Gastric cancer is the second leading cause of cancer deaths worldwide.[1] Early detection and accurate diagnosis of depressed gastric mucosal cancer are effective ways to decrease mortality because the depressed type is the predominant morphology among gastric mucosal cancers.[2-4] Moreover, detection of mucosal cancers ≤ 20 mm in diameter is ideal because they are curable with minimally invasive treatments such as EMR and endoscopic submucosal dissection (ESD).[5,6] However, these approaches have proven difficult when using conventional white-light imaging (C-WLI) endoscopy because depressed-type cancer shows subtle morphologic changes. Accurate diagnosis is hampered by the lack of reliable diagnostic criteria. A novel endoscopic technology, magnifying narrow-band imaging (M-NBI), is a powerful tool for characterizing gastric mucosal lesions because it can visualize the microvascular architecture as well as morphology of such lesions.[7]

We performed a multicenter, prospective, randomized, controlled trial and reported that M-NBI was more useful than C-WLI in terms of the ability to diagnose small, depressed gastric cancerous lesions (UMIN-CTR 000001072).[8] In this randomized controlled trial, 2 criteria,[9,10] the presence of a demarcation line (DL) and an irregular microvascular pattern (IMVP), were used for the endoscopic evaluation of lesions using M-NBI, whereas the presence of an irregular margin (IM) and spiny depressed area (SDA) were used for C-WLI evaluation. However, the endoscopic findings that contribute to the accurate diagnosis of small, depressed gastric cancerous lesions have not been fully identified. Moreover, M-NBI still leads to misdiagnosis of some lesions, and the reasons for these misdiagnoses are unclear. Therefore, the aim of this study was to identify an efficient diagnostic strategy using the most reliable endoscopic findings to diagnose early gastric cancers and propose an ideal diagnostic approach to these cancers.

Take-home Message

- The diagnostic performance of magnifying narrowband imaging (M-NBI) after conventional white-light imaging (C-WLI) using a demarcation line (DL) and an irregular microvascular pattern (IMVP) was significantly high for small, depressed gastric lesions.
- In M-NBI after C-WLI, it is ideal to identify the DL first to diagnose small, depressed gastric cancer, and the subsequent IMVP inspection efficiently excludes false-positive lesions by the DL. The reasons for misdiagnoses include technical and cognitive factors; thus, training should involve both aspects.

METHODS

Study design and endoscopic procedure

This study was conducted as a post-hoc analysis of data collected in our randomized controlled trial.[8] The protocol of the trial was approved by the Ethics Committee of the Kyoto University Graduate School of Medicine on February 14, 2008. The UMIN Clinical Trials Registry identification number for this study is 000001072 on March 15, 2008. In the trial, 1353 patients with concomitant gastric cancer or a history of endoscopic resection of early gastric cancer were enrolled and underwent endoscopic screening with C-WLI between June 2008 and May 2010. The target lesions were "newly detected and undiagnosed" small, depressed gastric lesions ≤ 10 mm in diameter. Only the first lesion detected in each patient was selected for examination.

Among all patients 353 previously undiagnosed lesions were found in 362 patients that were randomly assigned to the M-NBI (n = 177) and C-WLI (n = 176) groups. The diagnosis for the target lesion was made on-site by 1 endoscopist according to predetermined diagnostic criteria for C-WLI and M-NBI, and the result was recorded on a case report form. For the C-WLI group, M-NBI examination was performed after completion of a diagnosis based on C-WLI (M-NBI after C-WLI) to evaluate the effect of using M-NBI in conjunction with C-WLI. At least 2 endoscopic images of the target lesion in each mode were captured and stored in a computer server during the diagnosis. After compilation of all endoscopic diagnoses, at least 1 biopsy specimen was obtained from the target lesion. Lesions diagnosed as cancer or suspicious for cancer were removed by EMR/ESD to obtain a final histologic diagnosis. The demographics of the study samples are summarized in Table 1.

The biopsy and EMR/ESD specimens were evaluated based on the revised Vienna classification. Category C4 (mucosal high-grade neoplasia) and C5 (submucosal invasion by neoplasia) were diagnosed as cancerous lesions, and C1 (negative for neoplasia), C2 (indefinite for neoplasia), and C3 (mucosal low-grade neoplasia) were diagnosed as noncancerous lesions. When indeterminate lesions were encountered, we consulted with a main expert pathologist as a central review system to obtain a final diagnosis. The lesions in the M-NBI group comprised 20 cancerous and 157 noncancerous lesions, and those in the C-WLI group comprised 20 cancerous and 156 noncancerous lesions (Fig. 1). The prevalence rate was almost identical in both groups (11. 2% and 11.3%, respectively).

As described in the previous trial,[8] this study was conducted according to the Standards for the Reporting of Diagnostic Accuracy Studies initiative[11] and the Declaration of Helsinki. Randomization and masking were strictly enforced. Thirty-one endoscopists from 9 institutions in Japan participated after being trained in the acquisition of C-WLI and M-NBI images of small, depressed lesions to minimize diagnostic variation among observers. Ethical concerns were fully addressed.

Endoscopy system and setting

The video endoscopy system used in this study comprised a video processor (EVIS LUCERA CV-260SL; Olympus Medical Systems, Tokyo, Japan) and a light source

TABLE 1. Demographics of the study sample

	C-WLI group (n = 176)	M-NBI group (n = 177)	P
Median age, y	69	69	.56
Gender			
Male	138	140	.79
Female	38	37	
Mean SDL size, mm	5.6	5.6	.97
SDL location (longitudinal)			
Upper third	39	27	.21
Middle third	40	49	
Lower third	97	101	
SDL location (circumferential)			
Anterior wall	29	32	
Lessor curvature	47	68	.06
Posterior wall	60	41	
Greater curvature	40	36	
Endoscope			
GIF-Q240Z	71	65	
GIF-FQ240Z	1	3	.83
GIF-H260Z	104	109	
Histology			
Noncancerous	156	157	1.00
Cancer	20	20	

SDL, Small, depressed lesion; *M-NBI*, magnifying narrow-band imaging; *C-WLI*, conventional white-light imaging.

(EVIS LUCERA Olympus CLV-260SL; Olympus Medical Systems) that worked in both the C-WLI and NBI modes. In the NBI mode, narrow-banded short-wavelength lights (400-430 nm and 525-555 nm) were used to contrast the microvascular architecture and mucosal surface of the superficial mucosa.[12-14] High-resolution magnifying endoscopy with a capability of 80-fold optical magnification was used (GIF-Q240Z, GIF-H260Z, and GIF-FQ260Z; Olympus Medical Systems). A soft black hood (MB162 or MB46; Olympus Medical Systems) was attached at the tip of the endoscope. The structure enhancement of the endoscopic video processor was set to B-mode level 4 or 6 for C-WLI and to B-mode level 8 for M-NBI. The color mode was fixed at level 1.

Endoscopic criteria used to diagnose cancers

The 2 criteria[9,10] used in the endoscopic evaluation of lesions using M-NBI were the presence of a DL and an IMVP (Fig. 2). An IMVP refers to microvessels that differ in shape, take the shape of a closed or open loop, or are tortuous, branched, or bizarrely shaped. The vessels differ in both size and diameter, and the distribution of the microvessels is asymmetric with an irregular arrangement. The criteria used in the endoscopic evaluation of lesions using C-WLI were the presence of an IM and an SDA (Fig. 3). These findings were independently assessed and documented on a 3-point scale (present, absent, or indeterminate). Endoscopic diagnoses using both C-WLI and M-NBI were determined according to the combined visibility of the 2 findings. (1) If both findings were present, the diagnosis was cancer. (2) In the event of a combination other than pattern (1), the diagnosis was a noncancerous lesion.

Outcome measurements

Using the outlined criteria from C-WLI, M-NBI alone, and M-NBI after C-WLI, we compared the endoscopic diagnosis with the histologic diagnosis to determine the positive numbers of endoscopic findings in cancerous and noncancerous lesions, accuracy, sensitivity, specificity, positive predictive value, and negative predictive value. The diagnostic performance of each diagnostic criterion among C-WLI, M-NBI alone, and M-NBI after C-WLI was analyzed.

To clarify the reasons for incorrect diagnoses after reviewing the M-NBI findings and to extract the information that can be efficiently used in the training for M-NBI examination of early gastric cancers, the 2 experienced endoscopists who had analyzed more than 3000 endoscopic procedures using M-NBI reviewed the electronic images recorded in an image database for all facilities.

Statistical analysis

Demographics of the study samples between the C-WLI group and M-NBI group were compared using the Mann-Whitney U test for age and lesion size and the χ^2 test for gender, lesion location, endoscopy system, and histologic findings. Analyses of differences in the association between each endoscopic finding and cancer as well as analyses of differences in the diagnostic performance of the endoscopic findings provided by C-WLI, M-NBI alone, and M-NBI after C-WLI were compared using Pearson's χ^2 test and data from subjects with histopathologically confirmed diagnoses. Positive numbers of endoscopic findings in cancerous and noncancerous lesions were calculated with respect to relative risk.

In addition, all lesions diagnosed incorrectly using M-NBI were analyzed in terms of their endoscopic findings together with their histologic findings. The differences in the characteristics between correct and incorrect diagnoses were compared using the Mann-Whitney U test for lesion size and inspection time and using the χ^2 test for lesion location.

All P values were 2-sided and were not adjusted for multiple tests. $P < .05$ were considered statistically significant. All statistical analyses were performed using the Dr. SPSS II statistical software package (SPSS Japan Inc., Tokyo, Japan).

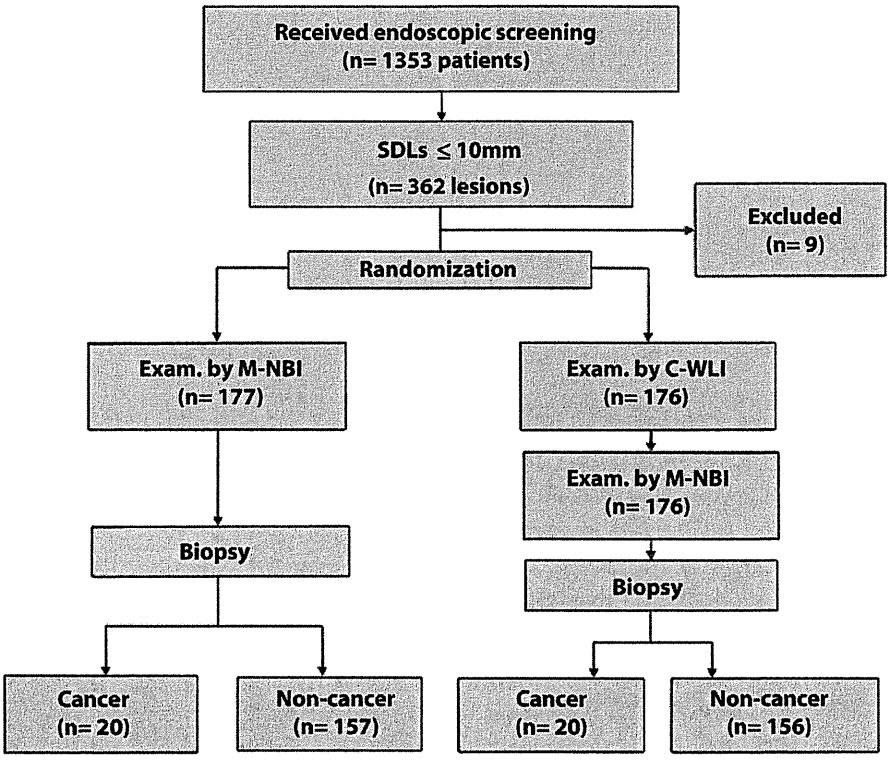

Figure 1. Trial profile, randomization, and examination. In this study, 1353 patients with concomitant gastric cancer or a history of endoscopic resection of early gastric cancer were enrolled and underwent endoscopic screening with C-WLI. Among these patients, 353 previously undiagnosed lesions were found in 362 patients that were randomly assigned to the M-NBI (n = 177) and C-WLI (n = 176) groups. *SDLs*, Small, depressed lesion; *M-NBI,* magnifying narrowband imaging; *C-WLI,* conventional white-light imaging.

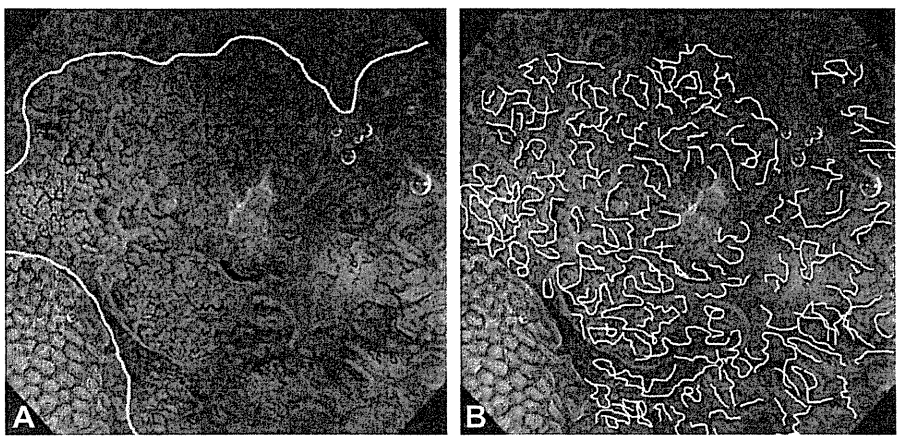

Figure 2. Endoscopic findings from magnifying narrowband imaging (M-NBI), **A,** A demarcation line (DL) is present between a depressed lesion and the surrounding mucosa *(yellow lines)*. **B,** An irregular microvascular pattern (IMVP), is diagnosed if the vessels differ in shape or are a closed loop, open loop, tortuous, branched, or bizarrely shaped. The size of the vessels also varies, and their arrangement and distribution are irregular and asymmetric, respectively *(white lines)*.

RESULTS

Association between each endoscopic finding and histologic result

Table 2 shows the association between each endoscopic finding in the cancerous and noncancerous lesions as diagnosed by C-WLI, M-NBI alone, and M-NBI after C-WLI. All endoscopic findings in C-WLI showed no significant differences between cancerous and noncancerous lesions. However, all endoscopic findings (DL, IMVP, and both a DL and an IMVP) in both the M-NBI alone and M-NBI after C-WLI groups were significantly associated with the diagnosis of cancerous lesions ($P < .01$ for all). In particular, IMVP and both a DL and an IMVP had strong

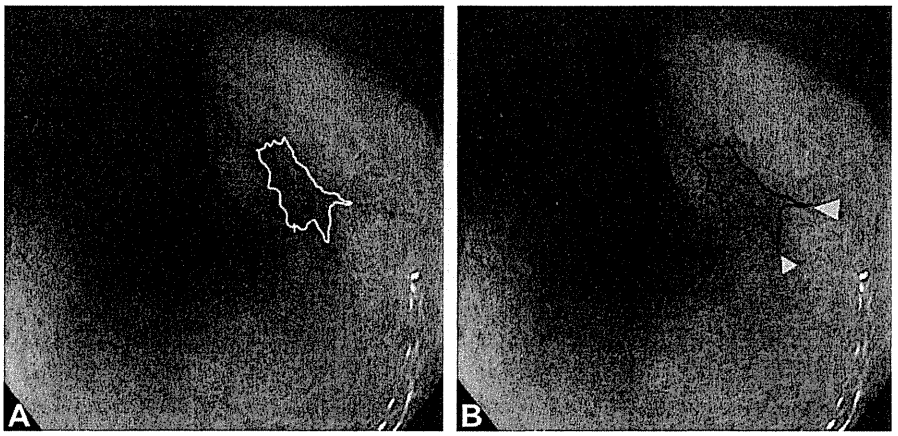

Figure 3. Endoscopic findings from conventional white-light imaging (C-WLI). **A,** An IM indicates the presence of an IM between a small, depressed lesion and the surrounding mucosa (*yellow line*). **B,** A spiny depressed area (SDA) indicates the presence of an SDA at the edge of a small, depressed lesion (*yellow arrowheads*).

associations with a cancer diagnosis (relative risks of 7.9 and 10.5 in M-NBI alone and 24.7 and 29.6 in M-NBI after C-WLI, respectively).

Diagnostic performance of each endoscopic finding

Table 3 shows the diagnostic performance according to each endoscopic finding from C-WLI, M-NBI alone, and M-NBI after C-WLI.

Accuracy. The accuracy of both M-NBI alone (90%; 95% confidence interval [CI], 85%-94%) and M-NBI after C-WLI (97%; 95% CI, 93%-99%) using a DL and an IMVP was significantly better than that of C-WLI using an IM and an SDA (65%; 95% CI, 57%-72%).

Sensitivity and specificity. Figure 4 shows the results of the comparison of the sensitivity and specificity of individual endoscopic findings provided by C-WLI, M-NBI alone, and M-NBI after C-WLI. The endoscopic findings of C-WLI were low in sensitivity (75% [95% CI, 51%-91%] for IM, 40% [95% CI, 19%-64%] for SDA, and 40% [95% CI, 19%-64%] for IM and SDA) and specificity (44% [95% CI, 36%-52%] for IM, 64% [95% CI, 56%-72%] for SDA, and 68% [95% CI, 60%-75%] for IM and SDA). The endoscopic diagnostic performance increased for C-WLI, followed by M-NBI alone; M-NBI after C-WLI ultimately showed the best diagnostic performance.

The sensitivity of an IMVP was low (60%; 95% CI, 36%-81%) in M-NBI alone, indicating that IMVP evaluation using M-NBI alone could lead to misdiagnosis of some cancers, and the sensitivity of an IMVP did not improve by combining it with evaluation of a DL (60%; 95% CI, 36%-81%). The sensitivity of an IMVP and both a DL and an IMVP in M-NBI alone significantly improved when they were evaluated after C-WLI (95% [95% CI, 75%-100%] and 95% [95% CI, 75%-100%]; $P = .02$ and $P = .02$, respectively). The specificity of an IMVP and both a DL and an IMVP was high in M-NBI alone (92% [95% CI, 87%-96%] and 94% [95% CI, 89%-97%], respectively) and M-NBI after WLI (96% [95% CI, 92%-99%] and 97% [95% CI, 93%-100%], respectively), suggesting that the presence of an IMVP indicates a high probability of cancer.

The sensitivity of a DL in M-NBI alone and M-NBI after C-WLI was high (85% [95% CI, 62%-97%] and 95% [95% CI, 75%-100%], respectively), whereas the specificity of these findings (53% [95% CI, 45%-61%] and 49% [95% CI, 41%-58%], respectively) was significantly lower than that of an IMVP (92% [95% CI, 87%-96%] and 96% [95% CI, 92%-99%]; $P = .000$ and $P = .000$, respectively). The specificity of a DL in M-NBI alone and M-NBI after C-WLI improved significantly when evaluated in combination with an IMVP (94% [95% CI, 89%-97%] and 97% [95% CI, 93%-100%]; $P = .000$ and $P = .000$, respectively). This suggests that DL is a reliable finding for identification of cancer but it needs to be evaluated with C-WLI findings and the presence of an IMVP to exclude false-positive lesions.

Positive predictive value and negative predictive value. The positive predictive value of a DL in M-NBI alone and M-NBI after C-WLI were 19% (95% CI, 11%-28%) and 19% (95% CI, 13%-29%), respectively, and were similar to those in C-WLI (15% [95% CI, 8%-23%] for IM, 13% [95% CI, 6%-23%] for SDA, and 14% [95% CI, 6%-25%] for IM and SDA). The low positive predictive value of a DL in M-NBI alone and M-NBI after C-WLI improved significantly when evaluated with IMVP ($P < .01$ and $P < .01$, respectively).

The negative predictive value of all endoscopic findings of M-NBI alone exceeded 95% (97% [95% CI, 90%-98%] for DL, 95% [95% CI, 90%-98%] for IMVP, and 95% [95% CI, 90%-98%] for both DL and IMVP). The negative predictive value of all endoscopic findings of M-NBI after C-WLI was 99% (99% [95% CI, 93%-100%] for DL, 99% [95% CI, 96%-100%] for IMVP, and 99% [95% CI, 96%-100%] for both DL and IMVP). This indicates that M-NBI findings, especially when M-NBI is performed after C-WLI, could be good markers to exclude cancer from small, depressed gastric lesions that were detected with C-WLI.

TABLE 2. Association between each endoscopic finding and cancer

Method	Endoscopic findings	Pathologic diagnosis		RR [95% CI]	P
		Cancer	Noncancerous		
C-WLI	IM	15	88	1.3 [1.0-1.8]	.11
	SDA	8	56	1.1 [.6-2.0]	.72
	IM and SDA	8	50	1.3 [.7-2.2]	.48
M-NBI alone	DL	17	74	1.8 [1.4-2.3]	<.01
	IMVP	12	12	7.9 [4.1-15.1]	<.01
	DL and IMVP	12	9	10.5 [5.1-21.7]	<.01
M-NBI after C-WLI	DL	19	79	1.9 [1.6-2.3]	<.01
	IMVP	19	6	24.7 [11.2-54.5]	<.01
	DL and IMVP	19	5	29.6 [12.4-70.6]	<.01

M-NBI, Magnifying narrow-band imaging; *C-WLI,* conventional white-light imaging; *DL,* demarcation line; *IMVP,* irregular microvascular pattern; *IM,* irregular margin; *SDA,* spiny depressed area; *RR,* relative risk; *CI,* confidence interval.

TABLE 3. Diagnostic performance according to endoscopic findings

Method	Endoscopic finding	Accuracy (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
C-WLI	IM	47	75	44	15	93
	SDA	61	40	64	13	89
	IM and SDA	65	40	68	14	90
M-NBI	DL	57	85	53	19	97
	IMVP	89	60	92	50	95
	DL and IMVP	90	60	94	57	95
M-NBI after C-WLI	DL	55	95	49	19	99
	IMVP	96	95	96	76	99
	DL and IMVP	97	95	97	79	99

M-NBI, Magnifying narrow-band imaging; *C-WLI,* conventional white-light imaging; *DL,* demarcation line; *IMVP,* irregular microvascular pattern; *IM,* irregular margin; *SDA,* spiny depressed area; *PPV,* positive predictive value; *NPV,* negative predictive value.

Analysis of lesions incorrectly diagnosed by M-NBI

We experienced 23 incorrect diagnoses (false positive, 14; false negative, 9) and 330 correct diagnoses for both M-NBI alone and M-NBI after C-WLI. There were no significant differences in characteristics (lesion size and location and time to establish diagnosis) between the correctly and incorrectly diagnosed lesions (Table 4). Two reviewers with experience in endoscopic diagnosis of more than 3000 cases using M-NBI reviewed the images of lesions that were misdiagnosed by M-NBI and identified the reasons for misdiagnosis as follows.

Technical factors. In 10 cases (5 false positives and 5 false negatives), the findings were judged to be indeterminate because the images were at a low magnification and/or out of focus.

Cognitive factors. Eleven cases had originally been misdiagnosed despite adequate examination. Eight cases were diagnosed as false positives, and 3 were diagnosed as false negatives. Reviewers correctly diagnosed lesions in 5 of the 8 false positives and 2 of the 3 false negatives. Thus, the reviewers posited that 1 reason for misdiagnosis was a lack of interpretive skill on the part of the endoscopist. The cases that were misdiagnosed despite adequate examination included 3 false positives and 1 false negative that the reviewers also misdiagnosed. This was presumably because of the limitations of the endoscopic criteria for diagnosing cancers used in this study.

Others. One diagnosis was mistakenly entered as a histologic noncancerous lesion on the case report form. One patient was misdiagnosed with a noncancerous lesion because of a sampling error as a result of forceps biopsy.

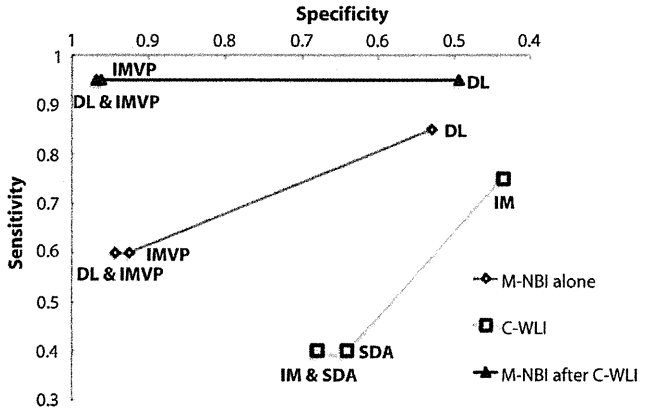

Figure 4. Comparison of the sensitivity and specificity of individual endoscopic findings in magnifying narrow-band imaging (M-NBI) alone, conventional white-light imaging (C-WLI), and M-NBI after C-WLI. Among all endoscopic findings, diagnostic performance improved significantly for C-WLI alone, followed by M-NBI alone, and then by M-NBI after C-WLI.

TABLE 4. Characteristics of correct and incorrect diagnosis by M-NBI

	Correct diagnosis (n = 330)	Incorrect diagnosis (n = 23)	*P*
Mean SDL size, mm	6	6	.67
SDL location (longitudinal)			
Upper third	61	5	
Middle third	82	7	.45
Lower third	187	11	
SDL location (circumferential)			
Anterior wall	57	4	.34
Lesser curvature	106	9	
Posterior wall	93	8	
Greater curvature	74	2	
Inspection time			
Average(s)	72	100	.15

M-NBI, Magnifying narrow-band imaging; *SDL*, small, depressed lesion.

DISCUSSION

In this study, we found that the endoscopic diagnostic performance increased for C-WLI, followed by M-NBI alone; M-NBI after C-WLI ultimately showed the best diagnostic performance for each diagnostic criterion (Fig. 4). Therefore, M-NBI should generally be performed after evaluation of C-WLI findings. The combination of DL and IMVP characterized by M-NBI after C-WLI contributed to the most reliable diagnosis for small, depressed gastric lesions and can be an ideal, simple, standard diagnostic strategy for small, depressed gastric lesions.

Based on the current results, we herein propose an efficient endoscopic diagnostic strategy for small, depressed gastric lesions, as indicated in Figure 5. In the M-NBI after C-WLI technique, both a DL and an IMVP had a high negative predictive value (99% and 99%, respectively), indicating that both criteria were sufficiently sensitive for exclusion of noncancerous lesions. A DL is technically easier to identify than an IMVP.[15] Therefore, the identification of a DL should be the first step in the diagnosis of gastric cancer because the absence of a DL alone allows for the exclusion of a noncancerous lesion. If a DL is absent, a noncancerous lesion can be diagnosed without any additional findings. Next, the presence of an IMVP is evaluated within a DL. If both a DL and IMVP are present, cancer is strongly suggested because an IMVP is sufficiently specific in the diagnosis of cancer (ie, it has a high positive predictive value) and an additional procedure with curative intent is indicated. If an IMVP is absent, the lesion can be diagnosed as a noncancerous lesion without a target biopsy sample because the negative predictive value of an IMVP is also very high. This strategy will provide a high level of accurate diagnosis for small, depressed gastric lesions. In particular, because the negative predictive value of both a DL and an IMVP is very high in M-NBI after C-WLI, the benefits of this strategy include reductions in the risk of hemorrhage, number of biopsy specimens for pathologic analysis, procedure time, and medical expenses, especially when a noncancerous lesion is diagnosed.

Analysis of lesions incorrectly diagnosed by M-NBI revealed reasons for misdiagnosis. The reasons for misdiagnosis included both technical and cognitive factors; thus, training should involve both aspects. Technical errors were mainly caused by difficulty in observation of a DL and an IMVP at maximum magnification. Attachment of a rubber cap is very helpful for capturing in-focus magnified images, but we failed to obtain sufficient images in some cases. To improve the performance of these techniques, 1 of the authors has published a book with a DVD[16] explaining the techniques necessary to perform M-NBI at maximum magnification. The role of videos in transferring information relating to endoscopic technique will be more important than that of text from now on. Cognitive factors included the lack of interpretative skill on the part of the endoscopist. In their review of the images, the 2 reviewers revised the diagnosis in 7 cases, indicating a false-positive rate of 4.5% and false-negative rate of 22.5%. These numbers could have improved with better mastery of interpretative skill. An e-learning system was developed to improve interpretative skill using M-NBI, and a multicenter study, entitled "Learning curve with an e-learning system on magnifying narrow-band imaging in endoscopic diagnosis of gastric lesions: A randomized

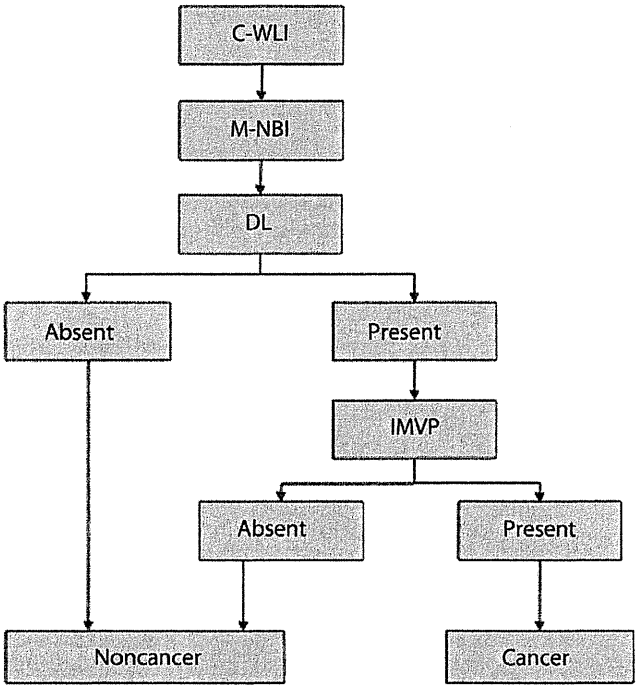

Figure 5. Strategy of using simplified criteria to make an endoscopic diagnosis of small, depressed lesions using magnifying narrowband imaging (M-NBI). When a small, depressed gastric lesion is detected by conventional white-light imaging (C-WLI), the presence or absence of a demarcation line (DL) should be the first step in diagnosing gastric cancer. If the DL is absent, a noncancerous lesion can be diagnosed. If the DL is present, the presence of an irregular microvascular pattern (IMVP) should be used for diagnosis. If an IMVP is absent, a noncancerous lesion can be diagnosed without a target biopsy sample and/or EMR/ESD. Finally, if both a DL and an IMVP are present, cancer is strongly suggested, and additional procedures are indicated.

study" (UMIN-CTR 000008569), was begun to examine the system's usefulness. After the review of recorded images by the 2 experts, there were limits of diagnosing lesions with M-NBI in 4 cases. The lesions did not fulfill the endoscopic cancerous or noncancerous diagnostic criteria of a DL and an IMVP according to M-NBI. For these lesions, development of other diagnostic equipment or another method is required to further improve the diagnostic performance.

This study has limitations. The study samples were limited to $\leq$10– mm depressed lesions. A diagnosis based on the microvascular pattern and a DL, but not the microsurface pattern, is not universally applicable to all macroscopic types of lesions. However, all small, depressed lesions in this study had a microvascular pattern that could be visualized, allowing the lesions to be diagnosed. Thus, the current authors are prospectively studying (UMIN-CTR 000004045) the ability of M-NBI to diagnose all macroscopic types of lesions using the vessel-plus-surface classification system[7] put forth by Yao et al without size or macroscopic type limitation. The vessel-plus-surface classification system uses the microsurface pattern, microvascular pattern, and DL as indices.

In conclusion, the current study suggests that although M-NBI alone provided good diagnostic performance, it is important to conduct a C-WLI evaluation before M-NBI diagnosis. When using M-NBI, identification of a DL is the first step in the diagnosis of cancer, and the subsequent identification of an IMVP is useful for excluding noncancerous lesions among the lesions that were identified to have a DL. Training in both techniques and knowledge is important to improve M-NBI diagnosis.

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ORIGINAL ARTICLE

The preventive effects of low-dose enteric-coated aspirin tablets on the development of colorectal tumours in Asian patients: a randomised trial

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ABSTRACT

Objective To evaluate the influence of low-dose, enteric-coated aspirin tablets (100 mg/day for 2 years) on colorectal tumour recurrence in Asian patients with single/multiple colorectal tumours excised by endoscopy.

Design A double-blinded, randomised, placebo-controlled multicentre clinical trial was conducted.

Participants 311 subjects with single/multiple colorectal adenomas and adenocarcinomas excised by endoscopy were enrolled in the study (152 patients in the aspirin group and 159 patients in the placebo group). Enrolment began at the hospitals (n=19) in 2007 and was completed in 2009.

Results The subjects treated with aspirin displayed reduced colorectal tumourigenesis and primary endpoints with an adjusted OR of 0.60 (95% CI 0.36 to 0.98) compared with the subjects in the placebo group. Subgroup analysis revealed that subjects who were non-smokers, defined as those who had smoked in the past or who had never smoked, had a marked reduction in the number of recurrent tumours in the aspirin-treated group. The adjusted OR for aspirin treatment in non-smokers was 0.37 (CI 0.21 to 0.68, $p<0.05$). Interestingly, the use of aspirin in smokers resulted in an increased risk, with an OR of 3.44. In addition, no severe adverse effects were observed in either group.

Conclusions Low-dose, enteric-coated aspirin tablets reduced colorectal tumour recurrence in an Asian population. The results are consistent with those obtained from other randomised controlled trials in Western countries.

The clinical trial registry website and the clinical trial number http://www.umin.ac.jp (number UMIN000000697).

Significance of this study

What is already known on this subject?

- A considerable amount of evidence regarding the utility of aspirin as a cancer chemopreventive agent has been generated in Western populations.
- The evidence regarding aspirin as a cancer chemopreventive agent in Asian populations is limited. Moreover, no cancer chemopreventive drugs have been approved in Japan.
- The advantages of aspirin as a cancer chemopreventive agent are well recognised, as it has been in clinical use for a long period of time. In addition, aspirin's adverse effects and cost-effectiveness are well known.

What are the new findings?

- We are the first to report the efficacy of low-dose, enteric-coated aspirin tablets in the suppression of colorectal tumour recurrence in Asian patients; these findings are consistent with the observations of other aspirin adenoma trials in Western populations.
- We report the safety of low-dose, enteric-coated aspirin tablets administered to patients as a cancer chemopreventive agent for 2 years.

How might it impact on clinical practice in the foreseeable future?

- The evidence that aspirin is effective in the reduction of colorectal tumour recurrence in Asian patients may impact cancer preventive strategies in Japan and other Asian countries, including Korea and China.

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INTRODUCTION

Among chemopreventive interventions, aspirin (acetylsalicylic acid) has been examined in numerous trials that support its suppressive effect on colorectal cancer (CRC) development. Aspirin is a synthetic medicine based on the structure of salicylates, which are commonly found in fruits and vegetables. Aspirin's antineoplastic effects have been mechanistically explained by its cyclooxygenase (COX) inhibitory activity. The use of aspirin as a cancer chemopreventive agent is advantageous because it has a long history of clinical use

and its adverse effects are well known. Moreover, the cost-effectiveness of aspirin administration to prevent other diseases, such as cardiovascular disease, has also been demonstrated.[1]

An early prospective cohort study of 662 424 adults (the Cancer Prevention Study II cohort) demonstrated that the CRC death rate decreased with frequent aspirin use. The decreased relative risk (RR) of CRC among frequent aspirin users (≥16 times/month for at least 1 year with doses greater than 160 mg) was 0.60 (95% CI 0.4 to 0.89) in men and 0.58 (95% CI 0.37 to 0.9) in women.[2] An updated analysis of this cohort (the Cancer Prevention Study II Nutrition cohort) demonstrated that long-term daily aspirin use (≥325 mg/day for ≥5 years) is associated with reduced incidence of CRC compared with non-users (RR=0.68, 95% CI 0.52 to 0.90 among men and women collectively).[3 4] The factors that may affect the impact of aspirin include the population, dose of aspirin and duration of intervention.[4] In the general population, trials of 75–325 mg/day aspirin for 3 years reduced the risk of recurrent of colorectal adenoma by 17%.[5] Moreover, the use of aspirin for 5 years or longer reduced the incidence and mortality of CRC by 30%–40% after 20-year follow-up.[6]

A considerable amount of evidence on the utility of aspirin has been generated in Western populations; however, the evidence for aspirin as a cancer chemopreventive agent in Asian populations is limited. Thus, it is important to present evidence that aspirin is also effective as a cancer chemopreventive agent in Asian populations.

We recently reported a double-blinded, randomised, placebo-controlled clinical trial of a high-risk CRC group, familial adenomatous polyposis, to evaluate the effect of low-dose, enteric-coated aspirin tablets. Secondary endpoint data from the trial revealed that subjects with a mean baseline polyp diameter of <2 mm administered aspirin displayed a significant reduction in mean polyp size.[7]

We investigated the effects of low-dose, enteric-coated aspirin tablets administered for 2 years in a double-blinded, randomised, placebo-controlled clinical trial in patients with a single/multiple colorectal adenomas and/or adenocarcinomas with invasions confined to the mucosa and excision by endoscopy. This population was considered to be a high-risk colorectal tumour group. Low-dose, enteric-coated aspirin tablets (100 mg/day) were chosen for the study because low-dose aspirin may circumvent the risk of upper GI toxicity.[8] In addition, the enteric coating may decrease gastric mucosal damage, as demonstrated in the MAJIC study targeting high-risk cardiovascular Japanese patients[9] as well as other short-term endoscopic studies.[10]

Here, we report the efficacy and safety of low-dose, enteric-coated aspirin tablets in the suppression of colorectal tumour recurrence in Asian patients with colorectal adenomas and/or adenocarcinomas with confined mucosal invasions that were excised by endoscopy.

METHODS

Trial methodology

In this double-blinded (both subjects and investigators), randomised, placebo-controlled trial using low-dose, enteric-coated aspirin tablets, the subjects received either 100 mg/day aspirin or placebo for 2 years. Each case was randomised by investigators using a computer-aided system from the Medical Research Support website. Using a minimisation algorithm, the primary examination selection was balanced with respect to three stratification variables: institution, age (≤60 and >60 years) and sex (male or female). The website was only available to the trial investigators. Subject enrollment and intervention assignment began at each hospital in January 2007, and the trial ended in July 2009. To further evaluate the effects of aspirin, follow-up for more than 2 years after the randomised trial was also planned. An Ethics Monitoring Committee was established for this multicentre trial (n=19) that was primarily based at Osaka Central Hospital. A system to ensure continuous follow-up of adverse events was also established. All hospitals participating in this trial obtained approval from their own ethics committees. This trial is registered and details are available at http://www.umin.ac.jp (number UMIN000000697), where the full trial protocol can be accessed.

Trial population

The trial population (n=389) consisted of patients with single/multiple colorectal adenomas and/or adenocarcinomas with invasions confined to the mucosa. The colorectal tumours of all subjects participating in the trial were excised by endoscopy before the trial start. An endoscopic examination was performed twice before the start of the trial; the examinations occurred at an average of 488.4 ±472 (mean±SD) days apart to confirm that all colorectal tumours were excised. All of the subjects were Asian men or women 40–70-years-old living in Japan. The following are exclusion criteria for the trial: (1) patients with familial adenomatous polyposis, Lynch syndrome or colorectal resection; (2) patients currently taking an antithrombotic or anticoagulant, including aspirin; (3) individuals with a history of stroke or gastric/duodenal ulcers (with the exception of patients with confirmed scars resulting from the successful eradication of *Helicobacter pylori*); (4) patients with IBD, haemorrhagic diverticulitis or haemorrhagic tendency; (5) patients with a platelet count of ≤100 000/mm^3 or abnormal prothrombin time; (6) patients with a known aspirin allergy; (7) patients currently taking an anticancer drug; (8) pregnant patients or those who planned to become pregnant during the trial period; and (9) patients taking non-steroidal anti-inflammatory drugs (NSAIDs) for pain relief more than thrice weekly. We calculated that 266 randomised patients would achieve an 80% power (with a 5% type I error) to detect a 40% difference in the recurrence rate of adenoma given a 40% risk of recurrence in the placebo group.[11] However, data were unavailable to calculate an appropriate number of individuals to recruit from the Asian population; therefore, we set our recruitment goal in the initial aspirin protocol to 700 randomised patients.

Consent interviews were performed individually, and written informed consent was obtained from all patients.

Investigational drug

Low-dose, enteric-coated aspirin tablets (100 mg per tablet) and the placebo tablets were kindly provided by Bayer Pharma AG (Leverkusen, Germany) and imported into Japan. The trial was financed by research funding from the Ministry of Health, Labor and Welfare, not by Bayer Pharma AG. We signed an agreement to certify that no conflicts of interest with Bayer Pharma AG existed. The investigational drugs were placed in blister packages (calendar sheets of 31 tablets), and both sides of the package were aluminium-laminated.

Trial questionnaire

At the time of trial enrolment, the height, body weight, medical history, smoking history, alcohol ingestion and use of NSAIDs were investigated for each patient using a questionnaire. In addition, data regarding everyday meals were collected using a self-administered food-frequency questionnaire developed by the Department of Health Promotion and Preventive Medicine,

Nagoya-City University Graduate School of Medical Science, Aichi, Japan.[12] Non-smokers were defined as people who had smoked in the past or never smoked. Occasional drinkers were defined as people who drank less than twice a week.

To ensure the accurate characterisation of adverse effects and evaluation of tolerability, the subjects were asked to keep a treatment diary that documented their conditions during treatment, such as drug compliance and medical conditions, and a blister sheet was sent to the data centre every month.

Trial endpoints

Colonoscopy was performed at least three times, twice before the start of the trial and once at the end of the trial. All the patients were given an oral lavage solution for colonic cleansing at the time of the colonoscopy for clear imaging, and a medical colonoscopy specialist carefully examined the patients from the rectum to the cecum. The final endoscopy examination was performed 2 years after the start of the trial. Recurrent tumours were further diagnosed by histology after tumour excision. The primary endpoint was the incidence of adenoma or adenocarcinoma recurrence. The data were analysed using logistic regression and ORs, and general factors, such as sex, age and the tumour number before the trial, were used to adjust occasional deviation during the randomised allocation. Each tumour was removed and examined histologically by a pathologist. Tumours were classified as adenomas or adenocarcinomas according to the 'Japanese Classification of Colorectal Carcinoma' criteria. The secondary endpoints included recurring tumour number, size and histology as well as the effects of lifestyle, such as smoking and alcohol drinking, and the frequency of adverse effects.

Statistical analysis

The baseline characteristics of the two arms were compared using the χ^2 test or the t test. The adverse effect rates of both arms were compared using the χ^2 test. If needed, Fisher's exact probability was applied due to sparse data in a table. To adjust for potential confounding effects at baseline, logistic regression was performed.

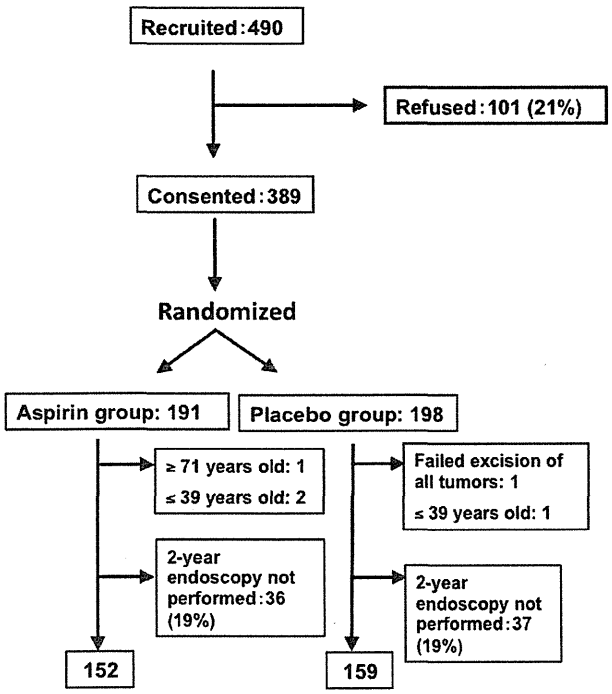

Figure 1 Flowchart of subject recruitment.

We also examined the effect modification (interaction) of several factors, such as (1) sex, (2) age, (3) smoking and (4) alcohol drinking, on the main effect of aspirin by adding an interaction term to the logistic regression. In this analysis, we determined the ORs of the subgroups of the above factors and the difference in the ORs of the subgroups.

All statistical analyses were based on the intention-to-treat and performed using PC-SAS (V.9.3; SAS Inc., Cary, North Carolina, USA), with $p<0.05$ considered statistically significant.

RESULTS

Characteristics of the trial subjects

A total of 490 patients were screened, and 389 patients provided informed consent. Subject enrolment began in January 2007, and the trial ended in July 2009. Subject recruitment ended according to the planned time schedule. After randomisation, the aspirin and placebo group consisted of 191 and 198 subjects, respectively. At the end of the trial, 152 subjects from the aspirin group and 159 subjects from the placebo group underwent a 2-year follow-up endoscopy examination (figure 1). The characteristics of the subjects in the aspirin and placebo groups after randomisation are displayed in table 1. No significant differences between the two groups were observed with regard to the following characteristics: age, sex, smoking status, alcohol drinking status, height, weight, body mass index, tumour number upon entry into the trial, past history of CRC with invasion confined to the mucosa, treatment period, compliance (ie, whether patients correctly take medicine and follow the doctors' instructions (data not shown)), surgical history (data not shown) and family history of CRC (data not shown). The serum concentrations of alanine transaminase, aspartate amino transferase, γ-glutamyl transpeptidase and triglycerides were almost identical between the groups (data not shown).

Colorectal tumour recurrence as the primary endpoint

In total, 96 patients did not experience colorectal tumour recurrence in the aspirin group (total 152), and 86 patients in the placebo group (total 159) did not recur. In crude analyses, the subjects in the aspirin group tended to demonstrate a reduced number of colorectal tumours, which was the primary endpoint, compared with subjects in the placebo group. The OR was 0.69 (95% CI

Table 1 Characteristics of the two groups

	Aspirin		Placebo	
Number	152		159	
Age	60.0	±7.3*	60.5	±6.6
Sex				
Male	121	(79.6%)	125	(78.6%)
Smoking				
Current smokers	45	(29.6%)	34	(21.4%)
Alcohol				
Drinker†	83	(54.6%)	92	(57.9%)
Height	164.7	±6.8	165.5	±7.3
Weight	64.3	±9.7	65.6	±10.1
BMI‡	23.6	±2.7	23.9	±2.8
Number of tumours upon trial entry	5.3	±5.7	5.1	±7.0
Past CRC history	40	(26.3%)	39	(24.5%)
Treatment period	751	±67 days	764	±90 days

*SD.
†Alcohol drinker: drinks more than three times a week.
‡Body mass index (BMI)=Weight (kg)/height (m) squared.
CRC, colorectal cancer.

Table 2 The effects of aspirin on colorectal tumour development in smokers

Subanalysis	No. of subjects with (+) or without (–) colorectal tumour			Adjusted OR (95% CI)
	–	+	Total	
Current smoker				
Placebo group	26	19	45	1
Aspirin group	14	20	34	3.45 (1.12 to 10.64), p=0.03
Non-smoker*				
Placebo group	60	54	114	1
Aspirin group	82	36	118	0.37 (0.21 to 0.68), p=0.01

Adjusted OR, OR is adjusted by sex, age and the number of tumours prior to the trial.
*Non-smoker: never smoked and former smokers.

0.44 to 1.08); despite a marginal difference, the value was not statistically significant. To adjust for potential confounders, such as sex, age and the number of recurrent tumours, we performed logistic regression and obtained a significant OR value of 0.60 (95% CI 0.36 to 0.98). The OR for the number of recurrent tumours <4 in the aspirin group was 0.34 (0.09 to 1.26), and the OR for a tumour >3 mm in longitudinal diameter was 0.86 (0.63 to 1.16), but the value was not statistically significant.

The effects of smoking and drinking on colorectal tumour recurrence

Using a logistic regression with smoking as the interaction term and aspirin as the effect, we observed that smoking displays strong effect modification on the main effect of aspirin (p for interaction=0.004). Namely, the OR for non-smokers was 0.37 (95% CI 0.21 to 0.68), and this value was significantly different from the OR for smokers (OR 3.44, 95% CI 1.12 to 10.64) after adjustment for age, sex and the number of tumours (table 2). In contrast, no significant effect modification for sex (p=0.68), age (p=0.53) or alcohol consumption (p=0.32) was observed. With regard to sex, the OR was 0.48 (95% CI 0.15 to 1.55) and 0.63 (95% CI 0.36 to 1.08) among men and women, respectively. For age, the OR was 0.68 (95% CI 0.36 to 1.28) and 0.49 (95% CI 0.22 to 1.08) for subjects aged <60 and ≥60 years, respectively. For alcohol consumption, the OR was 0.72 (95% CI 0.37 to 1.40) and 0.44 (95% CI 0.21 to 0.95; p<0.05) for drinkers and occasional drinkers, respectively.

In addition, no severe adverse effects, such as cardiovascular events, were reported in either group. GI bleeding was not observed. Of note, colorectal adenocarcinomas were observed in four subjects: two cases from the aspirin group (one adenocarcinoma with invasion confined to the mucosa, and one adenocarcinoma with muscularis propria invasion) and two in the placebo group (two adenocarcinomas with invasion confined to the mucosa). The remaining tumours were tubular adenomas; villous adenomas were not identified. In addition, three high-grade dysplasias were detected; one case was observed in the aspirin group, and two cases were noted in the placebo group. The adenocarcinomas were 10–20 mm in diameter. The lesions were localised to the transverse colon (n=2), the descending colon (n=1) and the sigmoid colon (n=1).

DISCUSSION

In the present trial, we enrolled subjects with single/multiple colorectal adenomas and/or adenocarcinomas with invasions confined to the mucosa that were excised by endoscopy. Patients treated with low-dose, enteric-coated aspirin tablets for 2 years were shown to have a low risk of incidental colorectal tumour development, and this appeared to be reduced after adjustment for sex, age and the number of baseline tumours. Moreover, smoking significantly modified the preventive effect of aspirin.

In a meta-analysis of subjects with a history of colorectal adenoma or cancer in four randomised adenoma prevention trials (nearly 3000 patients), aspirin reduced the occurrence of advanced lesions (ie, tubulovillous adenomas, villous adenomas, adenomas ≥1 cm in diameter, adenomas with high-grade dysplasia or invasive cancer) by 28% (adenoma 17%; RR=0.83; 95% CI 0.72 to 0.96).[3] Our trial also demonstrated reduced adenoma occurrence (OR=0.69), and similar effects were obtained compared with the meta-analysis by Cole *et al*.[5] However, the ORs we used have a predictable effect on the comparison of the two sets of analyses. Regarding the limitations of our trial, the number of subjects enrolled is rather small, but the tumour recurrence results are consistent with previous studies. Thus, our data demonstrate that aspirin is also useful as a CRC chemopreventive agent in an Asian population. Of note, the first Asian adjuvant study (ASCOLT, NCT00565708) is ongoing, wherein patients with Dukes C or high risk Dukes B CRC are treated with aspirin (200 mg/day for 3 years).

Although the daily aspirin doses administered for vascular disease prevention are as effective as high-dose (1200 mg/day) aspirin,[11] analyses comparing moderate (300–325 mg/day) and lower (81–160 mg/day) aspirin doses trials (AFPPS[11] and APACC[13]) revealed that the reduced risk of all adenoma recurrence was only observed with lower doses.[5] Our trial using low-dose, enteric-coated aspirin tablets (100 mg/day) was designed in light of these trials, thereby confirming that low-dose, enteric-coated aspirin tablets effectively reduce recurrence. In addition, low-dose regimens may have an advantage in that the lower doses potentially reduce adverse effects. Aspirin has been reported to induce GI bleeding at a rate of 1–2 GI bleeds per 1000 person-years.[14] In our trial, no severe adverse effects due to aspirin treatment were observed.

Aspirin's antineoplastic effects are explained by COX-dependent and -independent mechanisms. In humans, aspirin inhibits COX-1 and COX-2 at high doses[15] and appears to effectively inhibit prostaglandin synthesis in the colon.[16] COX-independent mechanisms underlying aspirin's antineoplastic effects are attributed to the modulation of nuclear factor κ B: the induction of spermidine/spermine N1-acetyltransferase, caspase-8 and -9; and the activation of 5′ adenosine monophosphate-activated protein kinase, Erk and β-catenin.[17–23]

Despite copious information regarding aspirin's functions, the mechanism by which smoking negates aspirin's CRC chemopreventive effects remains unclear. A strong association between antiplatelet therapy resistance (aspirin resistance) and smoking has been reported. Specifically, a statistically significant interaction exists based on the multivariate analysis (risk ratio 11.47, CI 6.69 to 18.63, p<0.0001),[24] which is likely due to smoking-induced platelet hyperactivity and chronic inflammation.[25] In addition, smoking-induced decreased basal GI blood flow may also be involved.[26] Thus, it is suggested that smoking negated aspirin's chemopreventive effects in CRC. However, the evidence is limited. It is important to review and generate additional aspirin trial data to examine the association between NSAIDs use and smoking history and to determine whether the benefits of aspirin are limited to non-smokers.

In conclusion, although the size of this trial is small, the results are consistent with the observations of other aspirin adenoma trials; thus, aspirin may be useful for chemoprevention in Asian

patients with single/multiple colorectal tumours and no antecedent risk of GI bleeding. Several years of follow-up after a randomised trial are necessary to evaluate the effects of aspirin as proposed by the CAPP2 randomised trial.[27] Moreover, it would be informative to test aspirin in combination with other chemopreventive agents that have demonstrated effectiveness and agents that prevent GI bleeding (eg, proton-pump inhibitors).[28]

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Competing interests None.

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Ethics approval All hospitals participating in this trial obtained approval from their own ethics committees to conduct the trial.

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The preventive effects of low-dose enteric-coated aspirin tablets on the development of colorectal tumours in Asian patients: a randomised trial

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Original Article

Intestinal Peyer's patches prevent tumorigenesis in *Apc*$^{Min/+}$ mice

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Peyer's patches are nodules that play a central role in intestinal immunity. Few studies demonstrate the relationship between the number of Peyer's patches and intestinal polyps. Here we identify a statistically significant inverse correlation between the quantity of Peyer's patches and of the development of intestinal polyps in *Apc*$^{Min/+}$ mice, which are a useful model to clarify the role of Peyer's patches in intestinal tumorigenesis. Using this model, we increased the number of Peyer's patches using 0.1% and 1% corn husk arabinoxylan through feed. Intestinal polyp formation significantly decreased, concomitant with an increase in Peyer's patches development (n = 12/group). In *Aly*$^{-/-}$*Apc*$^{Min/+}$ mice (negative control; no Peyer's patches) there was no change in the amount of intestinal polyps (n = 10/group). Immune reaction following corn husk arabinoxylan treatment was measured by cytokine array. Increasing the number of Peyer's patches decreased interleukin-17 production, which showed a dose dependent correlation with transcription factor/lymphoid enhancer-binding factor. This study identified a relationship between levels of Peyer's patches and intestinal polyp formation, partly explained by the involvement of interleukin-17 production and β-catenin signaling in *Apc*$^{Min/+}$ mice.

Key Words: **Peyer's patch, corn husk arabinoxylan, intestinal polyp, cancer prevention, *Apc*$^{Min/+}$ mouse**

Colorectal cancer is the third most common malignant neoplasm worldwide and the first leading cause of cancer deaths (irrespective of gender) in Japan.[1] According to the Ministry of Health, Labour and Welfare, the colorectal cancer mortality rate has increased from 1955 to the present.[2–4] Thus, relationship between food and intestinal tumors has generated attention because it is proposed that this disease is not only caused by hereditary factors but also environmental factors like change in diet.

Nutritive and foreign substances, such as germs, simultaneously enter the intestinal tract. The gut immune system normally protects against foreign invasion.[5] Peyer's patches (PP) are a central lymphoid organs of this immune response mechanism in the intestines, and several important reports demonstrate an association between PP and allergy, intestinal bacteria, etc.[6–9] However, few studies address whether or not PPs are linked to intestinal tumorigenesis and an underlying mechanism has not been elucidated.

The *adenomatous polyposis coli* (*Apc*) Min heterozygous knockout (*Apc*$^{Min/+}$) mouse, lacking a functional *Apc* gene product, is a model of human familial adenomatous polyposis, which spontaneously generates numerous intestinal polyps.[10–12]

In this study, we observed an inverse relationship between the number of PP and intestinal polyps in *Apc*$^{Min/+}$ mice. Therefore, we artificially increased the amount of PP in murine intestines using corn husk arabinoxylan (CHAX) as an adjuvant. We examined the effects of PP development on intestinal polyps following ingestion of CHAX in *Apc*$^{Min/+}$ and *Aly*$^{-/-}$*Apc*$^{Min/+}$ mice, which have no PP in the intestine.[13] Furthermore, we tried to clarify the molecular mechanism for the prevention of polyp formation in the mice.

Materials and Methods

Mice and feed. Wild type (WT; C57B1/6J) mice and *alymphoplasia* knockout (*Aly*$^{-/-}$, C57B1/6J) mice, were obtained from Clea Japan, Inc. (Tokyo, Japan). The *Apc*$^{Min/+}$ (C57B1/6J) mice were supplied by Jackson Laboratories (Bar Harbor, Maine, State). The mice were maintained under specific pathogen-free conditions at the Animal Center of Nagasaki International University. (Nagasaki, Japan) Female *Apc*$^{Min/+}$ and male *Aly*$^{-/-}$ mice were mated to obtain *Aly*$^{+/-}$*Apc*$^{Min/+}$ mice. The mouse feed was compounded with two different doses of CHAX (NIHON SHOKUHIN KAKO CO., LTD, Tokyo, Japan): 0.1 and 1% mixed (i.e., 0.2 and 2 g/kg (body weight)/day each). Preparation of CHAX is described elsewhere.[14] According to standard dietary intake guidelines provided by The Ministry of Health, Labour and Welfare, we calculated the appropriate dose for mice. We analyzed 10–12 mice in each feeding group: CHAX-free, 0.1, and 1% CHAX for each genotype (WT, *Apc*$^{Min/+}$, and *Aly*$^{-/-}$*Apc*$^{Min/+}$). Approximately 100 mice were used in the ingestion experiment. Mice were sacrificed at 12, 24, and 32 weeks of age for the analysis of intestinal polyps and PP. All animal experiments were conducted according to the Guidelines for Animal Experiments in the Faculty of Pharmaceutical Sciences, Nagasaki International University.

Genotyping. Mouse tails were genotyped using a Genomic DNA Purification Kit (QIAGEN, Hilden, Germany) according to the manufacture's protocol. Allele-specific polymerase chain reaction (PCR) primers for the *Apc* gene were produced according to previously published methods.[15] Forward and reverse primers for *Aly*$^{-/-}$ mice had complimentary annealing temperatures and a product length of 302 base pairs. Genomic DNA was used as a template for PCR to amplify fragments containing forward Aly_Wild 5'-CTGACATCCCGAGCTACTTCAACG, forward Aly_Mutant 5'-CTGACATCCCGAGCTACTTCAACA, and reverse 5'-GCCTAGGATCGGCCATTTTCTTCC primers using the AmpliTaq Gold 360 Master Mix (Applied Biosystems, California, CA). Touchdown PCR consisted of one cycle of 94°C for 9 min for the initial denaturation step. This was followed by 10 cycles each of denaturation at 94°C for 1 min, varying annealing conditions for 1 min, and extension at 72°C for 1 min. Annealing temperatures for the touchdown portion were as follows: starting annealing temperatures of 72°C decreasing by 0.5°C decrements

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per PCR cycle down to 67°C. Further 25 cycles consisted of the following: 94°C for 1 min, 68°C for 1 min, and 72°C for 1 min.

Counts of intestinal polyps and statistical analysis. Intestinal polyps and statistical analysis were examined according to previously published methods.[16] All statistical analyses were carried out using the GraphPad Prism 5 program. (GraphPad Software Inc., San Diego, CA) The correlation coefficient of the number of PP and intestinal polyps were analyzed using Pearson's correlation coefficient. We analyzed of two-group by using Mann–Whitney *t* test, and three or more groups were analyzed simultaneously by using Dunnett Comparison test.

Cytokine array. Serum was collected from the heart when mice were dissected. We pooled serum samples from four WT mice fed with or without CHAX, respectively. Similarly, we pooled serum samples from *Apc*$^{Min/+}$ or *Aly*$^{-/-}$*Apc*$^{Min/+}$ mice and we analyzed using Multiplex Suspension Array (Genetic Lab Corp., Hokkaido, Japan) The total number of samples was six, and the experiment was repeated two times using new samples.

Enzyme-linked immunosorbent assay (ELISA). All serum samples were stored in a –80°C deep freezer until use. The levels of interleukin (IL)-17 were measured by IL-17 Mouse ELISA Kit (Abcam, Cambridge, UK), according to the manufacturer's protocol. We measured fluorescence using a Model 680 Microplate Reader (Bio-Rad, Hercules, CA).

Quantitative real-time PCR analysis. All tissue samples from intestinal polyps of mice fed with or without CHAX were rapidly soaked in RNA later solution (Qiagen). Total RNA was isolated from tissue using the RNeasy Mini Kit (Qiagen). Complementary DNA (from reverse transcribed total RNA) and real-time PCR were examined according to previously published methods.[17] Primers for mouse cyclin D1 (5'primer-CCATGGAACACCAGCTCCTG and 3'primer-CGGTCCAGGTAGTTCATGGC) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 5'primer-TGTCAGCAATGCCTGCA and 3'primer-TTACTCCTTGGAGGCCATGT) were employed. The expression levels of cyclin D1 were normalized based on GAPDH levels.

Histology and immunohistochemistry. Small intestines were fixed, embedded, and sectioned as Swiss rolls for further immunohistochemical examination with the avidin–biotin complex immunoperoxidase technique and monoclonal mouse anti-β-catenin antibody (Ab; BD Transduction Laboratories, Franklin Lakes, NJ) at 100× dilution. The secondary Ab, biotinylated anti-mouse immunoglobulin G (Vector Laboratories, California, CA), was used at a 200× dilution. Staining was performed using avidin–biotin reagents (Vectastain ABC reagents; Vector Laboratories), 3,3'-diaminobenzidine and hydrogen peroxide, and the sections were counterstained with hematoxylin to facilitate orientation. As a negative control, consecutive sections were immunostained without exposure to the primary Ab.

Reporter assays. Caco-2 cells were plated at a concentration of 5×10^5 cells/well in 12-well plates and cultivated for 24 h. Furthermore, cells were transfected with pTOP or pFOP-Flash (Merck Millipore, Darmstadt, Germany) luciferase reporter plasmids (1.0 μg each) and Simian virus 40 (SV40)–RenillaLuc (pRL-SV40; 15:1 ratio) for 24 h. All samples were normalized for transfection efficiency using the SV40–RenillaLuc expression plasmid (Promega, Madison, WI) as a transfection control. Cells were harvested and lysed in 200 μl of lysis buffer 48 h after transfection. We measured four points using 20 μl of sample, and luciferase and Renilla luciferase activity were assayed using the Bright-Glo Luciferase Assay System and Renilla-Glo Luciferase Assay System respectively (Promega) using a GENios Microplate Reader (Tecan, Männedorf, Switzerland). Transfections were repeated three times.

After correction of measurements, we calculated the average of four measurements, and transcription factor/lymphoid enhancer-binding factor (TCF/LEF) activity was calculated by TOP divided by FOP. The relative transcriptional activity was calculated for the aforementioned value was divided by the measurement of the control cell.

Results

The number of PP correlates significantly with the number of intestinal polyps in *Apc*$^{Min/+}$ mice over 24 weeks in age. We counted the number of intestinal polyps and PP in *Apc*$^{Min/+}$ mice >24 weeks in age. Mice that developed many intestinal polyps tended to have been fewer PP in the intestine. A statistically significant inverse relationship existed between the number of intestinal polyps and PP ($r = -0.62$, $n = 20$, $p = 0.0035$; Fig. 1). In addition, the amount of PP in the intestines decreased in older mice groups (Fig. 2). The quantity of PP in the intestines was significantly reduced in *Apc*$^{Min/+}$ mice >21 weeks of age compared with 12-week-old *Apc*$^{Min/+}$ mice. Moreover, the number of polyps significantly increased in older mice groups.

CHAX increases the number of PP in *Apc*$^{Min/+}$ mice. It remains unclear whether or not PP suppresses polyp formation, and what factors affect the development of each condition. However,

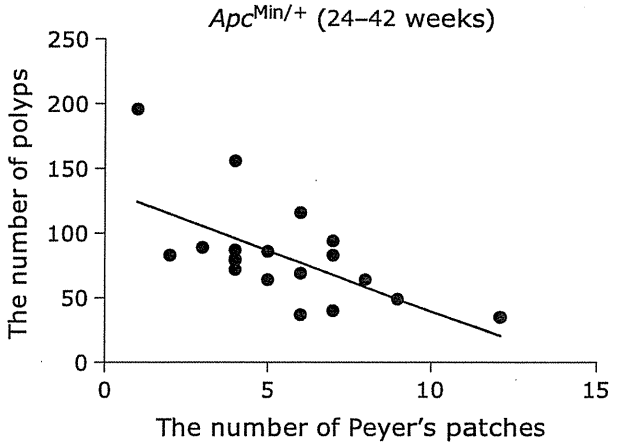

Fig. 1. The correlation between the number of intestinal polyps and Peyer's Patches ($r = -0.62$, $n = 20$, $p = 0.0035$).

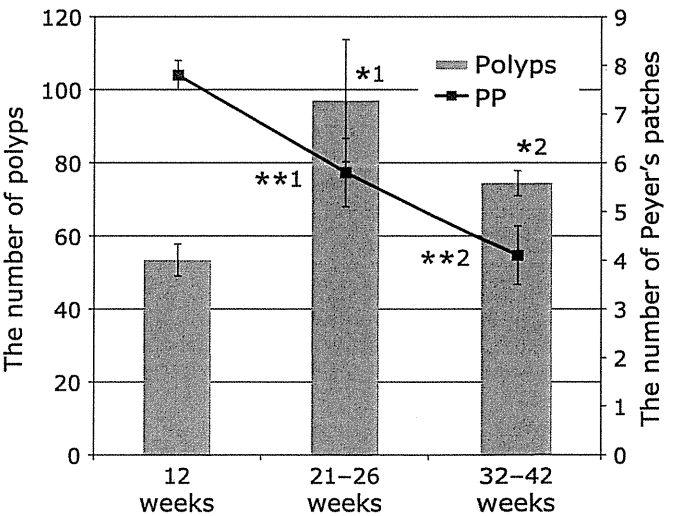

Fig. 2. The number of intestinal polyps and Peyer's Patches according to age. The *p* values are *1 = 0.0385 and *2 = 0.0046 for the number of polyps. The *p* values are **1 = 0.0099 and **2 = 0.0004 for the number of PP (Mann–Whitney *t* test). *n* = 12 per group.

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Table 1. The number of PP in *Apc*$^{Min/+}$ mice fed CHAX

CHAX dosage (%)	Small intestine			
	Proximal	Middle	Distal	Total
0	2.8 ± 0.3	1.6 ± 0.2	3.4 ± 0.2	7.8 ± 0.3
0.1	3.0 ± 0.2	2.3 ± 0.2*	3.9 ± 0.3	9.3 ± 0.3**
1	2.9 ± 0.2	2.8 ± 0.3**	3.8 ± 0.3	9.5 ± 0.2***

Values are mean ± SEM (n = 12/group). *p<0.05, **p<0.01, ***p<0.001; Mann–Whitney t test.

Table 2. The number of intestinal polyps in 12-week-old *Apc*$^{Min/+}$ mice

CHAX dosage (%)	Small intestine				Large intestine	Total
	Proximal	Middle	Distal	Total		
0	3.9 ± 0.7	22.3 ± 2.2	27.2 ± 2.7	53.4 ± 4.4	0.5 ± 0.2	53.8 ± 4.3
0.1	2.1 ± 0.5	11.0 ± 1.6**	16.2 ± 2.3*	29.3 ± 3.8**	0.3 ± 0.2	29.6 ± 3.9**
1	3.0 ± 0.4	10.0 ± 1.7**	17.9 ± 2.5*	30.9 ± 3.3**	0.4 ± 0.2	31.3 ± 3.4**

Values are mean ± SEM (n = 12/group). *p<0.05, **p<0.01, ***p<0.001; Mann–Whitney t test.

Table 3. The number of intestinal polyps in 12-week-old *Aly*$^{-/-}$*Apc*$^{Min/+}$ mice (negative control group)

CHAX dosage (%)	Small Intestine				Large intestine	Total
	Proximal	Middle	Distal	Total		
0	4.3 ± 2.9	13.7 ± 1.6	11.3 ± 1.4	29.3 ± 2.9	0.2 ± 0.1	29.6 ± 3.0
0.1	2.0 ± 0.3	8.4 ± 1.6	10.6 ± 2.8	21.1 ± 3.7	0.1 ± 0.1	21.1 ± 3.7
1	3.4 ± 0.9	11.1 ± 3.2	11.3 ± 2.7	25.8 ± 5.9	0	25.8 ± 5.9

Values are mean ± SEM (n = 10/group).

Table 4. Serum cytokine levels in WT, *Apc*$^{Min/+}$ and *Aly*$^{-/-}$*Apc*$^{Min/+}$ mice fed with or without CHAX

	WT mice		Rate of change	*Apc*$^{Min/+}$ mice		Rate of change	*Aly*$^{-/-}$*Apc*$^{Min/+}$ mice		Rate of change
	– (without CHAX)	+ (with CHAX)		–	+		–	+	
IL-1β (pg/ml)	<0.64	<0.64	—	4.38	<0.64	6.84	<0.64	<0.64	–
IL-5 (pg/ml)	10.68	4.37	2.44	8.58	7.11	1.21	22.64	129.94	5.74
IL-10 (pg/ml)	3.47	7.55	2.18	4.87	4.87	1	4.18	17.56	4.2
IL-17 (pg/ml)	3.86	1.05	3.68	3.24	0.93	3.48	<0.64	<0.64	—

The values are averages of extracted cytokines from double experiments of the cytokine array.

CHAX has been reported to increase the number of PP; therefore, we used this adjuvant to elucidate the effects of PP on polyp formation.

Mice were fed a diet of 0.1, 0.5, 1, or 4% CHAX to determine the optimal concentration that leads to the greatest volume of PP in WT mice. There were 7.7 ± 0.5 (n = 14) PP in 12-week-old WT mice administered 0.1% CHAX. The number significantly increased to 8.9 ± 0.5 when the mice were fed 0.5% CHAX and higher. (p = 0.0246, Mann–Whitney test) We chose the 0.1% and 1% concentrations of CHAX as optimal doses for PP formation. Furthermore, we fed 0.1% and 1% CHAX to *Apc*$^{Min/+}$ and WT mice for 4 weeks. The number of PP significantly increased in the *Apc*$^{Min/+}$ and WT groups (Table 1). Moreover, the number of intestinal polyps was significantly decreased in *Apc*$^{Min/+}$ mice fed both 0.1% and 1% CHAX (Table 2). There were no significant differences between *Aly*$^{-/-}$*Apc*$^{Min/+}$ mice fed or not fed CHAX (Table 3). *Aly*$^{-/-}$*Apc*$^{Min/+}$ mice have no PP in the intestine.

Increasing the number of PP caused a decrease in IL-17 production. PP plays a central role in immunity; therefore, we examined the changes in cytokine levels in serum. We identified four cytokines, IL-1β, IL-5, IL-10, and IL-17, in which concentration levels were increased or decreased more than three-fold (Table 4). IL-1β and IL-17 levels in serum diminished when *Apc*$^{Min/+}$ mice were fed CHAX.

Using ELISA, we measured IL-17 concentration in the serum of *Apc*$^{Min/+}$ mice fed or not fed CHAX based on the number of PP in the small intestine. IL-17 levels gradually decreased with increasing number of PP (Fig. 3).

CHAX leads to a decrease in polyp development through the β-catenin pathway. Furthermore, we examined immunohistochemical staining of paraffin-embedded intestinal polyps for β-catenin and proliferation cell nuclear antigen (PCNA). Although β-catenin is well known for accumulating in the nucleus of intestinal polyps in *Apc*$^{Min/+}$ mice, this accrual was not observed in mice fed a 1% CHAX diet (Fig. 4a and b). In addition, the staining intensity of PCNA was weaker in mice fed 1% CHAX (Fig. 4c and d). Furthermore, we measured the messenger RNA expression level of cyclin D1, which is downstream of Wnt signaling, in intestinal polyps by quantitative real-time PCR. We found that the expression of cyclin D1 tended to decrease as CHAX concentration increased (Fig. 5).

IL-17 promotes TCF/LEF transcriptional activation *in vitro*. We examined TCF/LEF-dependent transcriptional activity after 24-h treatment with IL-17 in Caco-2 cell transfected with pTOP-Flash or pFOP-Flash and SV40-RenillaLuc, and differentiated with 5 mM sodium butyrate (Fig. 6). Caco-2 cells are an estab-

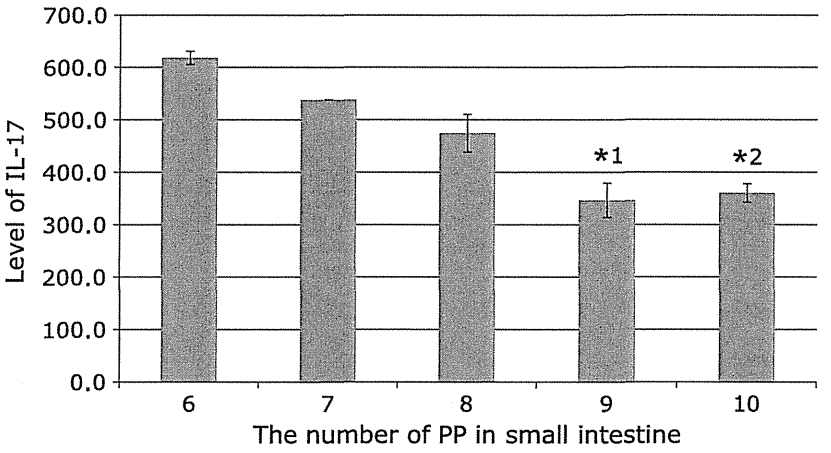

Fig. 3. The level of IL-17 in serum and the number of Peyer's Patches in 12-week-old *Apc*$^{Min/+}$ mice. The *p* values are *1 = 0.0058 and *2 = 0.0294 for IL-17 levels compared in mice which have six Peyer's Patches (Mann–Whitney *t* test). *n* = 2, 2, 6, 8, and 5 from the left.

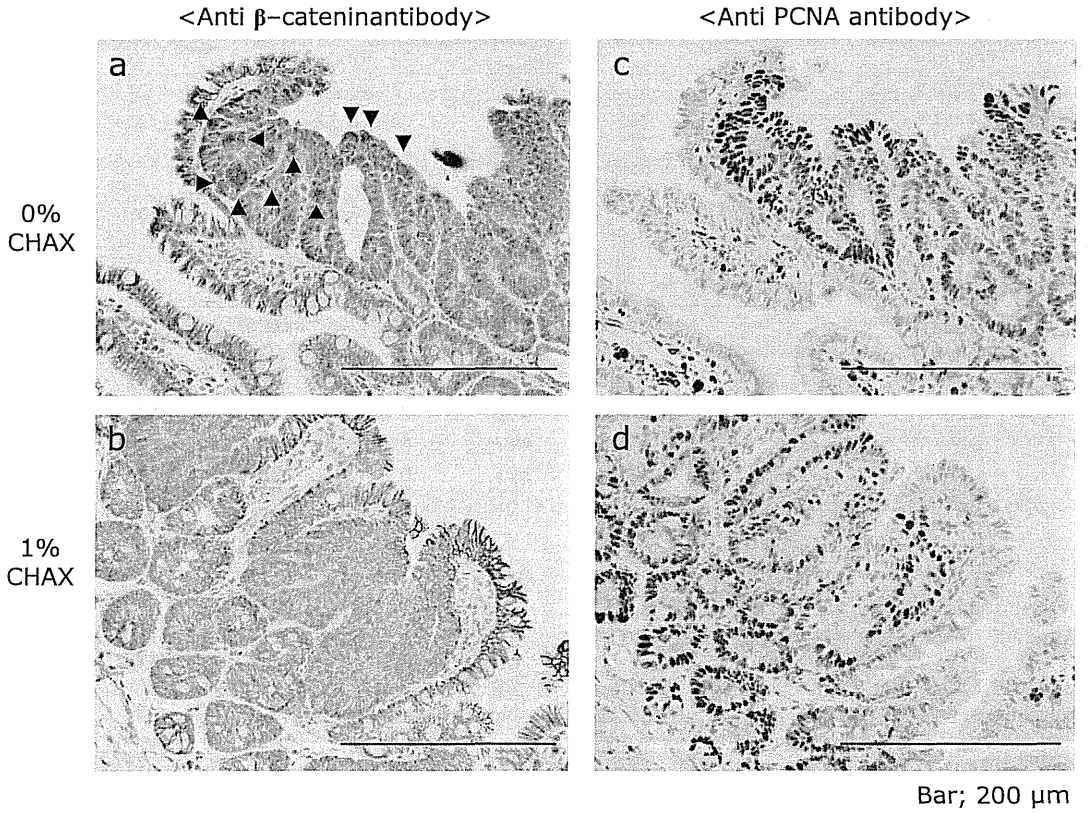

Fig. 4. Immunostaining of formalin-fixed paraffin-embedded polyps of the small intestine of *Apc*$^{Min/+}$ mice fed with (b and d) or without (a and c) CHAX. ▼ shows β-catenin aggregation in the nucleus.

lished cell line derived from colorectal tumors with loss of *APC* gene function.

There was no significant increase in TCF/LEF-dependent transcriptional activity with 0.2 ng/ml of IL-17, but this was observed at 0.5 ng/ml ($p<0.001$, Dunnett's multiple comparison test). TCF/LEF activity was activated by IL-17 in a dose-dependent manner. The control, 0.2, and 0.5 ng/ml IL-17 values for TOP/FOP were 1 ± 0.15, 1.13 ± 0.03, and 1.40 ± 0.12, respectively.

Discussion

With continuing observation, we noticed that the number of intestinal polyps in *Apc*$^{Min/+}$ mice varied. Usually, it is assumed that intestinal polyps appear in *Apc*$^{Min/+}$ mice at 8 weeks of age, and we confirmed several polyps at this age. Furthermore, it is known that the number of polyps will increase with age; the life span of *Apc*$^{Min/+}$ mouse is approximately 24 weeks because of bleeding caused anemia from intestinal polyps and other factors.[18–20] We

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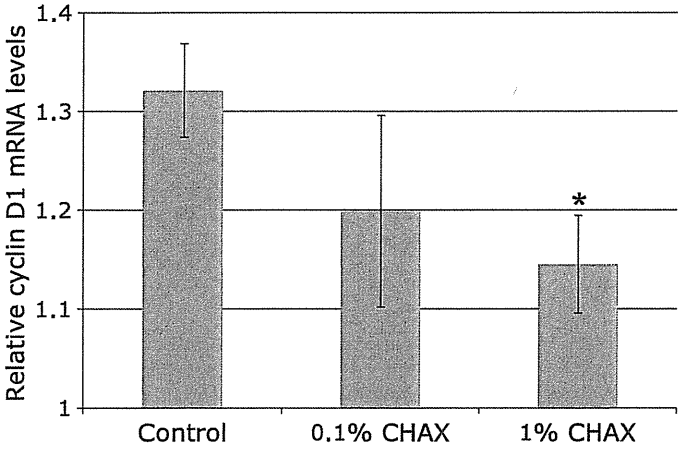

Fig. 5. Quantitative real-time PCR of cyclin D1. The p value is * = 0.0426 (Mann–Whitney t test). The number of polyps used: n = 6–8, respectively. cyclin D1 mRNA levels were corrected using GAPDH as a control.

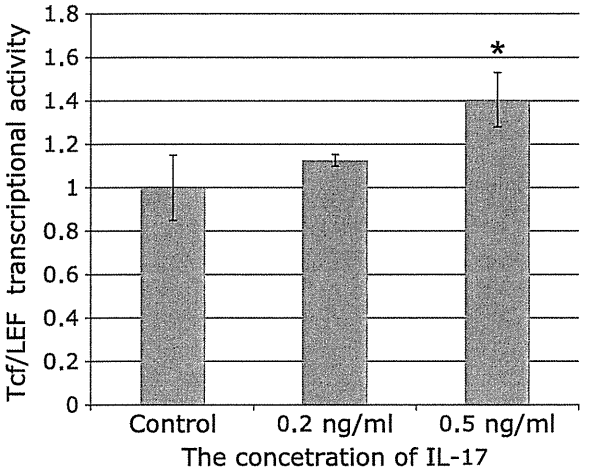

Fig. 6. TCF/LEF transcriptional activity was induced by IL-17 in Caco-2 cells. The cells were stimulated with 5 mM sodium butyrate. Data are representative of at least there independent experiments. The TCF/LEF transcriptional activity was significantly increased with 0.5 ng/ml of IL-17 (*1 = p<0.001, Dunnett's multiple comparison test).

noticed the presence of numerous PP when the mice have few polyps in the small intestine, we sought to examine the correlation between the number of polyps and PP. Previous reports have shown that the number of PP in the intestines is increased when mice are fed CHAX and/or short chain fructooligosaccharide diet.[21,22] However, there were no reports that described this change in PP levels in detail.

Our findings show that intestinal polyp formation in $Aly^{-/-}Apc^{Min/+}$ mice is lower in comparison with $Apc^{Min/+}$ mice. $Aly^{-/-}$ mice are created by a point mutation in the tumor necrosis factor receptor associated factor (TRAF) binding domain, which is in the *nuclear factor-κB (NF-κB)-inducing* kinase (*Nik*) gene.[13] *NF-κB* is involved in many physiological phenomena such as acute and a chronic inflammation reactions, cell growth, and apoptosis. Furthermore, in many cases, *NF-κB* is activated in malignant tumors. Therefore, the decreased number of intestinal polyps in $Aly^{-/-}Apc^{Min/+}$ mice compared to $Apc^{Min/+}$ mice may be because of the TRAF family, which have tumor promoting properties, not binding Nik.[23,24]

We examined changes in immunoassociated cytokines using cytokine array, because PP are critical organs in intestinal immunity and may be involved in intestinal polyp formation. In this study, we focused on IL-17, which increased the number of PP in intestine; the levels of IL-1β remained unchanged in WT mice. IL-5 and IL-10 were excluded from analysis because the changes in concentration were only observed in $Aly^{-/-}Apc^{Min/+}$ mice that do not develop PP. IL-17 is known to be produced by T helper 17 cells (Th17) cells and plays an important role in allergic responses, such as contact hypersensitivity, delayed hypersensitivity, and airway hyper-reactivity, as determined by IL-17 gene knockout mouse studies.[25] We found an inverse correlation existed between IL-17 concentration and the number of PP, suggesting a relationship between IL-17 and the number of PP. Although the reason of this phenomenon is not known in detail, Hirota *et al.*[26] reported that expression of IL-17 decreases when Th17 cells induce B cell formation in PP.

Short chain fatty acids, such as sodium butyrate, are mainly produced by intestinal bacteria, and are largely involved in differentiation and nutrition of intestinal epithelial cells.[27–29] Our cytokine array revealed that IL-17 levels were low (Table 4). Moreover, our results demonstrate a role for IL-17 in the enhancement of Wnt signal in the intestine. There are several reports highlighting that IL-17 is involved in intestinal polyp formation in $Apc^{Min/+}$ mice or it can aggravate inflammatory bowel disease.[30–32] These findings support the hypothesis that the number of intestinal polyps is decreased due to an increase in PP in $Apc^{Min/+}$ mice fed CHAX. Moreover, reduced expression of IL-17 in serum possibly causes destabilization of Wnt signaling. In the future, we propose that quantification of intestinal PP will be a useful tool for the prevention of tumorigenesis.

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Conflict of interest

No potential conflicts of interest were disclosed.

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