At age 25 weeks, all the animals were killed, and their stomachs were resected. From the posterior wall of the pyloric region, GECs were isolated by the gland isolation technique (31) for DNA and RNA extraction. The anterior wall of the pyloric region was further cut into two pieces: one for RNA extraction from the mucosal and submucosal layers and the other for histological analysis. DNA and RNA were extracted as described previously (14). As controls in immunohistochemistry of DNA methyltransferases (Dnmts), adult male mice (C57BL/6N, 11 weeks of age; CLEA Japan, Tokyo, Japan) were purchased and stomachs were resected. The animal experiment protocols were approved by the Committee for Ethics in Animal Experimentation.

Histological analysis

After fixation with 10% neutral formalin, tissues were embedded in paraffin and sections at 3 μm thickness were prepared. For histological analysis, hematoxylin and eosin staining was performed by a routine method. The degrees of infiltration of mononuclear and polymorphonuclear cells, intestinal metaplasia and heterotopic proliferative glands were graded on a four-point scale (0–3; 0, no or faint; 1, mild; 2, moderate and 3, marked) as described previously (32). For immunohistochemical analysis, a rabbit anti-human Ki-67 (Clone SP6; Thermo Fisher Scientific, Fremont, CA) antibody was purchased. Rabbit anti-mouse Dnmt1 (33), Dnmt3a (34) and Dnmt3b (34) antibodies were kindly provided by Professor Shoji Tajima at Osaka University. Rehydrated sections were incubated in HistoVT one (Nacalai Tesque, Kyoto, Japan) at 80°C for 40 min to unmask the antigen. After blocking with 0.5% bovine serum albumin in phosphate-buffered saline, sections were incubated with each primary antibody overnight, and the immune complex was visualized by a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Microscopic images were captured using the BZ-9000 microscope system (Keyence, Osaka, Japan). To analyze the number of the positive cells, more than five gastric glands in at least three different optic fields were counted, and the labeling index was calculated as a percentage of the positive cells relative to the total counted cells.

Human clinical samples

Human gastric mucosae were obtained by endoscopic biopsy from 7 *H.pylori*-negative (4 men and 3 women; average age 70, ranging from 44 to 83) and 18 *H.pylori*-positive (8 men and 10 women; average age 64, ranging from 46 to 81) persons with informed consents and approval of Institutional Review Boards. Their *H.pylori* infection statuses were determined by the serum anti-*H.pylori* IgG test (SBS, Kanazawa, Japan). Endoscopic superficial gastritis was observed in six of the seven *H.pylori*-negative persons and atrophic gastritis was observed in 14 of the 18 *H.pylori*-positive cases. RNA was extracted with ISOGEN (Wako, Osaka, Japan).

Gene expression analysis

The number of complementary DNA molecules was quantified by quantitative reverse transcriptase–polymerase chain reaction (qRT–PCR) as described previously (14). The number of complementary DNA molecules obtained by gene-specific primers (supplementary Table 1 is available at *Carcinogenesis* Online) was normalized to *Gapdh* (*GAPDH*) expression.

Methylation analysis

Methylation levels of gerbil CGIs (HE6, HG2, SA9, SC3, SD2, SE3, SF12 and SH6) were analyzed by quantitative methylation-specific polymerase chain reaction (PCR) and were expressed as a percentage of methylated reference as described previously (14). Bisulfite sequencing was conducted after cloning of PCR products after bisulfite modification as described previously (14).

Statistic analysis

To evaluate significant difference between two independent groups of sample data, the Mann–Whitney *U*-test was employed.

Results

Characterization of five kinds of inflammation triggered by the inducers

Gerbils were treated with five kinds of inflammation inducers (*H.pylori* ATCC 43504, *H.pylori* SS1, *H.felis*, EtOH and saturated NaCl solution) and also with MNU (Figure 1A). By histological examination of the pyloric area, the ATCC group had marked infiltration of mononuclear and polymorphonuclear cells into mucosae and submucosae and glands with intestinal metaplasia and heterotopic proliferative glands were occasionally observed (Figure 1B and Table I). The SS1 and HF groups showed milder infiltration of polymorphonuclear and mononuclear cells, less heterotopic proliferative glands and no intestinal metaplasia. The EtOH group showed infiltration of almost only polymorphonuclear cells. The NaCl group showed no or little infiltration of inflammatory cells but had thickened lamina propria. The MNU group showed no histological inflammatory changes but also had thickened lamina propria.

The kinds of infiltrating inflammatory cells were also assessed by qRT–PCR analysis [*Cd3g* (T cell), *Emr1* (macrophage), *Ela2* (neutrophil) and *Ms4a1* (B cell)] of gastric tissues containing both mucosal and submucosal layers (Figure 1C). In the ATCC, SS1 and HF groups, expression of all the four inflammatory cell markers was markedly elevated and met the typical features of chronic inflammation, such as infiltration of mononuclear cells. The macrophage and neutrophil markers were very high in the ATCC group. In the EtOH and NaCl groups, the neutrophil marker was in the same range as in the three *Helicobacter* groups, the macrophage marker was half, and the T- and B-cell markers were almost absent, showing that the inflammation in these groups was persistent acute inflammation. In the MNU group, none of the four markers were significantly elevated. These expression data were in accordance with the histological data, except for the polymorphonuclear infiltration in the NaCl group.

Induction of DNA methylation by the three Helicobacter strains but not by EtOH and NaCl

To assess methylation in GECs (not in infiltrating leukocytes), we used eight of the 10 CGIs known to be methylated in gerbil GECs as markers because these eight CGIs (HE6, HG2, SA9, SC3, SD2, SE3, SF12 and SH6) have been shown not to be methylated in peripheral blood cells (14). First, methylation levels of these CGIs were measured by quantitative methylation-specific PCR in GECs isolated by the gland isolation technique in each group (Figure 2A). The ATCC group had high methylation levels (significant in all the eight CGIs). The SS1 and HF groups also had high methylation levels (significant in six CGIs; HE6, HG2, SA9, SD2, SF12 and SH6) but lower than the ATCC group. The EtOH, NaCl and MNU groups had no increases of methylation in any CGIs.

To confirm the presence of densely methylated DNA molecules, bisulfite sequencing of HE6 was performed in one gerbil in each group (Figure 2B). Gerbils in the ATCC, SS1 and HF groups had densely methylated DNA molecule(s), and their fractions (3, 1–2, 1 of 24, respectively) were in accordance with the methylation level obtained by quantitative methylation-specific PCR. Gerbils in the EtOH, NaCl and MNU groups had no densely methylated molecules. These data showed that aberrant methylation of these CGIs was induced only by inflammation triggered by the three *Helicobacter* strains, most potently by *H.pylori* ATCC 43504-induced inflammation but not by EtOH- or NaCl-induced inflammation.

Insufficient role of cell proliferation in methylation induction

Cell proliferation was analyzed by immunohistochemistry of Ki-67 in gastric mucosae (Figure 3A) and counting the Ki-67 labeling indices (Figure 3B). All the treatment groups showed significant increases in Ki-67 labeling indices. The three *Helicobacter*-infected groups and the NaCl-treated group showed very high Ki-67 labeling indices. The NaCl-treated group, especially which did not show increased methylation levels, showed the highest Ki-67 labeling index. This result showed that induction of cell proliferation is not sufficient to induce DNA methylation.

Inflammation-related genes associated with methylation induction

To dissect inflammation components responsible for methylation induction, qRT–PCR analysis of 10 inflammation-related genes [*Cox2*, *Cxcl2* (*MIP-2*), *Ifng*, *Il1b*, *Il2*, *Il4*, *Il6*, *Il7*, *Nos2* (*iNos*) and *Tnf* (Tnf-α)] was performed using RNA collected from gastric tissues that contained both GECs and inflammatory cells (Figure 4A). In the three *Helicobacter*-infected groups, *Il1b*, *Nos2* and *Tnf* were significantly upregulated. *Ifng*, *Il2*, *Il4* and *Il6* were significantly upregulated in the

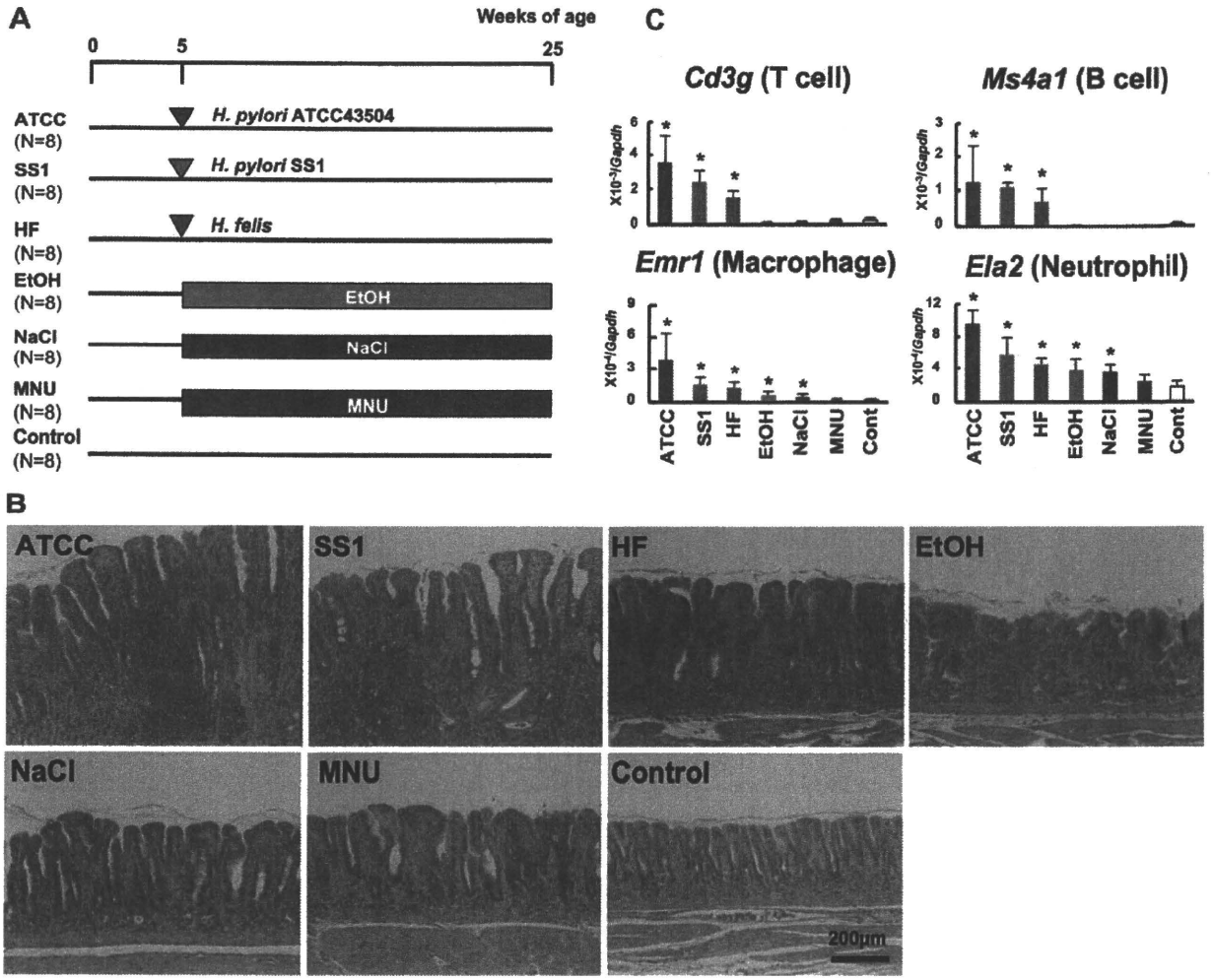

Fig. 1. Treatment of Mongolian gerbils by five inflammation inducers and MNU. (**A**) Experimental design. (**B**) Histology of gastric mucosa after treatment for 20 weeks. Transition of inflammatory cells was observed in the three *Helicobacter* groups. (**C**) Expression levels of inflammatory cell markers. Infiltration of T and B cells was prominent in the three *Helicobacter* groups. Values are shown as mean + SD. $^{*}P < 0.05$ compared with the control group.

Table I. Histological changes induced by the five inflammation inducers and MNU

Group	Infiltration of mononuclear cells	Infiltration of polymorphonuclear cells	Intestinal metaplasia	Heterotopic proliferative glands
ATCC	2.8 ± 0.5*	2.3 ± 0.7*	0.9 ± 0.6*	1.4 ± 0.9*
SS1	1.6 ± 0.5*	1.1 ± 0.7*	0.0 ± 0.0	0.3 ± 0.5
HF	1.6 ± 0.8*	0.7 ± 0.5*	0.0 ± 0.0	0.4 ± 0.8
EtOH	0.0 ± 0.0	0.9 ± 0.3*	0.0 ± 0.0	0.1 ± 0.3
NaCl	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
MNU	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Values are shown as mean ± SD.
$^{*}P < 0.01$ compared with control group.

SS1, HF, EtOH and NaCl groups but not in the ATCC group. Expression levels of these genes tended to be higher in the EtOH and NaCl groups than in the SS1 and HF groups. The MNU group did not show any significant changes compared with the control group. These results suggested that upregulation of *Il1b*, *Nos2* and *Tnf* was associated with methylation induction.

Expression of Dnmts

Dnmts are the final effectors that methylate DNA (35). To analyze the relation between expression of Dnmts and aberrant methylation induction, we conducted immunohistochemistry of Dnmts. Antibodies against mouse Dnmt1, Dnmt3a and Dnmt3b were tested in gerbils, and those against Dnmt1 and Dnmt3a were confirmed to have high sensitivity and specificity (supplementary Figure 1 is available at *Carcinogenesis* Online).

Dnmt1 protein was localized in the nuclei of GECs around the proliferative zone of gastric glands (supplementary Figures 1 and 2 are available at *Carcinogenesis* Online). In the ATCC, SS1, HF and NaCl groups, the number of GECs expressing Dnmt1 protein was markedly increased and the highest labeling index was observed in the NaCl group (Figure 4B). The profile of Dnmt1 expression was the same as that of Ki-67 (Figure 3B), indicating that Dnmt1 expression was elevated in association with increased cell proliferation. Dnmt3a protein was localized in the nuclei of most GECs except in some cells in the bottom of the glands. Although GECs expressing Dnmt3a protein significantly decreased in the ATCC, EtOH and MNU groups, the degree of decrease was small (Figure 4B and supplementary Figures 1 and 3 are available at *Carcinogenesis* Online). These results showed that the fractions of GECs expressing Dnmt1 and Dnmt3a in gastric glands were not associated with methylation induction.

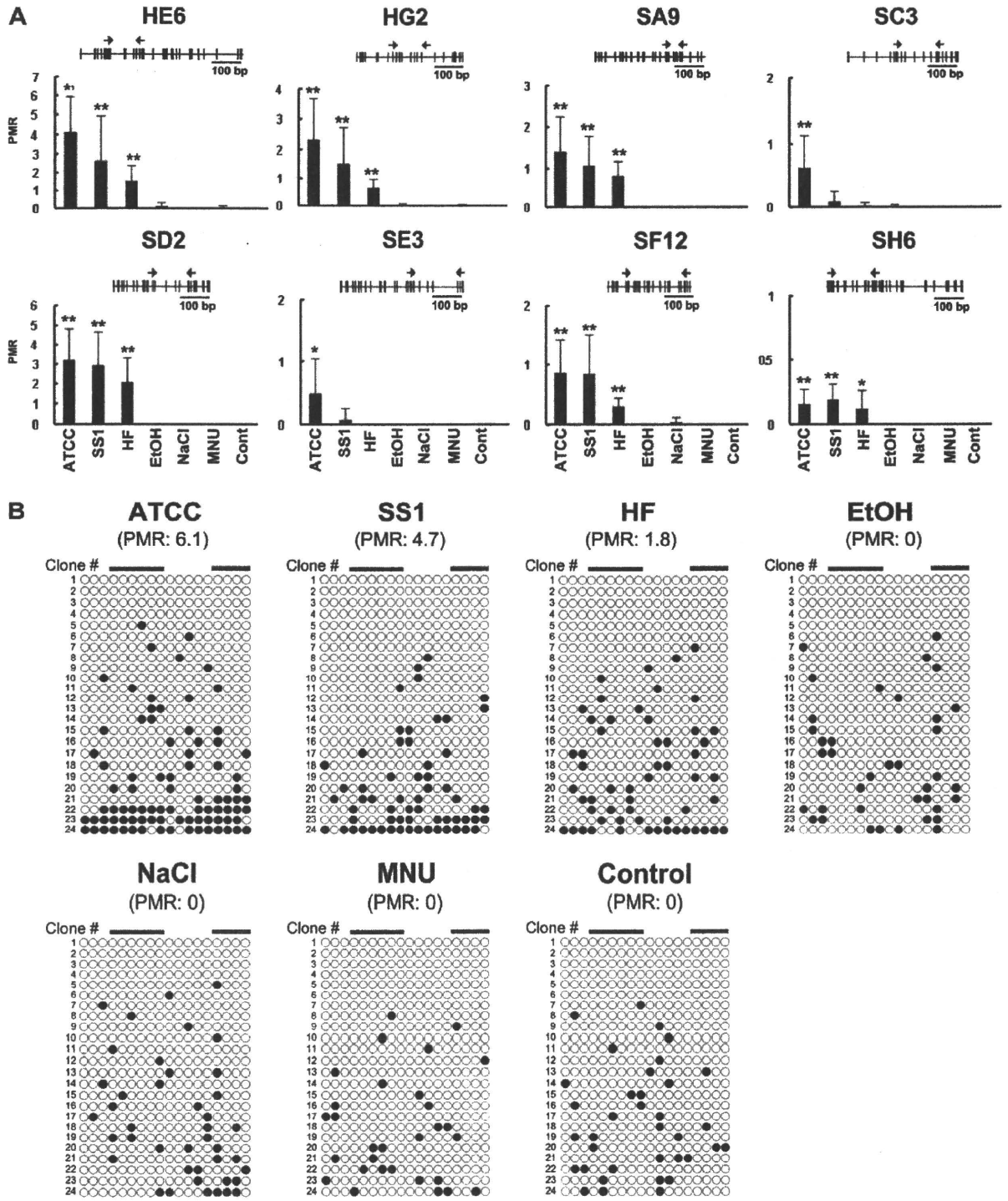

Fig. 2. Methylation induction in GECs by the three *Helicobacter*-induced inflammation but not by EtOH- or NaCl-induced inflammation. (**A**) Methylation levels of eight CGIs assessed by quantitative methylation-specific PCR. Upper panels show CpG maps, and lower panels show methylation levels in percentage of methylated reference. In the upper panel, vertical lines and arrows show individual CpG sites and positions of methylation-specific PCR primers, respectively. Values are shown as mean + SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group. (**B**) Bisulfite sequencing of HE6 in GECs. Numbers in parentheses indicate percentage of methylated reference of the sample assessed by quantitative methylation-specific PCR. Bars, CpG sites on quantitative methylation-specific PCR primers.

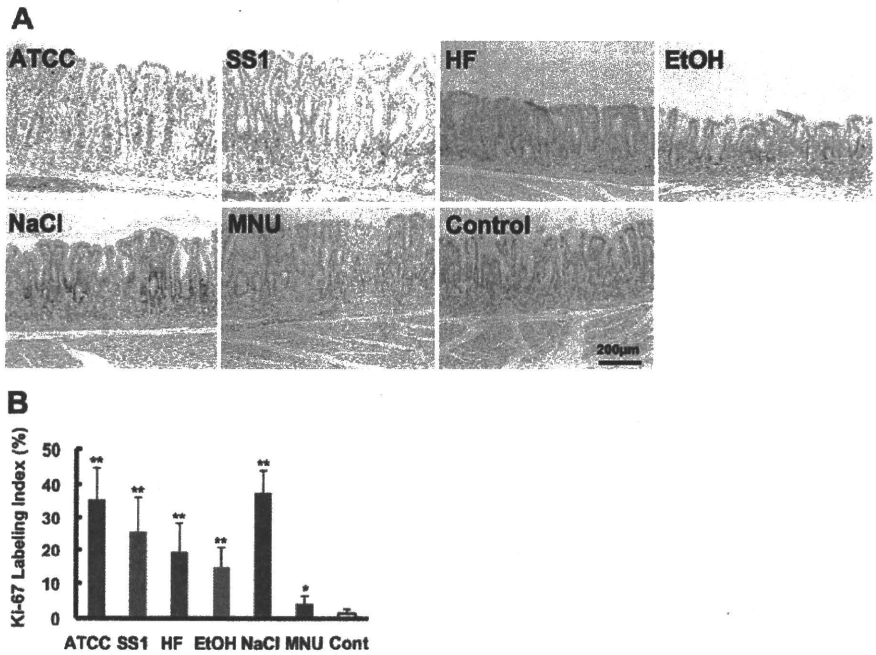

Fig. 3. Cell proliferation of gerbil GECs after the treatment. (**A**) Representative microscopic appearance of Ki-67 immunohistochemistry. (**B**) Ki-67 labeling index. Values are shown as mean + SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group. The NaCl group showed a marked increase of cell proliferation.

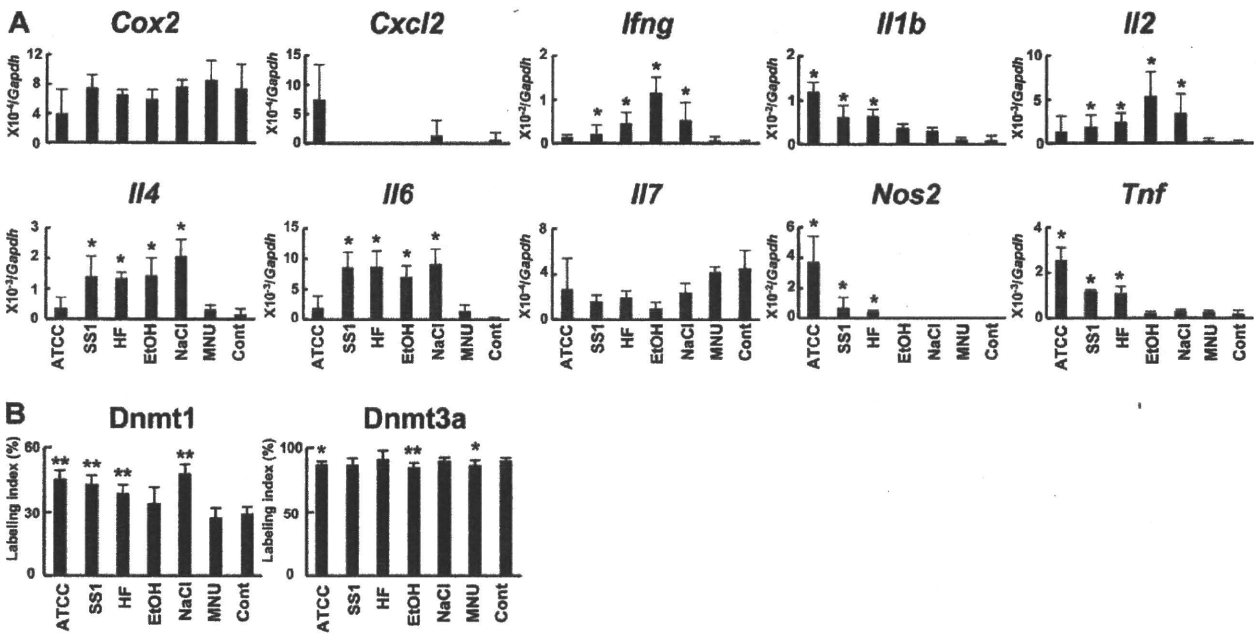

Fig. 4. Expression of inflammation-related genes and Dnmts in the gerbil stomach. (**A**) messenger RNA levels of inflammation-related genes in gerbil gastric tissues containing both mucosal and submucosal layers. Expression levels of *Il1b*, *Nos2* and *Tnf* were elevated only in the three *Helicobacter* groups. (**B**) The fractions of GECs expressing Dnmt proteins in gastric glands by immunohistochemistry. Values are shown as mean + SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control group.

Human relevance of inflammation-related gene expression

To address whether upregulation of specific inflammation-related genes are common in the human stomach, we conducted qRT–PCR of *COX2*, *IFNG*, *IL1B*, *IL6*, *NOS2* and *TNF* using human gastric mucosa samples with and without *H.pylori* infection. Expression levels of *NOS2* and *TNF* were markedly upregulated (27- and 3-fold,

respectively) also in human gastric mucosae (Figure 5). However, *IL1B* expression tended to be lower in gastric mucosae of *H.pylori*-infected individuals.

Discussion

Among the five groups with inflammation, aberrant methylation was induced only in the three *Helicobacter* groups, which showed inflammation with infiltration of mononuclear cells, increased expression of *Il1b*, *Nos2* and *Tnf* and increased cell proliferation. In the EtOH and NaCl groups, these agents were administered repeatedly for 20 weeks, and increased cell proliferation was present at the end of the experiment. The increased proliferation was considered to have persisted for this period because thickening of lamina propria was observed in these two groups. Nevertheless, aberrant methylation was not induced, at least in the CGIs analyzed here. This showed that cell proliferation alone is not sufficient for methylation induction and suggested that both specific types of inflammation and increased cell proliferation are necessary for induction of aberrant methylation.

The inflammation induced in the *Helicobacter* groups was characterized by infiltration of mononuclear cells (lymphocytes and macrophages). In our previous study, suppression of T-cell activation by cyclosporin A remarkably repressed inflammatory response and methylation induction triggered by *H.pylori* infection (14), showing that T-cell activation is involved in methylation induction in this system. However, our recent study in mouse colon demonstrated that aberrant methylation can be induced even in severe combined immunodeficiency mice, which lack functional T and B cells, by dextran sulfate sodium-induced colitis (Katsurano *et al.*, submitted for publication). It is known that, even in severe combined immunodeficiency mice, colitis with macrophage infiltration can be induced (36). If a common mechanism for methylation induction is present in *H.pylori*-infected gastric mucosae and dextran sulfate sodium-treated colonic mucosae, infiltration of macrophages is a candidate for the proximate effector that transmits signal for methylation induction to epithelial cells. It can be considered that, in *H.pylori*-infected gastric mucosae, activation of T cells is required only for the initiation or maintenance of inflammation capable of inducing aberrant DNA methylation.

Among the inflammation-related genes, *Il1b*, *Nos2* and *Tnf* were specifically upregulated in the three *Helicobacter* groups. These three genes are reported to be overexpressed also in human chronic inflammation associated with cancers, such as ulcerative colitis and hepatitis (37–40). *IL1B* promoter polymorphism is associated with risk of human gastric cancers (28) and aberrant methylation of multiple genes in gastric cancers (29). The lack of its upregulation in human gastric mucosae infected with *H.pylori* could be because most of them had superficial gastritis and had already increased *IL1B* expression. *NOS2*, which encodes nitric oxide synthase, was upregulated *in vitro* by administration of IL1B and nitric oxide donors induced methylation of *FMR1* and *HPRT* (41). These suggest that IL1B and NOS2 might be involved in methylation induction. On the other hand, *Ifng*, *Il2*, *Il4* and *Il6* were upregulated mainly in the EtOH and NaCl groups, in which no methylation was induced, and also in the SS1 and HF groups, in which methylation induction levels were lower than in the ATCC group. This suggested a possibility that some (one) of the genes could suppress methylation induction.

SS1 and *H.felis*, which lack CagA, were capable of inducing aberrant methylation although the capacity was weaker than the CagA-positive strain (*H.pylori* ATCC 43504). CagA-positive *H.pylori* strains are known to induce severe gastritis in Mongolian gerbils (16) as confirmed in this study, and this explains their stronger capacity to induce methylation. The three inflammation-related genes associated with methylation induction (*Il1b*, *Nos2* and *Tnf*) had the highest expression in the ATCC group among the three *Helicobacter* groups. CagA-positive *H.pylori* seems to promote methylation induction by maximizing expression of such genes and minimizing expression of genes that suppress methylation induction.

Dnmts are the final effectors to methylate DNA, and their overexpression was observed in various human cancers (35). Immunohistochemical analyses here revealed that Dnmt1 was upregulated in gastric mucosae of gerbils in the three *Helicobacter*-infected groups and the NaCl-treated group. However, the highest expression was observed in the NaCl group, where methylation was not induced. This result indicated that expression of Dnmt1 was not associated with methylation induction but with cell proliferation. Expression of Dnmt3a was significantly but slightly decreased in the ATCC group and this also suggested that the expression itself is not involved in aberrant methylation induction. However, due to the lack of an appropriate antibody, we were not able to exclude the possibility that upregulation of Dnmt3b is involved in methylation induction. Therefore, disturbance in the local balance between Dnmts and factors that protect DNA from aberrant methylation, such as the presence of RNA

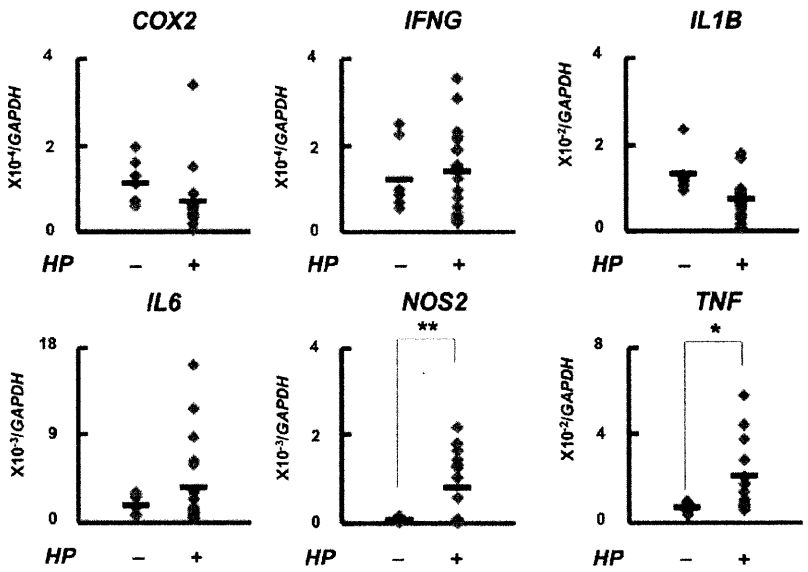

Fig. 5. Human relevance of expression changes in the gerbil stomach. Expression levels of inflammation-related genes were quantified in gastric mucosae of individuals without and with *H.pylori* infection. Bold horizontal bar, the mean expression level; $^{*}P < 0.05$ and $^{**}P < 0.01$.

polymerase II (42) and/or possible overexpression of Dnmt3b might be involved in methylation induction.

In conclusion, inflammation due to infection of *Helicobacter* strains had a high capacity to induce methylation in GECs, regardless of their CagA status. Increased cell proliferation was not sufficient for methylation induction. Therefore, specific types of inflammation, characterized by infiltration of mononuclear cells and expression of specific inflammation-related genes, along with increased cell proliferation were considered to be necessary for methylation induction.

Supplementary material

Supplementary Figures 1–3 and Table 1 can be found at http://carcin.oxfordjournals.org/

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Conflict of Interest Statement: None declared.

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JOURNAL OF CLINICAL ONCOLOGY ORIGINAL REPORT

Early Detection of Superficial Squamous Cell Carcinoma in the Head and Neck Region and Esophagus by Narrow Band Imaging: A Multicenter Randomized Controlled Trial

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ABSTRACT

Purpose
Most of the esophageal squamous cell carcinomas (ESCCs) and cancers of the head and neck (H&N) region are diagnosed at later stages. To achieve better survival, early detection is necessary. We compared the real-time diagnostic yield of superficial cancer in these regions between conventional white light imaging (WLI) and narrow band imaging (NBI) in high-risk patients.

Patients and Methods
In a multicenter, prospective, randomized controlled trial, 320 patients with ESCC were randomly assigned to primary WLI followed by NBI (n = 162) or primary NBI followed by WLI (n = 158) in a back-to-back fashion. The primary aim was to compare the real-time detection rates of superficial cancer in the H&N region and the esophagus between WLI and NBI. The secondary aim was to evaluate the diagnostic accuracy of these techniques.

Results
NBI detected superficial cancer more frequently than did WLI in both the H&N region and the esophagus (100% *v* 8%, $P < .001$; 97% *v* 55%, $P < .001$, respectively). The sensitivity of NBI for diagnosis of superficial cancer was 100% and 97.2% in the H&N region and the esophagus, respectively. The accuracy of NBI for diagnosis of superficial cancer was 86.7% and 88.9% in these regions, respectively. The sensitivity and accuracy were significantly higher using NBI than WLI in both regions ($P < .001$ and $P = .02$ for the H&N region; $P < .001$ for both measures for the esophagus, respectively).

Conclusion
NBI could be the standard examination for the early detection of superficial cancer in the H&N region and the esophagus.

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INTRODUCTION

Esophageal cancer is the eighth most common cancer worldwide, accounting for 462,000 new cases in 2002, and is the sixth most common cause of cancer-related death (386,000 deaths).[1] Squamous cell carcinoma (SCC) is the most common histologic type worldwide.[1] Head and neck (H&N) cancer accounted for 607,000 new cases and 261,000 deaths in 2002.[1] The most common histologic type of H&N cancer is also SCC.

The early detection of cancer offers the best prognosis. Currently, however, esophageal SCC (ESCC) and H&N SCC (HNSCC) are detected at a late stage and then have poor prognoses.[1] Early detection of these cancers is difficult by conventional endoscopic white light imaging (WLI). Lugol chromoendoscopy can be used to detect superficial ESCC, but it causes unpleasant adverse effects such as severe chest pain and chest discomfort,[2-4] and it cannot be used for HNSCC screening because of the risk of aspiration.

The narrow band imaging (NBI) system is an innovative optical image-enhanced technology that uses narrow bandwidth NBI filters.[5,6] The central wavelengths of the NBI filters are 415 and 540 nm and each has a bandwidth of 30 nm. This system is easily activated by pushing a button on the endoscope. NBI combined with magnifying endoscopy can clearly visualize the microvascular structure of the organ surface,[6,7] because the 415-nm light is well absorbed by hemoglobin. Surface microvascular irregularities provide useful landmarks for identifying an early neoplasm in the H&N region, bronchus,

and the GI tract.[7-15] We previously reported that NBI was useful for identifying HNSCC at an early stage.[8] Watanabe et al[16,17] also reported the usefulness of NBI rhinolaryngovideoscopy for the diagnosis of HNSCC. Yoshida et al[18] reported that NBI improves the accuracy of magnifying WLI in the assessment of ESCC.

However, the diagnostic yield of NBI in the early detection of superficial SCC has not been investigated. We conducted a prospective randomized study to directly compare WLI and NBI in the early diagnosis of SCC in the H&N region and the esophagus among high-risk patients.

PATIENTS AND METHODS

Study Rationale

Because ESCC patients frequently develop multiple intraesophageal SCC and second primary HNSCC synchronously and metachronously,[4,19-22] they provide a good cancer screening model. Whereas massively invasive SCC is easy to detect by endoscope, superficial cancer has been difficult. Furthermore, detection of high-grade intraepithelial neoplasia (HGIN) is clinically important because HGINs have the potential to become malignant invasive cancers.[23,24] Therefore, in this study, we targeted only macroscopic superficial cancer including HGIN that appeared as slightly elevated lesions lower than 5 mm, flat lesions, and lesions with a shallow depression. Lesions with an apparent elevation greater than 5 mm or those with apparent deeper ulceration were not evaluated.

The primary analysis of this study was a comparison of the detection rates of superficial cancer (HGIN, carcinoma in situ, and microinvasive SCC) using WLI and NBI. The secondary analysis was a comparison of the diagnostic accuracy (sensitivity and specificity) of the two imaging methods, size of the lesion detected, and the examination time. To evaluate diagnostic accuracy, we used the histologic diagnosis from a biopsy specimen as the gold standard diagnosis.

Study Populations

The protocol and consent form for this study were approved by the institutional review board at each participating institution, and written informed consent was obtained from all patients. The inclusion criteria were histologically confirmed present or previous ESCC and an age of 20 years or older. Although this study included patients with advanced ESCC, we evaluated only concomitant superficial cancer but not primary advanced cancer. Patients who had been previously treated for ESCC by endoscopic mucosal resection were included, because their esophagus was preserved with minimal damage. Patients with prior chemotherapy, radiotherapy, chemoradiotherapy, or surgical resection for ESCC or HNSCC were excluded, because their esophagus or pharynx was removed or too damaged to evaluate. Patients referred from another hospital with newly diagnosed ESCC were also included because they required more detailed examination (Fig 1) . The endoscopists were blinded to the endoscopic information. Patients with esophageal stricture, esophageal varices, or allergy to lugol dye solution were excluded.

Study Design

Patients were randomly assigned to receive primary WLI or primary NBI. 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, we performed both imaging methods in a back-to-back fashion so that primary WLI was followed by NBI and primary NBI was followed by WLI. To avoid affecting the first imaging results, the report of the first examination was completed before the second imaging was started.

To improve the quality of the reporting in the diagnostic accuracy study, we complied with the Standards for Reporting of Diagnostic Accuracy (STARD) initiative.[25] We set WLI as reference standard and NBI as index test.

Random assignment was performed in each case by an investigator using a computer-aided system on Medical Research Support Web site (Kyoto, Japan). This Web site was available only to the study participants. Using a minimization algorithm, the selection of the primary examination was balanced with respect to five stratification variables: institution, age (< 60 and ≥ 60 years), sex, alcohol consumption, and smoking habit.

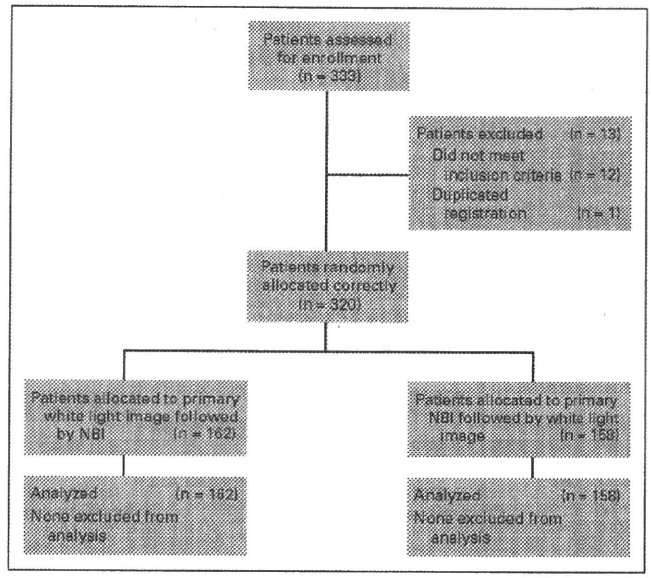

Fig 1. CONSORT diagram; overview of the study design. NBI, narrow band imaging.

Calculation of the Sample Size

For the purposes of this study, we set the probability for error (α) to .05 with a power of 0.80 (reflecting a β error of .2). Because there are no published comparative studies of NBI in ESCC patients, we estimated that the NBI system would increase the detection yield for superficial cancer by at least threefold compared with conventional WLI. This resulted in a calculated sample size of 250 patients (125 per group). Finally, we recruited an additional 50 patients in anticipation of instances of ineligibility or withdrawal during the examination because of discomfort (25 per group).

Endoscopic Examination

We used the same magnifying endoscope, with the capability for 80 times optical magnification (GIF-Q240Z, Olympus Medical Systems, Tokyo, Japan) for both WLI and NBI. The two imaging methods can be performed in a same video-endoscopy system (EVIS LUCERA system, Olympus Medical Systems, Tokyo, Japan). The details of the NBI system have been published elsewhere.[1,2,26,27] To maintain the quality of the endoscopic images, we used the same liquid-crystal color display for both imaging methods. Before the study started, all the participating endoscopists were trained using a central review of demonstrable NBI images of superficial squamous lesions (13 neoplasias and seven non-neoplastic lesions).

All endoscopic observations were made according to the protocol. During the first imaging, all parts of the oropharynx and hypopharynx were evaluated. The nasopharynx was not included the examination. After the first imaging was completed, an assistant physician immediately recorded the results on the case record form (CRF). After completion of the first imaging CRF, the second imaging of the oropharynx and hypopharynx was performed and the results were recorded on the CRF.

Next, all parts of the esophagus were evaluated using the same imaging as used for the H&N region. The endoscope was inserted to gain a view from the cervical esophagus to the esophagogastric junction, and the results were recorded on the CRF. The second imaging was performed on withdrawal of the endoscope, and the results were recorded on the CRF. During the procedure, we measured the examination time from start to finish of each imaging at each site. These procedure times included the evaluation of the lesion but not the biopsy procedure. The findings obtained by lugol chromoendoscopy are not included in this study.

Endoscopic Evaluation of Superficial Cancers

In this study, the real-time on-site diagnosis was evaluated because making an accurate diagnosis during an examination is clinically more important than a retrospective evaluation using a stored database. On WLI, if the lesion showed both a reddish color with uneven surface and disappearance of the vascular network pattern (Fig 2A), we diagnosed it as endoscopically suspected "superficial cancer." On NBI, if the lesion exhibited a well-demarcated brownish area as well as irregular microvascular patterns (Fig 2B), we diagnosed it as endoscopically suspected "superficial cancer." Details of these findings have been described previously.[7,8] If the lesion did not show these characteristics, the lesion was diagnosed as "non-cancer." Mucosal abnormalities were recorded with regard to endoscopic diagnosis, location, and size of the lesion.

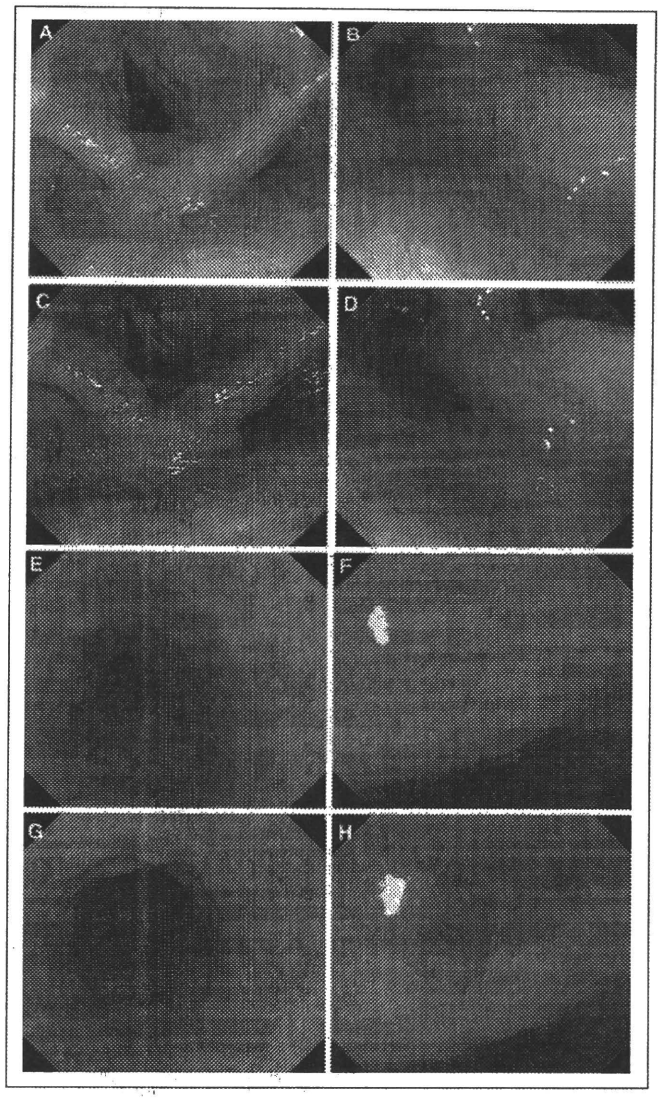

Fig 2. Superficial cancer in the head and neck region and esophagus. (A) White light imaging (WLI) shows a small reddish area (arrows) in the posterior wall of the hypopharynx. (B) Magnifying WLI shows a slightly reddish area with tiny microdots. (C) Narrow band imaging (NBI) shows a well-demarcated brownish area (arrows) in the posterior wall of the hypopharynx. (D) Magnifying NBI shows many tiny dots in the brownish area. This lesion was diagnosed histologically as squamous cell carcinoma in situ. (E) WLI shows a slightly reddish and depressed lesion (arrows) in the esophagus, although it is difficult to detect by WLI alone. (F) Magnifying WLI shows a slightly reddish area with an irregular microvascular pattern. (G) NBI shows a well-demarcated brownish area (arrows). (H) Magnifying NBI shows many tiny dots in the brownish area. This lesion was diagnosed histologically as high-grade intraepithelial cancer.

Pathologic Evaluation

Biopsy specimens were taken from each lesion after the completion of both types of imaging. Histologic evaluation was performed by central review by four experienced pathologists (H.S., A.O., T.S., and H.W.) who were blinded to the recorded endoscopic assessment. Histologic diagnoses were made according to WHO criteria[23] and were classified into two groups. One group included superficial cancers and the other group included non-cancers such as parakeratosis and inflammation. Microinvasion was estimated by the subepithelial invasion. The final pathologic diagnosis was made by the agreement of three of the four pathologists.

Statistical Analysis

The absolute and relative frequencies for qualitative variables were calculated for each group. Statistical analysis was performed using SPSS version

Table 1. Characteristics of Patients

Characteristic	Primary WLI (n = 162) No.	%	Primary NBI (n = 158) No.	%	P
Age, years					
Median	64		64		
Range	39-84		46-84		.99
Male sex	143	88	141	89	.86
Alcohol habit					
Drinking duration, years	157	97	148	94	.19
Median	41		40		.17
Range	10-63		5-60		
Favorite beverage					
Beer	61	38	59	37	1.00
Shochu	66	41	55	35	.30
Sake	43	27	48	30	.71
Whisky	22	14	24	15	.75
Wine	8	5	7	4	1.00
Others	1	0.6	0	0	1.00
Hot flashes					
Formerly had hot flashes	117	72	109	69	.62
Currently has hot flashes	75	46	70	44	.91
Smoking habit					
No. of smokers	145	90	142	90	1.00
Smoking duration, years					
Median	37		40		
Range	1-61		5-61		.41
No. of packs per day					
Median	1		1		
Range	0.05-4		0.125-4		.64
No. of packs per year					
Median	41		42		
Range	0.5-180		1.3-160		.89
Esophageal cancer					
No. of patients newly diagnosed	110	68	115	73	.39
Previously treated EMR	52	32	43	27	.39
Duration from previous EMR, years					
> 1	17	10	20	13	.60
1	45	28	33	21	.16
Depth of invasion					
Tis-T1a	74	46	67	42	.57
T1b	25	15	20	13	.27
T2	12	7	22	14	.07
T3	49	30	46	29	.90
T4	2	1	3	2	.68

Abbreviations: WLI, white light imaging; NBI, narrow band imaging; EMR, endoscopic mucosal resection.

17 software (SPSS, Chicago, IL). The continuous variables are expressed as medians and ranges. Continuous data were compared using the Mann-Whitney *U* test. Pearson's χ^2 test or Fisher's exact test was used to analyze categoric data to compare proportions. All *P* values were two-tailed, and a *P* value of $< .05$ was considered significant.

RESULTS

Between March 2005 and December 2005, 333 patients were enrolled onto this study (Fig 1). Twelve patients did not meet the inclusion criteria, and one was registered twice, so the remaining 320 patients were randomly assigned correctly into two groups: (1) 162 patients who underwent primary WLI followed by NBI, and (2) 158 patients who were examined by primary NBI followed by WLI.

The characteristics of the two groups are listed in Table 1. The two groups did not differ significantly in age, sex, alcohol consumption, smoking habits, or history of esophageal cancer treatment. In both groups, approximately 70% of the patients had newly diagnosed ESCC. Sixty-three (39%) patients in the primary WLI group and 71 (45%) patients in the primary NBI group had advanced ESCC deeper than the submucosal layer.

Table 2 provides the distribution of histologically confirmed superficial cancers. The total numbers of superficial cancer in the H&N region and the esophagus were 28 and 212, respectively. Total numbers of histologically confirmed non-cancer were 36 and 38 in each region. In all patients, superficial cancers were detected in 8% (26 of 320) in the H&N region and in 38% (121 of 320) in the esophagus. Multiple cancers were found in 0.6% of the patients in the H&N region and in 12% in the esophagus. The number of patients with superficial cancer, total number of superficial cancers, and their sizes and distribution did not differ between the two groups.

The diagnostic yields for superficial cancer using primary WLI and primary NBI detection are summarized in Table 3. The total numbers of superficial cancers detected by primary imaging differed between the two groups. In the H&N region, primary NBI detected all (100%; 15 of 15) of the superficial cancers, but primary WLI detected only one lesion (8%; 1 of 13). In the esophagus, only 58 (55%) lesions were detected by primary WLI, whereas 104 (97%) lesions were detected by primary NBI. All these differences were statistically significant ($P < .001$). The detection rate was significantly higher with primary NBI than with primary WLI, even for small lesions (< 10 mm in diameter) in both the H&N region ($P < .001$) and the esophagus ($P = .03$).

In the back-to-back analysis, secondary NBI after primary WLI significantly increased the detection rate in both the H&N region (8% *v* 77%; $P < .001$) and esophagus (55% *v* 95%; $P < .001$; Appendix Table A1, online only). In contrast, secondary WLI after NBI significantly decreased the detection rate (Appendix Table A1). Moreover, 16 (57%) superficial cancers in the H&N region and 48 (23%) superficial cancers in the esophagus were detected only by NBI (Appendix Table A2, online only). In contrast, no lesion was detected only

Table 2. Distribution of Histologically Confirmed Superficial Cancer According to Lesion in the Head and Neck Region and the Esophagus

Variable	Primary WLI (n = 162)			Primary NBI (n = 158)			*P*
	No.	%	95% CI	No.	%	95% CI	
Head and neck region							
No. of patients	12	7	3.3 to 11.4	14	9	4.4 to 13.3	.66
No. of lesions per patient							
1	12	7	3.3 to 11.4	14	9	4.4 to 13.3	> .999
≥ 2	1	0.6	−0.6 to 1.8	1	0.6	−0.5 to 1.9	
Total No. of superficial neoplasias	13			15			
Size threshold, mm							
< 10	7			10			.50
11-20	5			5			
≥ 21	1			0			
Histologic diagnosis							
High-grade intraepithelial neoplasia or carcinoma in situ	10			15			.09
Microinvasive cancer	3			0			
Esophagus							
No. of patients	58	36	28.4 to 43.2	63	40	32.2 to 47.6	.49
No. of lesions per patient							
1	39	24	17.4 to 30.7	43	27	20.3 to 34.2	> .999
≥ 2	19	12	6.7 to 16.7	20	13	7.4 to 17.9	
Total No. of superficial cancers	105			107			
Size threshold, mm							
< 10	18			18			.91
11-20	21			19			
≥ 21	66			70			
Histologic diagnosis							
High-grade intraepithelial neoplasia or carcinoma in situ	73			84			.16
Microinvasive cancer	32			23			

Abbreviations: WLI, white light imaging; NBI, narrow band imaging.

Table 3. Diagnostic Yield of Primary WLI and Primary NBI for Detection of Superficial Cancer in the Head and Neck Region and the Esophagus

Variable	Primary WLI (n = 162)			Primary NBI (n = 158)			P
	No.	%	95% CI	No.	%	95% CI	
Head and neck region							
No. of superficial cancers	1/13	8	0.2 to 36.0	15/15	100	78.2 to 100	< .001
Size of superficial cancer, mm							
< 10	0/7	0	0 to 41.0	10/10	100	69.2 to 100	< .001
11-20	1/5	20	0.5 to 71.6	5/5	100	48.7 to 100	.12
≥ 21	0/1	0	0.0 to 0.0	to			—
Esophagus							
No. of superficial cancers	58/105	55	45.2 to 65.0	104/107	97	92.0 to 99.4	< .001
Size of superficial cancer, mm							
< 10	7/18	39	17.3 to 64.3	17/18	94	72.7 to 99.9	.03
11-20	7/21	33	14.6 to 57.0	18/19	95	74.0 to 99.9	.02
≥ 21	44/66	67	54.0 to 77.8	69/70	99	92.3 to 100	< .005

Abbreviations: WLI, white light imaging; NBI, narrow band imaging.

by WLI, except one lesion of > 20 mm in the esophagus. No lesions were undetected by both WLI and NBI in either region.

Table 4 summarizes the diagnostic performance of primary WLI and primary NBI for detecting superficial cancer. The sensitivity of primary NBI was significantly higher than that of primary WLI in both the H&N region (100% *v* 7.7%; $P < .001$) and the esophagus (97.2% *v* 55.2%; $P < .001$). Accuracy was also significantly higher for primary NBI than for primary WLI in both regions (85.7% *v* 62.9%, $P = .02$ and 88.9% *v* 56.5%, $P < .001$, respectively). Specificity was not significantly different in the two regions ($P = .28$ and $P = .33$, respectively). The positive predictive value did not differ between the two imaging techniques, but the negative predictive value was significantly higher for primary NBI than for primary WLI in both the H&N region ($P = .02$) and the esophagus ($P < .002$).

The median procedure times of primary WLI and primary NBI for the H&N region were 120 seconds (range, 34 to 275 seconds) and 162 seconds (range, 30 to 525 seconds), respectively. Those for the esophagus were 95 seconds (range, 30 to 360 seconds) and 135 seconds (range, 30 to 616 seconds), respectively. These differences were statistically significant ($P < .001$). The procedure times in the secondary imaging in the back-to-back experiments also differed significantly between WLI and NBI in both regions (Appendix Table A3, online only). There were no serious adverse events related to examination with either procedure. All patients tolerated both procedures well.

DISCUSSION

This study clearly demonstrates that NBI is a more sensitive method for detecting and diagnosing superficial SCC in the H&N region and the esophagus. According to the concept of "field cancerization,"[28] patients with ESCC or HNSCC are at high risk for the development of multiple SCCs. In the clinical context, the early detection strategy for superficial SCC is the same between patients at high risk and those at risk because of heavy drinking, smoking, or aldehyde dehydrogenase 2 deficiency.[20-35] In addition, detection technique should not only be sensitive but should also be easily applicable. From this perspective, NBI is easily applied with a modicum of experience and will have a rapid learning curve compared with WLI. Thus, NBI is the ideal method for effectively detecting superficial SCC.

Table 4. Diagnostic Performance of Primary WLI and Primary NBI Observation for Detection of Superficial Cancer in the Head and Neck Region and the Esophagus

Variable	Primary WLI			Primary NBI			P
	No.	%	95% CI	No.	%	95% CI	
Head and neck							
Sensitivity	1/13	7.7	0.2 to 36.0	15/15	100	100	< .001
Specificity	21/22	95.5	77.2 to 99.9	11/14	78.6	54.6 to 98.1	.28
Accuracy	22/35	62.9	47.6 to 76.4	26/29	86.7	72.6 to 97.8	.02
PPV	1/2	50	1.3 to 98.7	15/18	83.3	58.6 to 96.4	.37
NPV	21/33	63.6	54.1 to 79.6	11/11	100	100	.02
Esophagus							
Sensitivity	58/105	55.2	45.2 to 65.0	104/107	97.2	92.0 to 99.4	< .001
Specificity	12/19	63.2	38.4 to 83.7	8/19	42.1	20.3 to 66.5	.33
Accuracy	70/124	56.5	47.3 to 65.3	112/126	88.9	82.1 to 93.8	< .001
PPV	58/65	89.2	79.1 to 95.6	104/115	90.4	85.3 to 95.1	.80
NPV	12/59	20.3	11.0 to 32.8	8/11	72.8	39 to 94	< .002

Abbreviations: WLI, white light imaging; NBI, narrow band imaging; PPV, positive predictive value; NPV, negative predictive value.

Detecting cancer at an early stage is an optimal strategy for preventing the development of advanced cancer and improving survival. Furthermore, early detection uses a minimally invasive treatment (eg, endoscopic resection) with curative intent.[8,36-38] In fact, in our study, 75% (21 of 28) of the superficial HNSCCs were completely removed by endoscopic resection or biopsy alone, while early detection of HNSCC had been quite difficult. These results provide us with new diagnostic and treatment strategies for ESCC patients, because the risk of development of HNSCC after esophagectomy is quite high.[21]

As the criteria for diagnosing superficial SCC by NBI, we used two endoscopic findings: a well-demarcated brownish area and an irregular microvascular pattern.[7-9] Using only these two findings, the sensitivity of primary NBI for the diagnosis of superficial SCC was 100% in the H&N region and 97.2% in the esophagus. The diagnostic accuracy was nearly 90%. These results indicate that these NBI findings are quite useful for the accurate diagnosis of superficial SCC.

Lugol chromoendoscopy is useful for the detection of superficial ESCC.[2-3] However, the administration of lugol solution is time-consuming, and accurate diagnosis by lugol chromoendoscopy is difficult[4] because the staining pattern shows wide variations.[2] This increases the incidence of false-positive lesions and leads to unnecessary biopsies. In contrast, NBI is easily manipulated and shows high sensitivity. Thus, NBI could reduce the number of unnecessary biopsies and shorten examination time. Furthermore, lugol chromoendoscopy is more invasive than both WLI and NBI, and WLI is still the gold standard for cancer screening. Therefore, we did not compare the diagnostic yield of NBI and lugol chromoendoscopy, and we used WLI as the standard reference to compared the diagnostic yield of WLI and NBI.

NBI required a significantly longer examination time than WLI. This might be related to the high detection rate and more frequent time spent in magnification during NBI, because if the lesions were not seen by WLI, no magnification was performed. The actual time difference between NBI and WLI was only 20 to 42 seconds. This is clinically acceptable, because the important time issue is not that NBI takes slightly longer than WLI, but rather that endoscopists spend more time in the careful observation of high-risk patients.

In this study, ESCC patients referred from another hospital were included. Even if the biopsies were previously done, the earlier biopsy sites were healed by the time of this study and were not generally detectable by either imaging method. Therefore, we thought that it was not a confounding factor.

The same endoscopists performed both imaging procedures in this study, whereas the endoscopists ideally should be separated and blinded to each imaging procedure. However, it was clinically impossible to change and blind the endoscopists during this series of examinations. Furthermore, the result produced with NBI first followed by WLI might underestimate the benefit of NBI because NBI is more sensitive than WLI. However, the detection and diagnosis of superficial SCC by NBI was significantly better than that using WLI in both the H&N region and the esophagus, regardless of whether NBI was primary or secondary. These results indicate that NBI should be the standard examination.

Significant detection results seen in this study were all achieved without the newest generation high-definition endoscope. If we use the newest high-definition endoscope with NBI, the rates of detection might increase compared with those found in this study. Furthermore, the endoscopy system used in this study and in most Asian countries was different from those used in North America and Europe.[26,27] However, we previously reported that even the nonmagnifying laryngoscope based on same system as that used in North America and Europe could dramatically improve the visualization of both the brownish area and irregular microvascular patterns.[39] Therefore, we believe that differences in the system are no longer as important as careful observation by NBI.

In conclusion, NBI combined with magnifying endoscopy significantly improved the detection rates for SCC with quite high sensitivity, and this new image-enhanced technology can be applied easily in clinical practice. Furthermore, early detection facilitates the potential of minimally invasive treatment, such as endoscopic resection or partial surgical resection.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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Excessive Fat Restriction Might Promote the Recurrence of Colorectal Tumors

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The incidence of colorectal cancer is rapidly increasing in Japan. This trend has been suggested to be caused by an increasing fat intake as a result of the Westernized diet among Japanese. We investigated whether dietary instruction optimizing the fat energy ratio suppresses the recurrence of colorectal tumors. The subjects, 373 men and women, were the participants in a randomized clinical trial of colorectal cancer prophylaxis. At entry, each participant completed a 3-consecutive-day food record on which dietary instruction was given to restrict fat energy ratio to 18–22%. Data obtained before and after the intervention were examined by cohort analysis. The primary endpoint was the presence or absence of colorectal tumor(s) at colonoscopy after 4 yr. Unexpectedly, the recurrence of tumor increased as the subjects reduced their fat intake. The lowest tumor recurrence among the men was observed in the group with 23.8–26.4% fat energy ratio after the intervention. Furthermore, in men, the risk of tumors decreased significantly as the intake of linoleic acids per body weight increased. For women, similar trends were observed. These results suggest that extreme fat restriction is highly likely to promote the recurrence of colorectal tumors, which may be partly attributable to linoleic acid deficiency.

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INTRODUCTION

Over the last several decades, the Japanese dietary habit has changed considerably. According to the National Nutrition Survey in Japan, fat intake, of animal fat in particular, has been increasing remarkably since around 1960. As compared to 1946, when the first National Nutrition Survey was conducted, fat intake had increased threefold by 1970 and even fourfold by 1995. Animal fat intake in 1995 was 4.6 times greater than that in 1955. At the same time, the disease structure in the Japanese population has been greatly changing, too. The incidence and mortality of colorectal cancer have been increasing rapidly (1,2), which resulted in colorectal cancer gaining the leading position in terms of cancer mortality among Japanese women in 2003 (1). It appears that the increase in colorectal cancer incidence followed the increase of fat intake with about a 20-yr lag (3).

Nakaji et al. (4) reported a significant correlation between fat intake and colon cancer in their cross-sectional analysis. Also, the incidence of colorectal cancer among Japanese migrants to Hawaii was, as their fat intake increased, reported to have increased much rapidly compared with that in Japanese people in Japan (5). On the other hand, Howe et al. (6) performed a meta-analysis of 13 case-control studies from all over the world. Their analysis suggested that there was no association between intakes of total fat or saturated fatty acids (SFA) and colorectal cancer (6). The second expert report issued by the World Cancer Research Fund and American Institute for Cancer Research listed only animal fat as a risk factor among fats, although judging it as "limited evidence" for its causal relation to colorectal cancer (7).

However, as far as Japanese studies are concerned, several recent results have suggested an association between fat intake and colorectal cancer. According to The Japan Public Health Center-based Prospective Study, the Western dietary pattern characterized by high intake of fat and meat was positively associated with colon cancer risk in women (8). Also, the Japan Collaborative Cohort Study indicated positive association of chicken and egg consumption with colon cancer (9).

A few clinical trials examining the preventive effects of fat-restricting dietary intervention on colorectal cancer have been reported (10–12). However, with regard to the Japanese, whose fat intake has been rapidly increasing, no such trials have ever been reported. Furthermore, most of the epidemiological studies on colorectal cancer (13–16) applied the Semi-Quantitative Food Frequency Questionnaire (FFQ). Thus far, no study has applied a 3-day diet record (DR) to all the subjects for this purpose. Since 3-day DR is based on actual intakes, it is considered to give better estimates of absolute amount of intakes for energy and fats than an FFQ (17). In addition, due to a large variety of the Japanese diet, FFQ might lack many food items actually consumed by Japanese subjects.

In this study applying a 3-day DR for dietary assessment, we investigated whether dietary instruction optimizing the fat intake could suppress the recurrence of colorectal tumors. The subjects were recruited to participate in a randomized clinical trial of colorectal cancer prophylaxis by administration of wheat bran and/or *Lactobacillus casei* performed at the Osaka Medical Center for Cancer and Cardiovascular Diseases (18).

SUBJECTS AND METHODS

Study Design and Population

The subjects were the participants of a randomized clinical trial of colorectal cancer prophylaxis by administration of wheat bran and/or a *Lactobacillus casei* preparation (18). The details of this study were previously reported (19). In brief, the subjects were 406 men and women aged 40 to 65 yr who had had at least two colorectal tumors (adenomas and/or early cancers) removed endoscopically within 3 mo before recruitment. Excluded were subjects with other malignant tumors, a history of intestinal or gastric resection (except appendectomy), familial adenomatous polyposis, and severe illness.

The subjects were randomly allocated into 4 groups to receive wheat bran, *Lactobacillus casei* preparation, both, or neither. Each subject received dietary instruction individually so that his or her fat intake would constitute 18–22% of the total energy intake.

The subjects were recruited at the Osaka Medical Center for Cancer and Cardiovascular Diseases between June 1993 and September 1997. The study protocol was approved by the Ethics Committee of this institution. All the subjects gave written informed consent.

Colonoscopy

Total colonoscopy for observation was performed 2 and 4 yr after the start of the regimen. All polyps discovered by this procedure were resected and examined histologically. The primary endpoint was the presence or absence of colorectal tumor(s) after 4 yr.

Dietary Assessment

Dietary assessments were conducted by means of a 3-day DR at entry, at 3 mo, and at 4 yr after the start of the intervention. Trained dietitians interviewed each subject individually for about 1 h to assess and record the contents of his or her meals. In principle, male participants were accompanied by a family member. Intakes of energy and nutrients were calculated using the Food Composition Database developed by the Osaka Medical Center for Cancer and Cardiovascular Diseases (19). The intake of wheat bran biscuit was included in dietary assessment after 3 mo and after 4 yr. Intakes of other health foods including food supplements were excluded from the assessment. Intake amounts of energy and nutrients before and after the intervention were represented by the mean values of 3 days at entry and those after 3 mo, respectively.

Dietary Intervention

The core of the dietary instruction was to optimize the fat intake of the subjects. Based on the results of the dietary survey at entry, each subject was advised on the selection of food items, food preparation methods, and so forth so that the energy from fat would constitute 18–22% of total energy intake. We recommended reducing both animal and vegetable fats equally. Those whose fat intake was too low were instructed to increase it. Dietary instruction was given during the interview immediately after the dietary survey at entry followed by additional comments and an individually calculated diet assessment sent by post. Compliance with the instructions was evaluated at dietary checkup 3 mo after the start of the intervention. If necessary, instruction was given again at that time. Furthermore, the subjects were encouraged to adhere to their dietary instruction by means of written dietary information handed over at the follow-up visit after 1 yr and sent by post after 2 yr.

Other Variables

Colonoscopic findings in the past, height, past medical history, medication history, and family history of the subjects were recorded at entry. Body weight was measured at entry and at each consultation. Subjects' information about drinking, smoking, physical activity, and use of health foods and/or supplements was recorded during the initial interview by the dietitian.

Statistical Analysis

Drinking and smoking status were represented by baseline values. Body weight was represented by baseline value (before) and the measurement after 3 mo (after). Crude nutrients were used in t-tests for intake of nutrients. For analysis of the rate of subjects in each category of fat energy ratio before and after the intervention, χ^2 test was used. Before relative risk analysis, nutrients were adjusted for total energy intake using the residual method (20). Energy and nutrient intakes were analyzed for men and women separately. Statistical significance was established at $P < 0.05$ for the 2-tailed test. Men were equally divided into quintiles, and women were divided into tertiles based on height, body weight, body mass index [(BMI); weight (kg)/height $(m)^2$], and energy and nutrient intakes. On the basis of unconditional logistic regression models, the lowest quintile/tertile was taken as the reference in estimating the relative risk as the odds ratio (OR) adjusted for age, BMI (<18.5, 18.5–25, and ≥25), amount of alcohol consumption (never, ≤23 g/day, and >23 g/day), current smoking status (smoker or nonsmoker), physical activity level (light or moderate), and randomization group. Linear trends in logistic regression analysis were evaluated using medians of each quintile/tertile. SPSS statistical analysis software version 15 (SPSS, Inc., Chicago, IL) was used.

RESULTS

The initial dietary survey was conducted between June 1993 and April 1998. Colonoscopy after 4 yr was completed in February 2002. Among 406 subjects who participated in the initial dietary survey, 373 subjects completed colonoscopic examination after 4 yr as well as dietary assessment/instruction after 3 mo.

Table 1 shows the baseline characteristics of the subjects consisting of 305 men and 68 women; approximately 80% were men. More than 80% of both sexes had low levels of physical activity. By colonoscopy after 4 yr, recurrence of colorectal tumors was diagnosed in 53.1% of men and 47.1% of women; that is, 51.7%–52.0% of subjects overall. Virtually all cases were precancerous lesions, including adenoma and intramucosal cancer, and only one case of colorectal cancer was diagnosed as adenoma with severe dysplasia. Elevated risk of colorectal cancer was associated with higher values of age, body weight, and height; whereas no significant correlation was found between the colorectal cancer risk and physical activity, drinking, or smoking.

For the data analysis after intervention, mean values of 3 days at 3 mo were used throughout. An alternative analysis using mean values of 6 days, 3 days each at 3 mo and at 4 yr was also performed with the similar but somewhat obscured results (data not shown). The number of subjects with fat energy ratio of 18–22% increased significantly from 97 (26.0%) at baseline to 112 (30.0%) after the intervention ($P = 0.01$) as shown in Fig. 1. Among the subjects with the highest fat energy ratio at baseline (>22%), the risk of developing colorectal tumors increased substantially in the subjects who reduced the ratio after the intervention. When the subjects with unchanged fat energy ratio (>22%) after the intervention was taken as reference, OR of those with the ratio reduced to 18–22% was 2.16 and OR of those with the ratio reduced to <18% was 4.45.

TABLE 1
Baseline characteristics of subjects

	Men ($n = 305$)	Women ($n = 68$)
Age (yr)[a]	54.8 ± 6.1	56.3 ± 6.3
Height (cm)[a]	166.5 ± 6.0	153.4 ± 4.5
Body weight (kg)[a]	65.4 ± 9.4	53.8 ± 6.7
Body mass index (kg/m^2)[a]	23.9 ± 2.6	22.9 ± 2.8
Physical activity[b]		
Light	256 (83.9)	55 (80.9)
Moderate	49 (16.1)	13 (19.1)
Current smokers[b]	151 (49.5)	12 (17.6)
Alcohol intake[b]		
Never	41 (13.4)	46 (67.6)
≤23.0 g/day	112 (36.7)	17 (25.0)
>23.0 g/day	152 (49.8)	5 (7.4)

[a]Values are means ± SD.
[b]Values are number; values in parentheses are percent.

Energy and major nutrient intakes before and after the intervention are shown in Table 2. In both men and women, the intake of dietary fiber was significantly increased after the intervention. In men, the intakes of energy, total fat, carbohydrate, calcium, iron, and vitamin C were significantly decreased; whereas in women, significant decreases were found in energy and carbohydrate intakes.

Table 3 and 4 show energy and energy-adjusted nutrient intakes at baseline in relation to tumor recurrence for men and women, respectively. A significant decrease in OR was found only in the third quintile for linoleic acid (LA) intake per body weight in men [OR = 0.36, 95% confidence interval (CI) = 0.17–0.77].

Energy and energy-adjusted nutrient intakes after the intervention in relation to tumor recurrence for men is shown in Table 5. OR decreased significantly in the fourth quintile, with fat energy ratio of 23.8–26.4% compared with the lowest quintile (OR = 0.23, 95% CI = 0.11–0.50). As for total fat, OR in the fourth quintile showed a significant decrease. Furthermore, OR decreased significantly in the fifth quintile for saturated fatty acids (SFA) and in the fourth and fifth quintiles for monounsaturated fatty acids (MUFA). For polyunsaturated fatty acids (PUFA), a

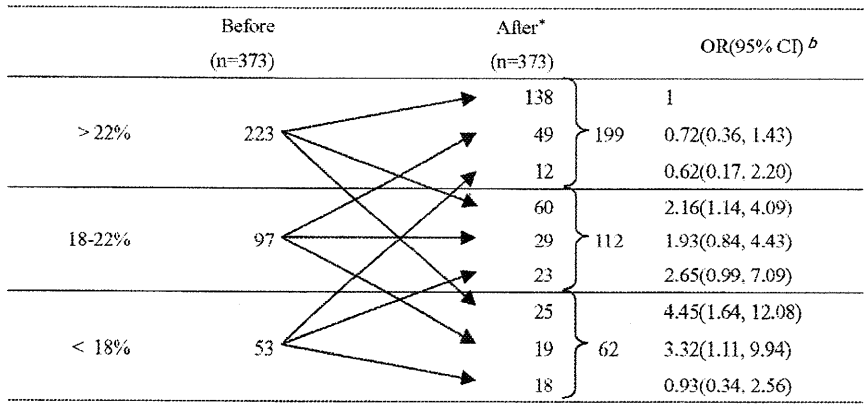

FIG. 1. Number of subjects at before and after dietary intervention according to fat energy ratio (%) (<18%, 18–22%, or >22%) in 373 subjects (305 men and 68 women) [a]. a: Statistical significance is as follows: *, $P < 0.01$ vs. before. b: Odds ratios (OR) adjusted for age, body mass index, physical activity, alcohol use, current smoking status and randomization group, with 95% confidences intervals (CIs) in parentheses.

significant decreasing trend was observed (P for trend = 0.04). Also for LA intake, a significant decreasing trend in OR was observed (P for trend = 0.02), with significantly lower risk in the highest quintile (OR = 0.42, 95% CI = 0.19–0.89). The greatest risk reduction in terms of fatty acid intakes per body weight was observed in LA. For LA intake per body weight, the second (OR = 0.38, 95% CI = 0.18–0.80), the fourth (OR = 0.46, 95% CI = 0.21–0.97), and the fifth (OR = 0.36, 95% CI = 0.17–0.78) quintiles had significantly decreased ORs, all of which were less than half compared with the lowest quintile.

Table 6 shows energy and energy-adjusted nutrient intakes after the intervention in relation to tumor recurrence for women. Significant decreases in OR were found in the highest tertile of SFA (OR = 0.17, 95% CI = 0.04–0.75) and MUFA (OR = 0.12, 95% CI = 0.02–0.60). For linolenic acid (ALA), a significant decreasing trend in OR was observed (P for trend = 0.03). As for LA, ORs for LA intake per body weight in the 2 highest tertiles decreased to less than half, although not significant, compared with the lowest tertile.

At 3 mo after the start of intervention, the mean value and SD of LA intake were 9.5 ± 2.9 g for men and 8.7 ± 2.6 g for women; 25.7% of the subjects overall had fat intakes of less than 7.5 g. Furthermore, no association was found between tumor recurrence risk and eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), or docosahexaenoic acid (DHA) in both men and women. In both sexes, strong correlations were found between LA and SFA, MUFA, PUFA, or ALA (r = 0.53, 0.75, 0.96, and 0.91, respectively, $P < 0.01$ for men; r = 0.62, 0.73, 0.92, and 0.92, respectively, $P < 0.01$, for women).

DISCUSSION

This study revealed that the relative risk of recurrence of colorectal tumor after 4 yr was higher in subjects who reduced their fat energy ratio.

TABLE 2
Energy and nutrient intakes of subjects before and after dietary intervention[a]

	Men (n = 305)		Women (n = 68)	
	Before	After	Before	After
Energy (kcal/day)	2,171 ± 371	2,078 ± 344**	1,770 ± 307	1,708 ± 230*
Protein (g/day)	85.9 ± 16.5	84.1 ± 15.6	72.5 ± 14.8	71.3 ± 11.1
Total fat (g/day)	55.3 ± 15.4	51.8 ± 14.3**	49.6 ± 12.2	48.0 ± 10.7
Fat energy ratio (%)	23.0 ± 5.2	22.4 ± 4.9	25.3 ± 4.7	25.3 ± 4.2
Carbohydrate (g/day)	278 ± 65	267 ± 55**	250 ± 51	239 ± 38**
Total fiber (g/day)	15.0 ± 4.0	15.6 ± 4.5**	16.1 ± 4.5	17.1 ± 4.4*
Calcium (mg/day)	636 ± 225	603 ± 211**	699 ± 290	681 ± 216
Iron (mg/day)	11.5 ± 2.8	11.0 ± 2.9**	10.9 ± 3.1	10.8 ± 2.5
Carotenoids (μg/day)	2,809 ± 1,607	2,715 ± 1,831	3,231 ± 1,727	3,075 ± 1,433
Vitamin C (mg/day)	126 ± 60	119 ± 60**	150 ± 64	145 ± 60

[a]Values are means ± SD. Statistical significance is as follows: *, $P < 0.05$, **, $P < 0.01$ vs. before.

TABLE 3
Odds ratios (ORs) and 95% confidence intervals (CIs) for tumor recurrence according to quintiles of energy and energy-adjusted nutrient intakes before dietary intervention in men

	1 (low; $n = 61$)	2 ($n = 61$)	3 ($n = 61$)	4 ($n = 61$)	5 (high; $n = 61$)	P^a
Energy intake (kcal/day)[b]	1,277–1,880	1,880–2,062	2,062–2,246	2,246–2,454	2,454–3,855	
No. of cases	35	27	33	29	38	
OR (95% CI)[c]	1.0	0.59 (0.28–1.23)	0.89 (0.43–1.84)	0.64 (0.30–1.35)	1.15 (0.54–2.44)	0.73
Fat energy ratio (%)[b]	10.1–18.6	18.6–21.6	21.6–24.2	24.2–27.0	27.0–49.5	
No. of cases	35	28	36	30	33	
OR (95% CI)[c]	1.0	0.66 (0.32–1.37)	1.18 (0.56–2.48)	0.83 (0.40–1.74)	0.97 (0.47–2.04)	0.89
Total fat (g/day)[b]	6.4–43.5	43.5–50.2	50.2–56.0	56.0–64.4	64.4–119.5	
No. of cases	35	26	38	36	27	
OR (95% CI)[c]	1.0	0.58 (0.28–1.20)	1.41 (0.67–2.97)	1.32 (0.62–2.81)	0.63 (0.30–1.32)	0.93
Saturated fatty acids (g/day)[b]	0.2–10.6	10.6–12.9	12.9–15.1	15.1–17.6	17.6–28.0	
No. of cases	35	33	32	27	35	
OR (95% CI)[c]	1.0	0.90 (0.43–1.87)	0.91 (0.44–1.89)	0.61 (0.30–1.28)	1.17 (0.55–2.46)	0.86
Monounsaturated fatty acids (g/day)[b]	0.0–14.1	14.1–17.1	17.1–19.8	19.8–23.1	23.1–57.3	
No. of cases	34	34	32	30	32	
OR (95% CI)[c]	1.0	1.05 (0.50–2.17)	0.97 (0.47–2.00)	0.81 (0.39–1.68)	0.96 (0.46–2.01)	0.41
Polyunsaturated fatty acids (g/day)[b]	4.3–10.9	10.9–12.6	12.6–14.3	14.3–16.2	16.2–30.2	
No. of cases	33	33	33	30	33	
OR (95% CI)[c]	1.0	1.03 (0.50–2.14)	1.09 (0.53–2.24)	0.96 (0.46–2.01)	1.03 (0.50–2.14)	0.99
Linolenic acids (g/day)[b]	0.1–1.3	1.3–1.5	1.5–1.9	1.9–2.3	2.3–4.0	
No. of cases	35	30	38	32	27	
OR (95% CI)[c]	1.0	0.69 (0.33–1.42)	1.27 (0.61–2.66)	0.87 (0.42–1.81)	0.58 (0.28–1.22)	0.43
Linoleic acids (g/day)[b]	3.0–7.8	7.8–9.3	9.3–10.5	10.5–12.0	12.0–26.4	
No. of cases	34	36	32	29	31	
OR (95% CI)[c]	1.0	1.17 (0.56–2.47)	0.95 (0.46–1.97)	0.75 (0.36–1.57)	0.87 (0.42–1.82)	0.24
Linoleic acids per body weight (mg/kg/day)[b]	61–117	117–144	144–171	171–201	201–336	
No. of cases	37	39	22	34	30	
OR (95% CI)[c]	1.0	1.22 (0.58–2.57)	0.36 (0.17–0.77)	0.88 (0.42–1.88)	0.71 (0.33–1.51)	0.45

[a]Test for linear trend.
[b]Values in parentheses are range.
[c]OR adjusted for age, body mass index, physical activity, alcohol use, current smoking status, and randomization group, with 95% CI in parentheses.

Around the period of this study, the Recommended Dietary Allowance (RDA) of fat energy ratio for the Japanese was 20–25% (21). Since we inferred that lower fat intakes would be more beneficial for a high-risk group for colorectal cancer, we took the lowest value of the RDA with 2% margins on both sides and determined our target fat energy ratio of 18–22%. In fact, risk reduction was observed in groups with higher fat energy ratio.

According to the Dietary Reference Intakes of the United States and Canada, based on the results of intervention studies, fat energy ratio of at least 20% is recommended to maintain normal levels of serum lipids such as HDL cholesterol (22). In an intervention study reducing fat energy ratio to 20%, McKeown-Eyssen et al. (10) reported a significant risk reduction (relative risk = 0.6; 95% CI = 0.4–0.9) in the male subjects with the highest fat energy ratio.

Two previous intervention trials have reported no effect of fat restriction on the risk of colorectal tumor recurrence (11,12,23). Compared with those studies, our study was clearly different in that the subjects had a relatively low fat energy ratio at baseline, that is, 23.0% and 25.3% for men and women, respectively. The average fat energy ratio of the Japanese population, even after a rapid increase, is reported to be about 25% (24), which is substantially lower than that of Western people. Besides, our

TABLE 4
Odds ratios (ORs) and 95% confidence intervals (CIs) for tumor recurrence according to tertiles of energy and energy-adjusted nutrient intakes before dietary intervention in women

	1 (low; $n = 22$)	2 ($n = 23$)	3 (high; ($n = 23$)	P^a
Energy intake (kcal/day)[b]	851–1,646	1,646–1,864	1,864–2,462	
No. of cases	10	11	11	
OR (95% CI)[c]	1.0	0.77 (0.20–2.98)	0.61 (0.15–2.58)	0.17
Fat energy ratio (%)[b]	15.5–23.2	23.2–27.7	27.7–36.1	
No. of cases	10	11	11	
OR (95% CI)[c]	1.0	1.13 (0.31–4.10)	1.09 (0.27–4.30)	0.55
Total fat (g/day)[b]	35.9–52.7	52.7–59.1	59.1–78.3	
No. of cases	10	11	11	
OR (95% CI)[c]	1.0	0.92 (0.25–3.42)	0.88 (0.22–3.49)	0.19
Saturated fatty acids (g/day)[b]	9.1–13.9	13.9–17.1	17.1–22.8	
No. of cases	11	14	7	
OR (95% CI)[c]	1.0	1.87 (0.48–7.29)	0.24 (0.05–1.14)	0.63
Monounsaturated fatty acids (g/day)[b]	11.8–17.6	17.6–21.6	21.6–28.7	
No. of cases	10	12	10	
OR (95% CI)[c]	1.0	1.06 (0.28–3.97)	0.76 (0.19–2.99)	0.40
Polyunsaturated fatty acids (g/day)[b]	8.6–12.8	12.8–14.8	14.8–23.4	
No. of cases	9	12	11	
OR (95% CI)[c]	1.0	2.29 (0.54–9.64)	1.13 (0.29–4.44)	0.91
Linolenic acids (g/day)[b]	1.0–1.5	1.5–2.0	2.0–3.6	
No. of cases	9	9	14	
OR (95% CI)[c]	1.0	1.19 (0.30–4.75)	2.24 (0.58–8.62)	0.21
Linoleic acids (g/day)[b]	5.0–9.3	9.3–11.4	11.4–19.2	
No. of cases	9	13	10	
OR (95% CI)[c]	1.0	2.88 (0.69–11.97)	0.82 (0.20–3.29)	0.23
Linoleic acids per body weight (mg/kg/day)[b]	62–154	154–193	193–358	
No. of cases	10	13	9	
OR (95% CI)[c]	1.0	1.39 (0.38–5.14)	0.40 (0.09–1.82)	0.52

[a]Test for linear trend.
[b]Values in parentheses are range.
[c]OR adjusted for age, body mass index, physical activity, alcohol use, current smoking status, and randomization group, with 95% CI in parentheses.

subjects had an even slightly lower energy intake and fat energy ratio compared with those of the overall Japanese population according to the National Nutrition Survey in 1999, conducted during this study period (24). This might be explained by our subjects' background; many of them had low levels of physical activity and probably had reduced their fat and meat intake after having been diagnosed with multiple colorectal tumors. Therefore, it is possible that restricting fat in subjects with originally low fat intakes made its harmful effect more evident. It should be noted that this study, as well as other clinical trials with fat restriction discussed here (11,12,22,23), has examined risk of recurrence among the high-risk group for colorectal cancer, namely, the patients who previously underwent multiple tumor resection. Therefore, our results should be interpreted with caution when discussing the initial development of tumors in the general population.

As to the question which specific fatty acid(s) might be involved in it, LA is suspected since we observed the clearest trend of increasing risk as LA intake decreased. With regard to the daily requirement of LA, Collins et al. (25) investigated patients with total parenteral nutrition, who were at risk of LA deficiency, and reported the need for at least 7.5 g/day for adult men. In our study, 25.7% of the subjects did not reach such levels of LA intake. On the other hand, some Japanese researchers have linked excessive LA intake to inflammatory bowel disease, atopy, and asthma, creating such a situation in Japan that food manufacturers have reduced LA content in their oil products (26). Partly because of this situation, it is possible that Japanese people are nowadays easily at risk of LA deficiency. Tuyns et al. (27) reported that LA consistently decreased the risk of colon and rectal cancer in their case-control study in which LA intake among the cases was as low as in our study. In contrast, 3

TABLE 5
Odds ratios (ORs) and 95% confidence intervals (CIs) for tumor recurrence according to quintiles of energy and energy-adjusted nutrient intakes after dietary intervention in men

	1 (low; $n = 61$)	2 ($n = 61$)	3 ($n = 61$)	4 ($n = 61$)	5 (high; $n = 61$)	P^a
Energy intake (kcal/day)[b]	1,107–1,806	1,806–1,972	1,972–2,148	2,148–2,342	2,342–3,240	
No. of cases	31	34	31	28	38	
OR (95% CI)[c]	1.0	1.20 (0.58–2.47)	0.99 (0.48–2.05)	0.80 (0.39–1.64)	1.53 (0.72–3.21)	0.44
Fat energy ratio (%)[b]	8.8–18.1	18.1–20.9	20.9–23.8	23.8–26.4	26.4–37.9	
No. of cases	40	38	35	20	29	
OR (95% CI)[c]	1.0	0.88 (0.41–1.85)	0.67 (0.32–1.41)	0.23 (0.11–0.50)	0.47 (0.22–0.98)	0.22
Total fat (g/day)[b]	12.8–40.6	40.6–46.7	46.7–53.2	53.2–58.9	58.9–89.0	
No. of cases	39	39	33	21	30	
OR (95% CI)[c]	1.0	0.97 (0.46–2.04)	0.62 (0.30–1.30)	0.27 (0.13–0.59)	0.50 (0.24–1.05)	0.08
Saturated fatty acids (g/day)[b]	3.1–10.2	10.2–11.8	11.8–13.4	13.4–15.9	15.9– 29.7	
No. of cases	40	34	30	34	24	
OR (95% CI)[c]	1.0	0.70 (0.33–1.49)	0.53 (0.25–1.13)	0.70 (0.33–1.47)	0.36 (0.17–0.75)	0.05
Monounsaturated fatty acids (g/day)[b]	2.0–12.7	12.7–15.3	15.3–18.0	18.0–21.0	21.0–35.1	
No. of cases	38	41	31	25	27	
OR (95% CI)[c]	1.0	1.22 (0.57–2.59)	0.60 (0.29–1.26)	0.41 (0.19–0.86)	0.47 (0.22–0.99)	0.11
Polyunsaturated fatty acids (g/day)[b]	4.8–9.9	9.9–11.7	11.7–13.0	13.0–15.2	15.2–26.3	
No. of cases	37	38	33	29	25	
OR (95% CI)[c]	1.0	1.13 (0.54–2.37)	0.80 (0.39–1.67)	0.63 (0.30–1.31)	0.48 (0.23–1.02)	0.04
Linolenic acids (g/day)[b]	0.1–1.1	1.1–1.4	1.4–1.7	1.7–2.1	2.1–4.7	
No. of cases	37	28	40	30	27	
OR (95% CI)[c]	1.0	0.58 (0.28–1.21)	1.31 (0.61–2.80)	0.68 (0.33–1.42)	0.55 (0.26–1.16)	0.46
Linoleic acids (g/day)[b]	2.0–7.0	7.0–8.4	8.4–9.6	9.6–11.3	11.3–20.7	
No. of cases	40	35	31	30	26	
OR (95% CI)[c]	1.0	0.75 (0.35–1.61)	0.54 (0.26–1.15)	0.56 (0.26–1.12)	0.42 (0.19–0.89)	0.02
Linoleic acids per body weight (mg/kg/day)[b]	32–107	107–128	128–151	151–181	181–337	
No. of cases	43	29	32	31	27	
OR (95% CI)[c]	1.0	0.38 (0.18–0.80)	0.49 (0.23–1.05)	0.46 (0.21–0.97)	0.36 (0.17–0.78)	0.18

[a]Test for linear trend.
[b]Values in parentheses are range.
[c]OR adjusted for age, body mass index, physical activity, alcohol use, current smoking status, and randomization group, with 95% CI in parentheses.

other case-control studies (28–30) and a cohort study (31) did not show such trends regarding LA in particular or n-6PUFA in general. In those studies, LA intake in the subjects was much higher than in our subjects, suggesting no concern regarding LA deficiency.

In addition, there is another factor in relation to the increased risk in the subjects who reduced their fat energy ratio. In one study, a possible role of stress in the development of tumors was suggested (32). If so, radical alteration of diet in our subjects might have given them stress, which promoted to some extent the recurrence of colorectal tumors.

Furthermore, the outcome of dietary instruction was not satisfactory; less than a half of the subjects met our target fat energy ratio of 18–22%. This could be explained as follows. First, the intake of hidden fat contained in meat or fish might not have changed, as subjects had no grasp of it. One study reported that the awareness of subjects regarding fat did not necessarily affect contents of their diet (33). Second, during the time of this study, little information for consumers was available regarding fat content of food items because very few products had fat content labeling (34). Third, our dietary instruction focused on fat restriction, whereas attention to increased intakes of protein and carbohydrate to substitute fat might have been insufficient. As a consequence, our subjects might have reduced the whole intake amount of diet, which affected optimization of fat energy ratio negatively.