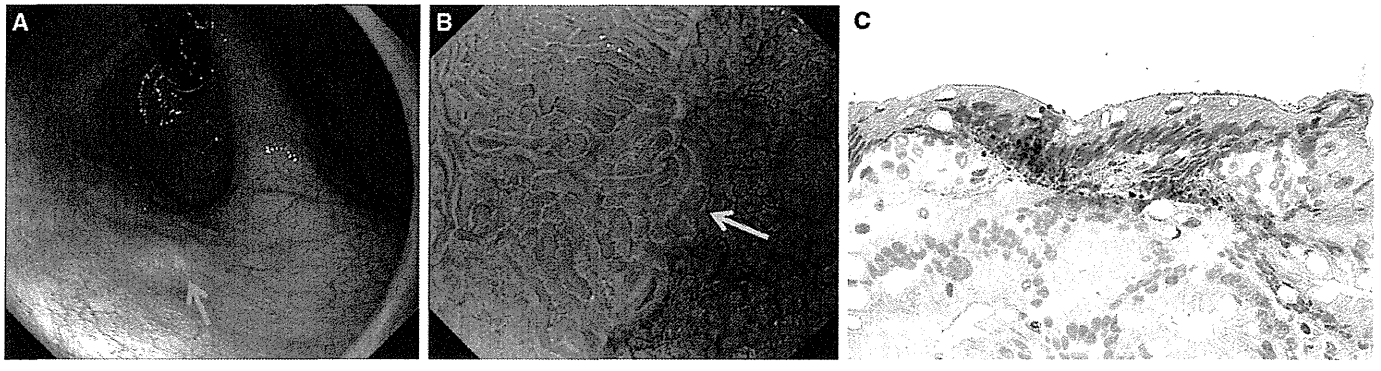

Fig. 2. (a) Standard endoscopic findings with white light of low-grade neoplasia (adenoma) of 0-IIa type. Superficial pale elevated lesion (arrow) can be seen at the lesser curvature of the gastric cardia. (b) Magnifying endoscopy (ME) with narrow-band imaging (NBI) findings. When we magnified the marginal part of the neoplasias, distinct brownish subepithelial capillaries were seen in the background mucosa. However, in the neoplastic mucosa within the margin of the lesion (arrow), the microvascular pattern could not be visualized because some WOS obscured the subepithelial microvascular architecture beneath the neoplastic epithelium. (c) Histopathological findings of the biopsied specimen from the neoplasia (oil red O staining, 100×). Oval amorphous LD in various sizes have accumulated within both the epithelium and the subepithelial part of the neoplastic tissue.

incorporation of nutrients into the biomass (nucleotides, amino acids, lipids etc.) needed to produce new cells. This is supported by the observation that certain cancer-associated mutations enable cancer cells to acquire and metabolize nutrients in a manner conducive to proliferation rather than to adenosine 5′-triphosphate (ATP) production.[24] We should also take this mechanism into consideration. Further intensive basic laboratory studies are required to investigate whether neoplastic cells synthesize LD.

The absorbed LD were located only at the surface of the mucosa of the relatively apical part between the crypts and there were no LD in the cryptal epithelium. This localization of LD to the apical part correlates well with previously reported endoscopic findings as visualized by ME with NBI (i.e. WOS is invariably detected within the epithelium of the intervening (apical) part between the cryptal epithelium).[7,9,11] The cause of this localization is unclear. We speculate that this might be because the surface epithelium at the apical parts has a greater chance to expose lipid emulsion than the cryptal epithelium, which is usually filled with rich mucus, or because the surface epithelial cells are more differentiated than the cryptal epithelium within the neoplasias. Further study is needed in order to clarify this characteristic localization of LD.

It has been suggested that oil red O, which detects lipid largely by its solubility in lipid materials, may have been unable to detect micellar lipid because micelle itself has a hydrophobic nature.[26] The intraepithelial LD as visualized by oil red O staining may be composed of triglycerides which have been resynthesized by neoplastic cells with an intestinal phenotype from the micellar fatty acid and monoglycerides, or may simply be chylomicron.

Even after the patients had fasted for more than 12 h, LD were retained within the epithelium and in the subepithelial part. In the case of the normal small intestine, chylomicron is drained into the lymphatics within the lamina propria at the tip of the intestinal villi. It is possible that this retention is not due to the lack of beta-lipoprotein, but rather is due to the