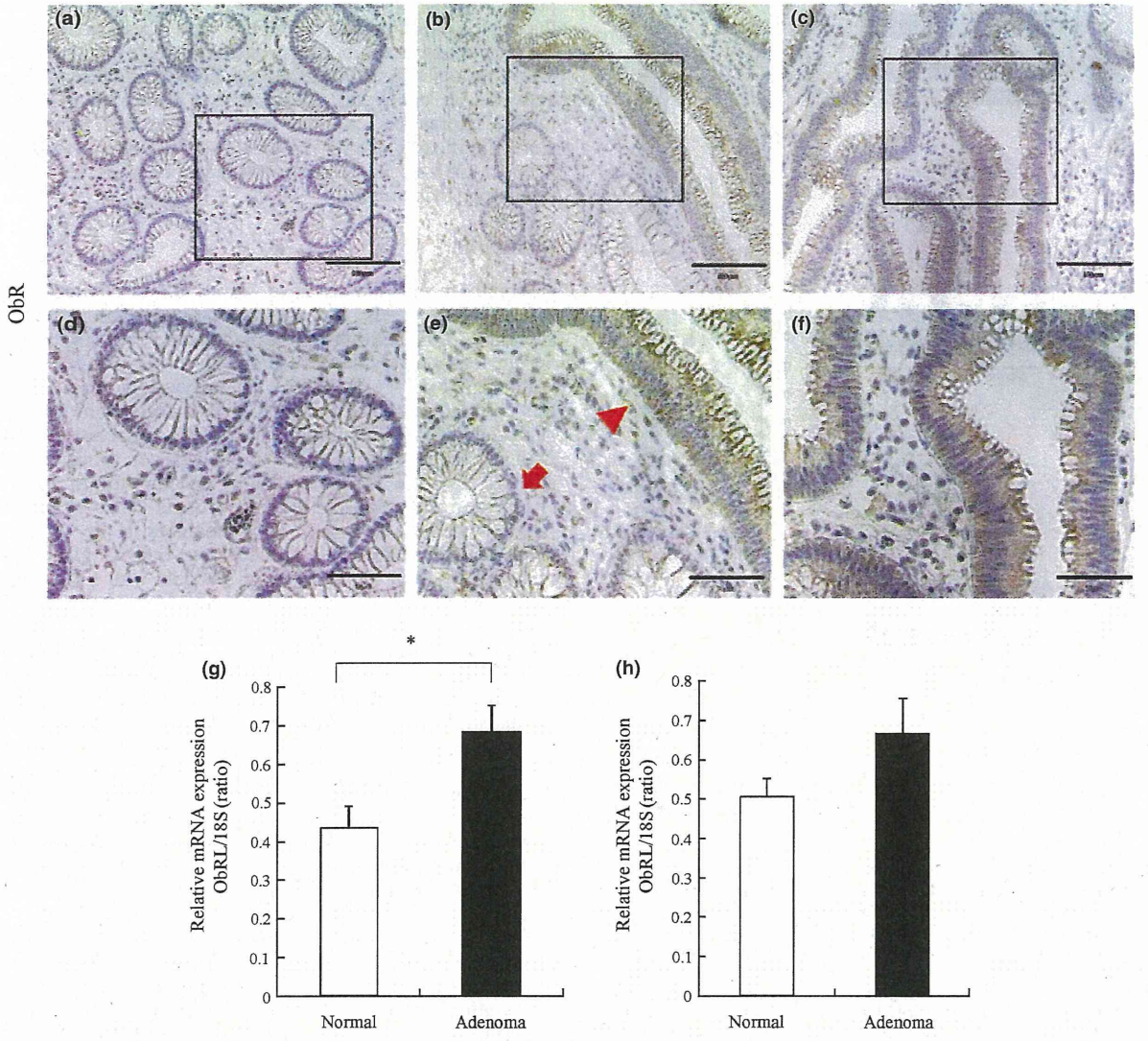

Fig. 2. Immunohistochemical staining for ObR and mRNA level of the leptin receptor. (a) Normal colorectal tissue. (b,c) Colorectal adenoma tissues. (d–f) magnified view of (a–c), respectively. The red arrowhead represents a normal gland and the red arrow points to an adenoma gland. The relative mRNA expressions of (g) ObRL and (h) ObRS in colorectal adenoma and normal colorectal tissues were expressed as the ratio relative to the expression of 18S. Each column represents the mean ± SEM from 12 patients. Statistical analysis was performed using the Mann–Whitney *U*-test. $*P < 0.05$. $**P < 0.01$. ObR, leptin receptor; ObRL, leptin receptor long variant; ObRS, leptin receptor short variant.

conducted gene expression analyses specific for ObRL and ObRS. The mRNA expression levels of ObRL and ObRS in the colorectal adenomas and normal colorectal tissues were investigated. The mRNA expression level of ObRL was significantly higher in the colorectal adenomas than in the normal colorectal tissues. In contrast, the expression of ObRS was slightly but not significantly higher in the colorectal adenomas than in the normal colorectal tissues (Fig. 2g,h). Furthermore, western blot analysis was performed to analyze the phosphorylation level of the cytoplastic domain of ObR to investigate the signaling pathway of the leptin receptor. Western blot analysis showed significant increase of ObR expression in the colorectal adenomas than in the normal colorectal tissues (Fig. 3a). Moreover, the Tyr 1141 phosphorylation level of ObR that is required for leptin-induced activation of STAT3[28] was significantly higher in the colorectal adenomas than that in the normal colorectal tissues. In contrast, no difference was observed in the Tyr 985 phosphorylation level of ObR that is required for activation of the extracellular-signal-regulated kinase (ERK) signaling pathway (Fig. 3b,c).[29] These results suggest that the phosphorylation of ObRL in adenomas might activate the JAK/STAT signaling pathway.

Phosphorylated STAT3 in colorectal adenoma. To investigate the activation of STAT3, immunohistochemical staining and western blot analysis of STAT3 phosphorylation (p-STAT3) status were performed. Expression of p-STAT3 was predominantly observed in the nuclei of the adenoma gland cells, but only faint expression was observed in the normal gland cells (Fig. 4). The percentage of cells showing positive staining for p-STAT3 in the examined tissue specimens of colorectal adenoma was 49.1% (30/61). The expression level of p-STAT3 was significantly higher in ObR-positive adenomas than in ObR-negative adenomas, as shown in Table 2. The western blot analysis showed that the levels of p-JAK2 and p-STAT3 were significantly higher in the adenomas than in the normal colorectal tissues (Fig. 5). In addition, the mRNA levels of the genes

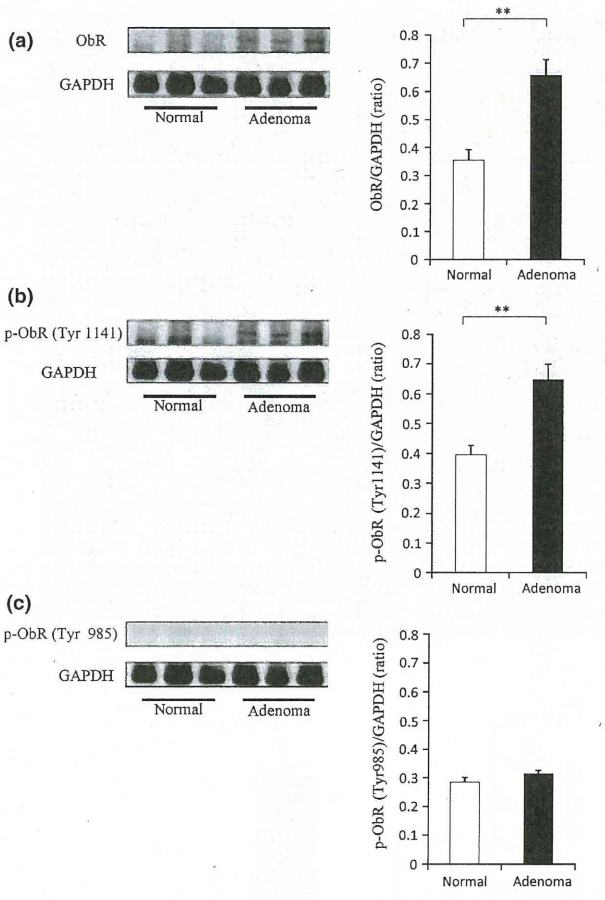

Fig. 3. Western blot analysis for leptin receptor (ObR) and phosphorylated ObR (Tyr 1141 or Tyr 985). (a) ObR, (b) Tyr 1141-phosphorylated and (c) Tyr 985-phosphorylated ObR. Left panels: representative western blot images for ObR, Tyr 1141-phosphorylated and Tyr 985-phosphorylated ObR. Lanes 1, 2 and 3, normal colorectal tissues; lanes 4, 5 and 6, colorectal adenoma tissues. Right panels: ratios of ObR, Tyr 1141-phosphorylated and Tyr 985-phosphorylated ObR expressions to the expression level of GAPDH are shown. Each column represents the mean with the SEM from 25 patients. Statistical analysis was performed using the Mann–Whitney *U*-test. $*P < 0.05$. $**P < 0.01$.

encoded by STAT3 were analyzed by real-time RT-PCR. The expression levels of the apoptosis-suppressing protein BclX, the late G1 to G1/S phase proteins cyclin D1 and c-Myc, the G2/M phase proteins cdc2 and cyclin B1[30] and the genes encoding the angiogenesis protein VEGF[31] were significantly higher in the adenomas than in the normal colorectal tissues (Fig. 6). These results suggest that the JAK/STAT signaling pathway is activated in colorectal adenomas. As shown in Table 3, the mRNA expression levels of BclX, c-Myc, cdc2 and cyclinB1 were significantly higher in ObR-positive colorectal adenomas than in ObR-negative colorectal adenomas, as evaluated by immunohistochemistry.

Discussion

Although recent studies have shown an association between serum leptin levels and the presence/absence of colorectal adenoma, the relationship still remains controversial.[16–23] Serum leptin levels have been shown to be strongly correlated with the BMI.[7–9] Therefore, as bodyweight might influence this correlation, the bodyweight differences should be carefully analyzed while interpreting the above correlation. Patients with cachexia were included in the cancer group in several studies.[18,19] We therefore suspect that this might have influenced the results and caused the conflicts in the results of the previous studies. To reduce the differences in the serum leptin levels caused by the effect of cancer on bodyweight, we investigated the serum leptin levels in patients with colorectal adenoma, which is regarded as a precancerous lesion,[32] not associated with bodyweight loss. Also in the present study, we observed a significant correlation between serum leptin levels and the BMI, consistent with previous reports;[7–9] we consider that this reflects the high reliability of our data. We observed no statistically significant differences in the serum leptin levels between patients with colorectal adenoma and normal control subjects in the present study. This result suggests the possibility of colorectal adenoma being associated with leptin receptor expression or activation, but not with serum leptin concentrations, assuming that leptin plays some role in colorectal adenoma growth. To elucidate this hypothesis, we investigated the expression and signal transduction mediated by leptin receptors in colorectal adenomas.

Several earlier studies have demonstrated, by immunohistochemical analysis, the expression of ObR in colorectal cancer and normal colorectal tissues.[33] In addition, recent studies have also confirmed the expression of ObR in colorectal adenomas and cancers.[34] However, none of the previous studies considered the expression of ObR isofoms, namely, ObRL and ObRS, in the colorectal tissues. In the present study, we showed, by immunohistochemical analysis, that ObR was clearly expressed in colorectal adenomas, but only weakly expressed in normal colorectal tissues. In addition to the immunohistochemical data, colorectal adenomas were also found to show significantly

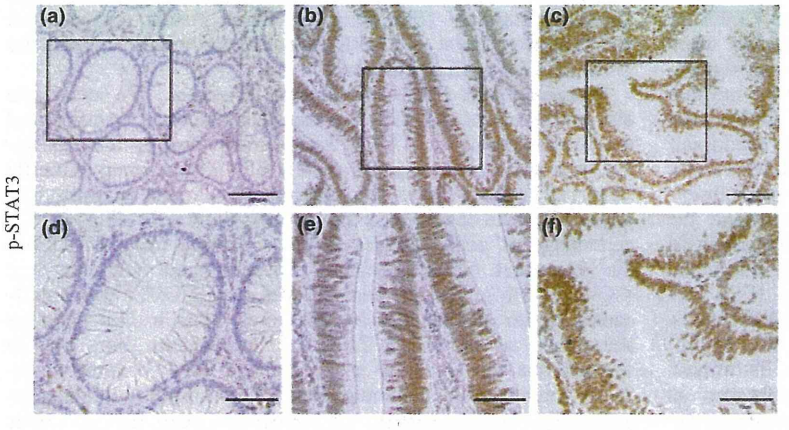

Fig. 4. Immunohistochemical staining for phosphorylated STAT3 in colorectal tissues. (a) Normal colorectal tissue. (b,c) Colorectal adenoma tissues. (d–f) Magnified view of (a–c), respectively.

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Table 2. Correlation between the expressions of ObR and p-STAT3 in colorectal adenomas by immunohistochemical analysis

	ObR-positive adenoma	ObR-negative adenoma	*P* value
p-STAT3-positive adenoma	58.8% (24/41)	30% (6/20)	<0.05*

Data are shown as the percentage and number of phospho-STAT3 (p-STAT3)-positive colorectal adenoma samples in ObR-positive and ObR-negative colorectal adenoma. Statistical analysis was performed using the chi-square test. *$P < 0.05$. **$P < 0.01$. ObR, leptin receptor.

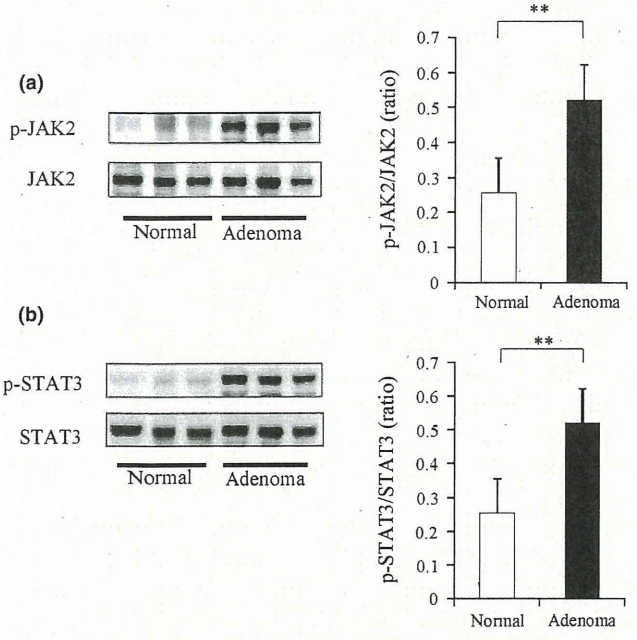

Fig. 5. Western blot analysis for phosphorylated JAK2 and STAT3. (a) Phosphorylated JAK2 and (b) phosphorylated STAT3 in normal colorectal and adenoma tissues. Left panels: representative western blot images for phosphorylated and total levels of JAK2 and STAT3. Lanes 1, 2 and 3, normal colorectal tissues; lanes 4, 5 and 6, colorectal adenoma tissues, respectively. Right panels: ratios of the phosphorylated protein levels compared with the total protein level. Each column represents the mean with the SEM from 25 patients. Statistical analysis was performed using the Mann–Whitney *U*-test. *$P < 0.05$. **$P < 0.01$.

higher expression levels of the gene for ObRL, but not for ObRS, than normal colorectal tissues. These results suggest that the expression of ObRL rather than ObRS might be important for the downstream signal transduction in colorectal adenomas. Therefore, we investigated the ObRL-mediated signaling pathways in colorectal adenomas. It is known that phosphorylation of Tyr 1141 of ObRL by leptin activates the JAK/STAT signaling pathway.[28] We demonstrated significantly increased phosphorylation of Tyr 1141, but not Tyr 985, in colorectal adenomas than in the normal colorectal tissues. Taken together, these results suggest that induction of ObRL gene expression in colorectal adenomas might augment phosphorylation of Tyr 1141 of ObRL by leptin, which might result in activation of the JAK/STAT signaling pathway. In fact, we showed enhanced activation of the JAK/STAT signaling pathway and higher gene expressions downstream of the STAT3 signaling pathway in colorectal adenomas than in the normal colorectal tissues.

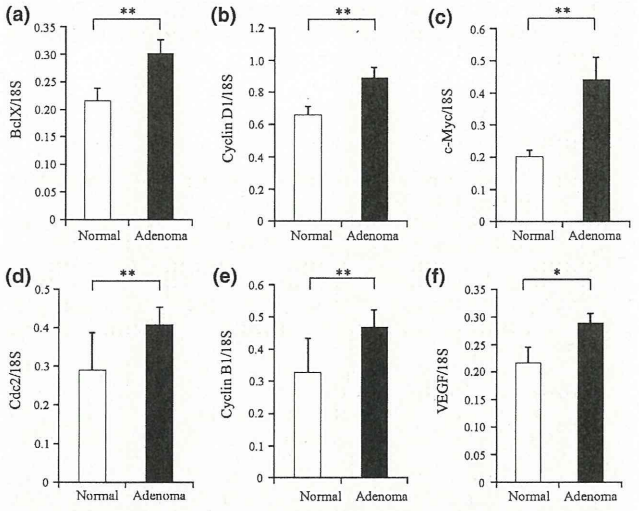

Fig. 6. The expression of downstream genes encoded by STAT3 transcriptional regulation in normal colorectal and adenoma tissues. The relative mRNA expressions of (a) BclX, (b) cyclinD1, (c) c-Myc, (d) cdc2, (e) cyclin B1 and (f) VEGF in colorectal adenoma and normal colorectal tissues were expressed as the ratios relative to the expression of 18S. Each column represents the mean with the SEM from 12 patients. Statistical analysis was performed using the Mann–Whitney *U*-test. *$P < 0.05$, **$P < 0.01$.

Table 3. The expression of downstream genes encoded by STAT3 transcriptional regulation in ObR-positive adenomas and ObR-negative adenomas

	ObR-positive adenoma ($n = 7$)	ObR-negative adenoma ($n = 5$)	*P* value
BclX	0.34 ± 0.09	0.24 ± 0.07	<0.05*
cdc2	0.50 ± 0.12	0.24 ± 0.11	<0.01**
Cyclin D1	0.96 ± 0.17	0.76 ± 0.32	0.21
Cyclin B1	0.58 ± 0.17	0.28 ± 0.07	<0.01**
c-Myc	0.57 ± 0.23	0.21 ± 0.09	<0.01**
VEGF	0.29 ± 0.06	0.28 ± 0.08	0.46

Data are shown as mean ± standard deviation. Statistical analysis was performed using the Mann–Whitney *U*-test. *$P < 0.05$. **$P < 0.01$. ObR, leptin receptor; VEGF, vascular endothelial growth factor.

Although we could not show direct evidence of this signaling in human colorectal adenomas, our results provided evidence to suggest that leptin-mediated STAT3 signaling through activation of ObRL in colorectal adenoma might control the expression of genes involved in the cell cycle and apoptosis. Further investigations are required to clarify the growth mechanism of colorectal adenoma.

In conclusion, STAT3-mediated leptin signaling through the activation of ObRL in colorectal adenoma directly controls the expressions of genes involved in the cell cycle and apoptosis, resulting in the growth of adenoma cells.

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Disclosure Statement

The authors declare no conflict of interest.

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Research Article

Cancer Prevention Research

Metformin Suppresses Colorectal Aberrant Crypt Foci in a Short-term Clinical Trial

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Abstract

The biguanide metformin is widely used for treating diabetes mellitus. We previously showed the chemopreventive effect of metformin in two rodent models of colorectal carcinogenesis. However, besides epidemiologic studies, little is known about the effects of metformin on human colorectal carcinogenesis. The objective of this pilot study was to evaluate the chemopreventive effect of metformin on rectal aberrant crypt foci (ACF), which are an endoscopic surrogate marker of colorectal cancer. We prospectively randomized 26 nondiabetic patients with ACF to treatment with metformin (250 mg/d, $n = 12$) or no treatment (control, $n = 14$); 23 patients were evaluable for end point analyses (9 metformin and 14 control); the two groups were similar in ACF number and other baseline clinical characteristics. Magnifying colonoscopy determined the number of rectal ACF in each patient at baseline and after 1 month in a blinded fashion (as were all laboratory end point analyses). We also examined proliferative activity in colonic epithelium (via proliferating cell nuclear antigen labeling index) and apoptotic activity (via terminal deoxynucleotidyl transferase dUTP nick-end labeling). At 1 month, the metformin group had a significant decrease in the mean number of ACF per patient (8.78 ± 6.45 before treatment versus 5.11 ± 4.99 at 1 month, $P = 0.007$), whereas the mean ACF number did not change significantly in the control group (7.23 ± 6.65 versus 7.56 ± 6.75, $P = 0.609$). The proliferating cell nuclear antigen index was significantly decreased and the apoptotic cell index remained unaltered in normal rectal epithelium in metformin patients. This first reported trial of metformin for inhibiting colorectal carcinogenesis in humans provides preliminary evidence that metformin suppresses colonic epithelial proliferation and rectal ACF formation in humans, suggesting its promise for the chemoprevention of colorectal cancer. *Cancer Prev Res; 3(9); 1077–83.* ©2010 AACR.

Introduction

Colorectal cancer (CRC) is a major neoplasm worldwide (1), and its prevalence and mortality have been increasing (2). A paradigm shift from surveillance for early detection of cancer or adenomas (polypectomy) to new preventive strategies, including chemoprevention, is needed to lower the burden of this disease. Several large epidemiologic and/or clinical studies have evaluated the possible effects of more than 200 agents, including fiber, calcium, and nonsteroidal anti-inflammatory drugs including aspirin and selective cyclooxygenase-2 (COX-2) inhibitors, in protecting against CRC development (3). Nonsteroidal anti-inflammatory drugs, especially COX-2 inhibitors alone or in combination, have shown the most promise for CRC risk reduction (4), but reports have revealed an increased risk of serious cardiovascular events associated with COX-2 inhibitor use (5, 6). In light of the adverse cardiovascular effects of otherwise effective COX-2 inhibitors and ineffective clinical results with other agents that had shown promise in this setting, novel drugs that would be both safe and effective are needed for CRC prevention. CRC is associated with lifestyle-related diseases such as diabetes and obesity (7–10), and these conditions might represent new targets for CRC chemoprevention.

Metformin (1,1-dimethylbiguanide hydrochloride) is a biguanide derivative that has been long and widely used for treating diabetes mellitus (11). It decreases basal glucose output by suppressing gluconeogenesis and glycogenolysis in the liver and by increasing glucose uptake in muscle tissue. Because metformin does not directly stimulate insulin secretion, the risk of hypoglycemia associated with its use is lower than that associated with the use of other oral antidiabetes drugs (12). The molecular mechanism of metformin involves liver kinase B1–dependent

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activation of AMP-activated protein kinase (AMPK; ref. 13). Patients with type 2 diabetes taking metformin might be at a lower risk of cancer (including CRC) compared with those patients who do not take metformin (14, 15). This evidence suggests that metformin might be a candidate agent for the chemoprevention of CRC in diabetic patients.

Previously, we showed the chemopreventive effect of metformin in two rodent models of colorectal carcinogenesis: one a genetic cancer model, the other a chemically induced cancer model. We showed that metformin suppressed the development of intestinal polyps in adenomatous polyposis coli ($APC^{Min/+}$) mice, a murine model of familial adenomatous polyposis (16). Furthermore, we showed that metformin suppressed the azoxymethane-induced formation of colorectal aberrant crypt foci (ACF) by activating AMPK (17). Both studies were performed in nondiabetic mice, suggesting the chemopreventive potential of metformin in nondiabetic patients. Little is known, however, about the effect of metformin on human colorectal carcinogenesis.

The end points of CRC chemoprevention trials are, in general, the incidence of polyps or of the cancer itself. These end points usually require several years of evaluation, and therefore such studies can be fraught with compliance issues and a high dropout rate. To overcome these problems, shorter-term surrogate markers for cancer could be used to advantage. ACF are tiny lesions that develop in the earliest stage of colorectal carcinogenesis and consist of large, thick crypts identified by dense methylene blue staining (18–21). ACF have been shown to be precursor lesions of the adenoma-carcinoma sequence in humans (22) and have been suggested as a suitable surrogate end point for CRC chemoprevention trials. Therefore, we conducted a prospective pilot clinical trial to evaluate the chemopreventive effects and safety of metformin against ACF formation in nondiabetic patients. This is the first report of a clinical trial of metformin for inhibiting colorectal carcinogenesis in humans.

Materials and Methods

Patients and study design

Eligible patients had ACF determined by clinical and histologic criteria, did not have diabetes, and had a medical history and physical examination indicating that they were in acceptable health and had no severe systemic diseases that could affect the results of a colonoscopic examination. Major exclusion criteria included the following conditions: liver or renal dysfunction, pregnancy, history of drug allergy, lactic acidosis or colorectal cancer, and current nonsteroidal anti-inflammatory drug use. Eligible participants were randomized to metformin (250 mg/d) or control (untreated) groups. Each participant was treated (metformin) or followed (control/untreated) for 1 month by protocol, and the number of ACF per patient in the two groups was examined by magnifying colonoscopy before and after 1 month of study. The study was conducted in accordance with the Declaration of Helsinki (revised 1989) and with the approval of the Ethics Committee of Yokohama City University School of Medicine. Each participant provided written informed consent for study participation and the use of their data for research purposes.

Magnifying endoscopy and criteria for endoscopic diagnosis

Bowel preparation for colonoscopy was carried out using polyethylene glycol solution. A Fujinon EC-590ZW/M-H colonoscope was used to perform the magnifying colonoscopy (Fujinon Toshiba ES Systems, Co., Ltd.). Total colonoscopy was performed before imaging of the rectal

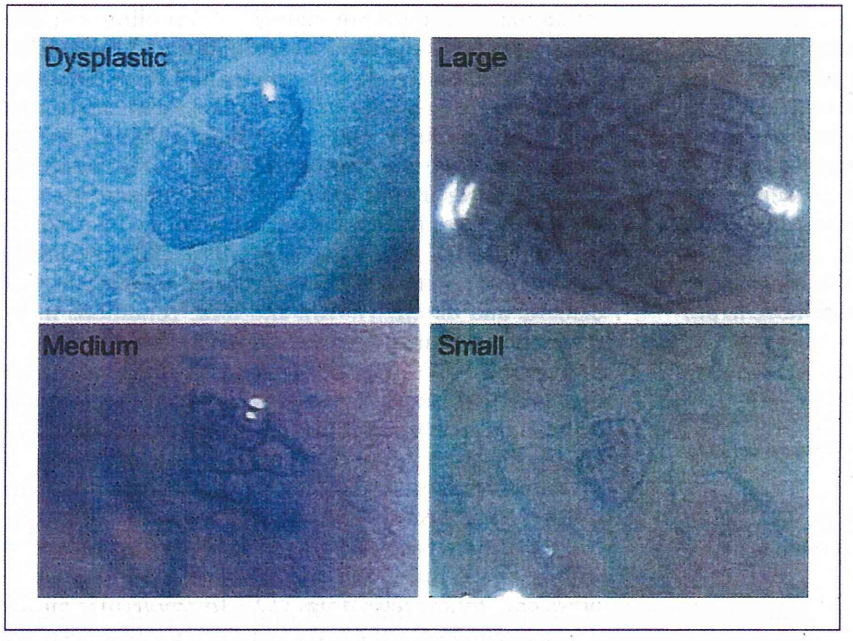

Fig. 1. Endoscopic features of ACF, as defined in Materials and Methods. Dysplastic, small, medium, and large ACF are shown.

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Table 1. Baseline clinical patient characteristics

	Metformin	Control	*P*
No. of patients	9	14	NS
Male/female	8:1	12:2	NS
Age (y)*	69.1 ± 5.95	64.2 ± 90.7	NS
BMI (kg/m^2)*	23.7 ± 3.21	24.3 ± 3.74	NS
HbA1c (%)*	5.8 ± 1.01	5.4 ± 0.56	NS
Adenomas (n, %)	4 (44.4)	6 (42.8)	NS
ACF (mean number per patient)*	8.78 ± 6.45	7.23 ± 6.65	NS

Abbreviations: NS, nonsignificant; BMI, body mass index; HbA1c, hemoglobin A1c.
*Data are expressed as mean ± SD.

ACF. Subsequently, 0.25% methylene blue was applied to the mucosa with a spray catheter, as described previously (22). Methylene blue was applied to the lower rectal region extending from the middle Houston valve to the dentate line. Lesions consisting of large, thick crypts in the methylene blue–stained regions of the colon were defined as ACF (19–21). Dysplastic ACF were defined as crypts in which each lumen was compressed or not distinct, with an epithelial lining that was much thicker than that of the normal surrounding crypts (21). Similarly, ACF were classified according to the number of crypts per focus (small, 1 to 9 crypts; medium, 10 to 19 crypts; and large, 20 crypts or more per focus; ref. 22; Fig. 1). All ACF were recorded photographically and evaluated by two independent observers (H. Takahashi and K. Hosono), who were blinded to the clinical history and study group of the patients.

Insulin resistance and plasma lipid levels

Metformin is widely used as an antidiabetes drug that improves insulin resistance. Therefore, we investigated the effect of metformin on insulin resistance and plasma lipid level by comparing these end points at baseline and after 1 month within the metformin group; the analyses were blinded as to the timing of the sample. The plasma levels of fasting glucose, insulin, cholesterol, and triglyceride were measured using an ELISA kit (Wako Pure Chemical). The degree of insulin resistance, as represented by the homeostasis model assessment of insulin resistance (HOMA-IR), was investigated (23); HOMA-IR was calculated using the following formula: HOMA-IR fasting = plasma immunoreactive insulin (μU/mL) × fasting plasma glucose (mg/dL)/405.

Assays for cell proliferative and apoptotic activities in rectal epithelium

To evaluate the effects of metformin on cell proliferative and apoptotic activities in rectal epithelium, we used immunohistochemical techniques, and the analyses, which compared baseline to 1-month results within the metformin group, were blinded as to the timing of the samples. Proliferative activity was evaluated by staining for the proliferative cell nuclear antigen (PCNA), and apoptotic activity was evaluated by the terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick-end labeling (TUNEL) method. Colonic epithelial samples were obtained from the same trial patients by biopsy before and after metformin treatment. Samples embedded in paraffin were sectioned at 4 μm thickness

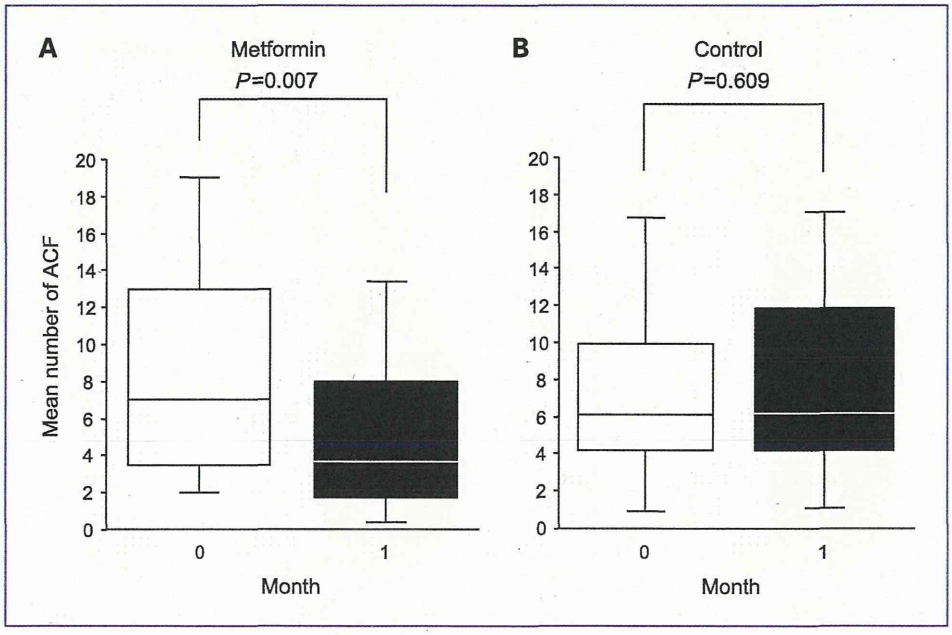

Fig. 2. Metformin effect on ACF. A, the mean number of rectal ACF per patient was significantly decreased at 1 mo (versus at baseline) in the metformin group. B, in contrast, the mean number of ACF per patient remained similar at 1 mo (versus at baseline) in the control group. Data are presented in a box-and-whisker plot. Each box includes the mean of all results falling between the 25th and 75th percentiles, and the median value is represented as a horizontal line inside each box.

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and mounted on slides coated with polylysine. Sections were deparaffinized in xylene and dehydrated through a series of graded alcohol solutions. Inhibition of endogenous peroxidase activity was performed with 3% hydrogen peroxide. The blocking of nonspecific interaction was accomplished by incubating the samples with a serum block for 30 minutes at room temperature. Sections were then incubated overnight at 4°C with the primary antibody, PCNA mouse monoclonal antibody (no. 2586, 1:4,000 dilution; Cell Signaling Technology). The antibody was applied to the sections and incubated with Histofine simple stain max PO kit for 30 minutes (Nichirei Laboratories), in accordance with the instructions of the manufacturer. The signals were counterstained with hematoxylin and visualized with diaminobenzidine (peroxidase substrate kit; Vector Laboratories). Six random microscopic fields per sample of ~250 cells were counted at a magnification of ×400 using a bright-field microscope. Results were expressed as the percentage of positively stained cells.

Cells were assessed for apoptosis by staining with a TUNEL staining kit, in accordance with the instructions of the manufacturer (Wako Pure Chemical). The apoptotic index (percentage of cells showing positive TUNEL staining relative to the total number of cells) was determined similarly to PCNA results.

Statistical analysis

Data were expressed as mean ± SD, unless otherwise indicated. All comparative results involve within-group comparisons of baseline with 1 month data in the metformin or control group (for ACF) or within the metformin group (for laboratory end points). The statistical significance was determined using a paired or unpaired t test using the StatView software (SAS Institute, Inc.). $P < 0.05$ was considered as denoting statistical significance.

Results

Patient characteristics

Trial recruitment and conduct took place between July 2008 and May 2010 at the Yokohama City University School of Medicine (UMIN clinical trial registry 000001241). During this period, 1342 patients were screened in our colorectal neoplasia screening clinic, and 629 patients had ACF and were assessed for trial eligibility. ACF occurred in 80.9% (430/531) of patients also found to have adenomas and in 24.5% (199/811) of patients without adenomas ($P < 0.001$), showing an association of ACF with presence of adenomas. Of the 629 ACF patients, 402 refused to participate and 201 were ineligible (Supplementary Fig. S1). Reasons for exclusion were NSAID use (54.7%), liver dysfunction (cirrhosis, hepatitis, elevated enzymes; 30.8%), renal dysfunction (e.g., elevated creatinine; 10.5%), pregnancy (1.5%), CRC (1.5%), and drug allergy (1.0%). Patients with adenomas removed during screening colonoscopy were eligible. Therefore, we prospectively randomized 26 eligible nondiabetic patients with ACF into the trial —12 into the metformin group and 14 into the control group. In the metformin group, one patient stopped treatment early and did not have a follow-up colonoscopy and two refused follow-up colonoscopy, leaving nine metformin group patients who were included in our end point analyses. Baseline patient characteristics

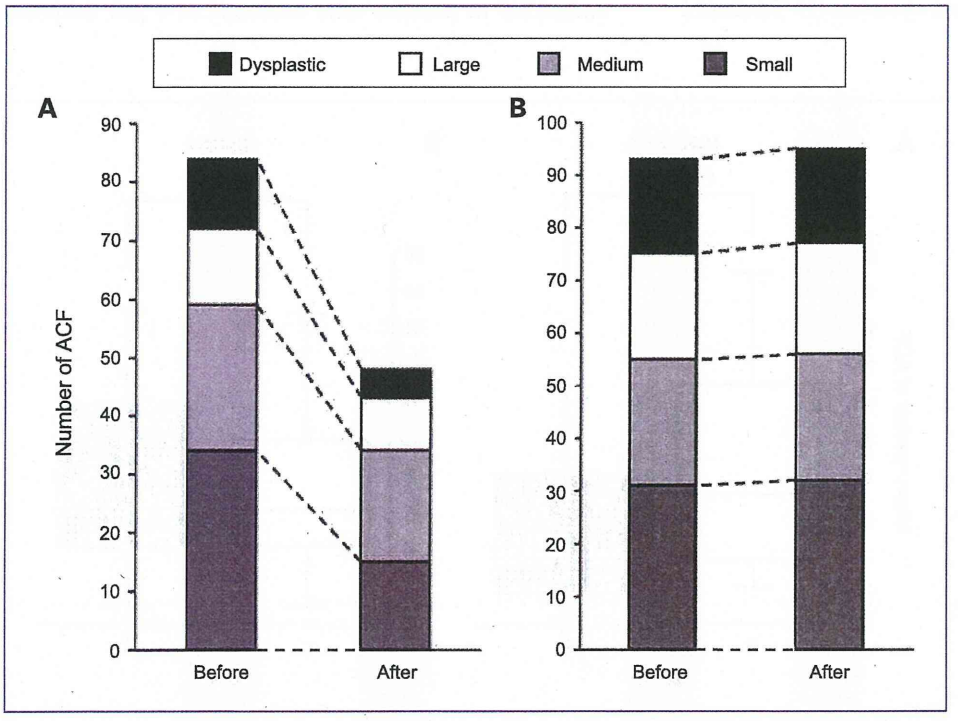

Fig. 3. The size and dysplasia distribution of ACF before (baseline) and after 1 mo in the two study groups. The overall numbers of small and dysplastic ACF were reduced by half in the metformin group (A); no significant changes occurred in the control (no treatment) group (B) in the distribution of dysplastic or large, medium, and small ACF. The dotted lines reflect the magnitude (or relative lack) of change.

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Table 2. Changes in serum laboratory data and ACF number in the metformin group between baseline and 1 mo of treatment

	Before*	After*	P
Blood glucose (mg/dL)	104.8 ± 14.5	101.3 ± 13.4	NS
HbA1c (%)	5.8 ± 1.01	5.7 ± 0.82	NS
HOMA-IR	1.21 ± 1.13	1.20 ± 0.586	NS
Total cholesterol (mg/dL)	179.2 ± 63.8	179.0 ± 66.2	NS
Triglyceride (mg/dL)	112.5 ± 29.8	107.0 ± 41.2	NS
ACF (mean number per patient)	8.78 ± 6.45	5.11 ± 4.99	0.007

Abbreviations: NS, nonsignificant; HbA1c, hemoglobin A1c.
*Data are expressed as mean ± SD.

were similar between the two study groups (Table 1), including the mean numbers of ACF per patient (8.78 ± 6.45 metformin and 7.23 ± 6.65 control).

Effect of metformin on ACF formation

In the metformin group, the mean number of ACF per patient decreased significantly from 8.78 ± 6.45 (baseline) to 5.11 ± 4.99 (at 1 month, $P = 0.007$). In contrast, there was no significant change in the mean number of ACF per patient in the control group (7.23 ± 6.65 baseline versus 7.56 ± 6.75 at 1 month, $P = 0.609$; Fig. 2). Regarding size and dysplasia distributions of ACF, the mean numbers of small and dysplastic ACF were reduced by half in the metformin group between baseline and the 1-month follow-up, whereas size and dysplasia distribution did not change significantly in the control group (Fig. 3).

Effect of metformin on insulin resistance and plasma lipid levels

Our HOMA-IR analysis found no significant differences in blood glucose levels or degree of insulin resistance in the metformin group between baseline and 1 month of treatment (Table 2). There were also no significant differences in the metformin group in plasma cholesterol or triglyceride levels between baseline and 1 month of treatment.

Effects of metformin on rectal epithelial cell proliferation

To gain insight into the mechanism of suppressive metformin effects on ACF, we investigated the PCNA labeling index of proliferative activity in the rectal epithelium. The PCNA index decreased significantly in the metformin group between baseline and the 1 month follow-up (Fig. 4A and B).

Effects of metformin on apoptotic activity in rectal epithelium

To investigate whether metformin induced apoptosis, we performed TUNEL staining of the rectal epithelium. Metformin did not significantly alter the percentage of apoptotic cells in the metformin group between baseline and at 1 month of treatment (Fig. 4C and D).

Drug safety

Metformin at a low dose of 250 mg/d did not produce any side effects, including lactic acidosis, hypoglycemia, or diarrhea in this 1-month study.

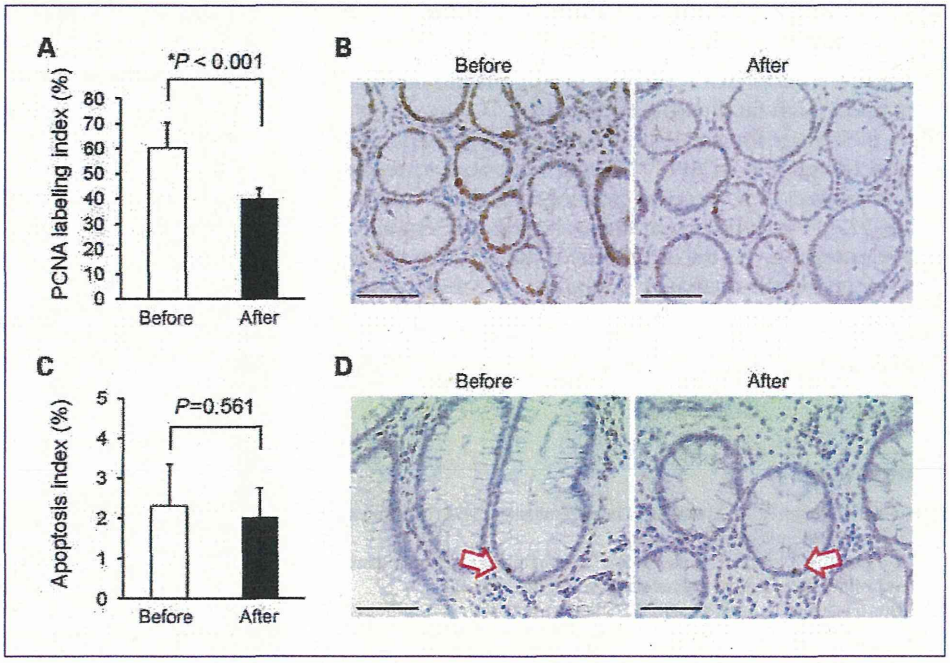

Fig. 4. PCNA staining to determine cell proliferation and TUNEL staining to determine apoptosis in the metformin group. The average PCNA index (A) and apoptotic index (C) are expressed as a percentage of positively stained nuclei out of the total number of nuclei counted; the indexes are indicated at baseline (open columns labeled "before") and after 1 mo (solid columns labeled "after"). Columns, mean; bars, SE. Representative immunohistochemical staining for PCNA (B) and TUNEL (D) is shown before and after 1 mo of study. The small dark spots indicated by arrows in D are apoptosed nuclei. The PCNA index was significantly decreased after versus before metformin treatment (A and B), whereas metformin did not induce any significant change in the percentage of apoptotic cells (C and D). Bars, 100 μm (B and D).

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Discussion

The present study clearly shows that metformin suppressed the formation of human colorectal ACF. Furthermore, metformin (at this study's dose for 1 mo), did not decrease blood glucose or affect insulin resistance, plasma cholesterol, or plasma triglyceride levels of nondiabetic ACF patients, indicating that the ACF suppression effect was direct rather than due to the attenuation of insulin resistance or hyperlipidemia.

We examined the potential direct effects of metformin on ACF via PCNA immunostaining for colorectal cell proliferative activity and TUNEL for apoptosis. The PCNA index decreased significantly following metformin treatment, although the apoptotic index did not change significantly. These data suggest that metformin effects in suppressing cell proliferation mediate suppressive effects on ACF formation.

Metformin activates AMPK, which inhibits the mammalian target of rapamycin (mTOR) pathway (13). The mTOR pathway plays an important role in protein translational machinery and cell proliferation (24). Inhibition of the mTOR pathway might be a direct mechanism of ACF suppression. The best-characterized downstream effector of mTOR is S6 kinase, which regulates the initiation and elongation phases of translation (25). Activation of the mTOR pathway accelerates cell cycle progression from G_1 to S in CRC DLD-1 cells (26). Therefore, AMPK activation may lead to the inhibition of cell growth and proliferation as a result of suppressed protein synthesis and thus might have a potent antiproliferative effect. Recent evidence indicates that metformin has a suppressive effect on tumorigenesis and cancer cell growth (27–29). Metformin activated AMPK and consequently decreased cellular proliferative activity and produced a general decrease in protein synthesis *in vitro* in human breast carcinoma cells (27). Metformin also inhibited the proliferation of human prostate cancer cells (29). Our group has shown that mTOR inhibition with rapamycin reduced ACF formation in adiponectin-deficient mice under high-fat diet conditions (30).

We previously showed that metformin suppressed intestinal polyp growth in $APC^{Min/+}$ mice (16) and colorectal ACF formation in a chemical carcinogen–induced murine model (17). We clarified that the molecular mechanism of ACF suppression was the inhibition of the mTOR pathway by the metformin-mediated activation of AMPK, which consequently led to the suppression of cellular proliferation (17). The present study extended these murine model studies to the human clinical setting, in which metformin also suppressed colorectal ACF formation. The relevance of ACF to the adenoma-carcinoma sequence in humans (22) and thus to CRC prevention is strengthened by our patient data showing that ACF were significantly associated with adenomas in the overall population of screened patients.

Metformin was re-evaluated as an antidiabetes drug with a cardioprotective effect based on evidence showing that the incidence of cardiovascular events was significantly lower in diabetics taking metformin than those taking other diabetes drugs (31). Our results similarly suggest that metformin should be re-evaluated as an antidiabetes drug with a chemopreventive effect against CRC. Our pilot trial, the first reported clinical study of the inhibitory effect of metformin on colorectal carcinogenesis, has shown that metformin, at 250 mg/d for 1 month, safely and directly suppressed both colorectal epithelial proliferation and ACF formation, supporting further, larger-scale studies of metformin in small doses for CRC chemoprevention in diabetic or nondiabetic patients. Determining some important issues, such as the potential optimal dose, schedule, and duration, will be necessary prior to conducting a long-term clinical trial of metformin for preventing colorectal polyps or cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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