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Involvement of JNK pathway in the promotion of the early stage of colorectal carcinogenesis under high-fat dietary conditions

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ABSTRACT

Background and aims: The molecular mechanisms underlying the promotion of colorectal carcinogenesis by a high-fat diet (HFD) remain unclear. We investigated the role of the insulin-signal pathway and the c-Jun N-terminal kinase (JNK) pathway, which reportedly play crucial roles in insulin resistance, during colorectal carcinogenesis in the presence of hyperinsulinaemia induced by a HFD.

Methods: Azoxymethane-induced aberrant crypt foci formation and cell proliferation in the colonic epithelium were compared between mice fed a normal diet (ND) and mice fed a HFD. A western blot analysis was performed to elucidate the mechanism affecting colorectal carcinogenesis by a HFD.

Results: The number of aberrant crypt foci and the colonic epithelial cell proliferative activity were significantly higher in the HFD group than in the ND group. While the plasma insulin level was significantly higher in the HFD group than in the ND group, a western blot analysis revealed the inactivation of Akt, which is located downstream of the insulin receptor, in the colonic epithelia of the HFD group. On the other hand, JNK activity was significantly higher in the HFD group than in the ND group. A JNK specific inhibitor significantly suppressed the increase in epithelial cell proliferation only under a HFD, but not under a ND.

Conclusions: Colonic cell proliferation was promoted via the JNK pathway in the presence of a HFD but not in the presence of a ND. This novel mechanism may explain the involvement of the JNK pathway in the effect of dietary fat intake on colon carcinogenesis.

Colorectal cancer is a major cause of morbidity and mortality worldwide.[1] Recently, associations between obesity and metabolic abnormalities, which are caused by a high intake of dietary fat and physical inactivity, and an elevated risk of colorectal cancer have been reported.[2 3] Many epidemiological studies have provided evidence of a relation between dietary fat intake and an increased risk of colorectal cancer.[4] Most animal experimental studies have shown that a high-fat diet (HFD) leads to an increased number of chemically induced aberrant crypt foci (ACF),[5] which are identifiable lesions in experimental colon carcinogenesis, and tumours[6] in the colon. The possibility that an elevated plasma insulin level promotes colorectal cancer has also been debated.[7] Some studies support the hypothesis that insulin, when exogenously administered, acts as an important growth factor for colonic epithelial cells.[8 9] However, whether an elevated plasma insulin level might enhance the proliferative state through the activation of an insulin signalling pathway downstream of the insulin receptor, such as the phosphatidylinositol 3-kinase (PI3K)/Akt signalling pathway, in the colon – a non-classical insulin target tissue – remains unclear.[10] Furthermore, adipocytokines also act as positive or negative modulators of colonic epithelium and tumours.[11 12] We previously reported that adiponectin suppressed colorectal carcinogenesis only under a HFD condition.[12] Despite accumulating evidence, the molecular mechanisms underlying the influence of a high intake of dietary fat on the promotion of colorectal carcinogenesis are not fully understood. The identification and evaluation of the relation between colorectal cancer and a high intake of dietary fat will be critical for preventive strategies against colorectal cancer in the near future.

Recent studies have demonstrated that c-Jun N-terminal kinase (JNK) plays a crucial role in obesity and insulin resistance.[13] JNK is activated in obesity, in part because of lipotoxic stress.[14] Hirosumi *et al*[13] reported abnormally elevated JNK activity levels in the liver, muscle and adipose tissues of mice fed a HFD. Most of the molecular mechanisms of JNK activity and their relation to insulin resistance have been studied in the liver and adipose tissues of various models of obesity, but little is known about the action of JNK in colonic epithelial cells. We postulated that JNK might provide a link between dietary fat intake and colorectal cancer.

The JNK pathway represents one subgroup of mitogen-activated protein kinases (MAPK) that is activated primarily by cytokines and exposure to environmental stress.[15] A major target of the JNK signalling pathway is the activator protein-1 (AP-1) transcription factor, which is activated, in part, by the phosphorylation of c-Jun and related molecules.[16] JNK has been implicated in the pathogenesis of cancer in various tissues in oncogenic transformation and cell proliferation.[15] Genetic and pharmacological approaches have been used to evaluate the potential importance of JNK in tumour formation and growth.[17 18] Growth inhibition has been observed in response to the JNK inhibitor and antisense oligonucleotides in multiple myeloma cells[19] and breast cancer cells.[20] Nateri *et al*[21] used a mouse model of intestinal tumorigenesis to show that the ablation of the c-Jun gene or the mutation of the JNK phosphorylation sites on c-Jun reduced the tumour number and size. These oncogenic functions of c-Jun are dependent on the N-terminal phosphorylation of c-Jun by JNK, implicating JNK as a potential oncogene in the intestine. Several studies

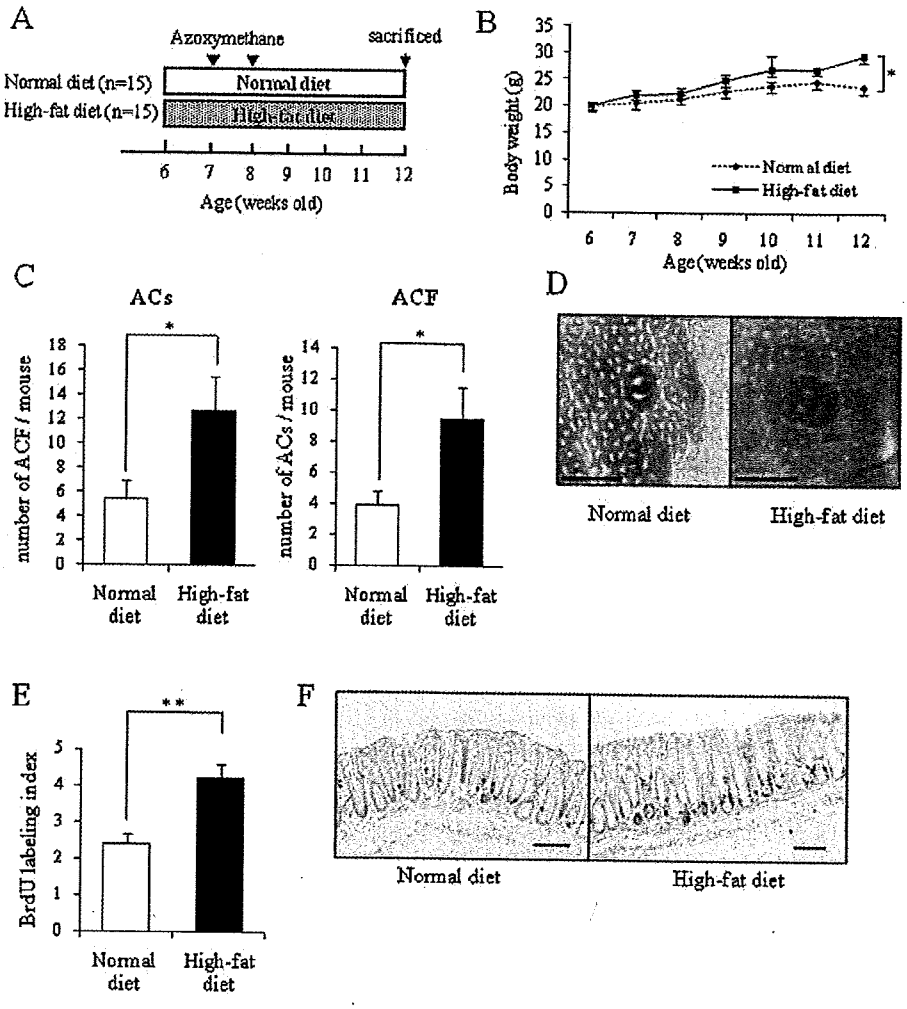

Figure 1 Enhanced proliferative activity of colonic epithelial cells in the presence of a high-fat diet. (A) ACF experiment protocol. Mice (6 weeks old) were divided into a normal diet group (n = 15) and a high-fat diet group (n = 15). Mice in each group were given two weekly intraperitoneal injections of 10 mg/kg of AOM. Six weeks after the start of the normal diet or high-fat diet, the mice were killed. (B) Changes in the body weights of mice fed a normal diet (broken line, n = 15) and mice fed a high-fat diet (solid line, n = 15) and treated with AOM. *Significant differences were observed between the normal diet and high-fat diet groups at all time points except for the initial measurement. *$p<0.01$. (C) Average numbers of ACs and ACF for mice fed a normal diet (n = 9) and mice fed a high-fat diet (n = 9). Each column represents the mean with the SEM; *$p<0.05$. (D) Stereomicroscopic observations of ACF in colon specimens from each group. Samples were stained with 0.2% methylene blue. Scale bar: 100 μm. (E) Average BrdU labelling index in each group in the ACF formation experiment. BrdU was administered intraperitoneally 1 h before the mice were killed. Both indices were expressed as the percentage of positively stained nuclei to the total number of nuclei counted in the crypts of the colon. Each bar represents the mean with the SEM of 9 mice/group. **$p<0.01$. (F) Representative immunohistochemical staining for BrdU in each group. Scale bar: 100 μm. ACs, aberrant crypts; ACF, aberrant crypt foci; AOM, azoxymethane; BrdU, 5-bromodeoxyuridine.

have demonstrated that colonic tumours of human origin exhibit an elevated expression of JNK/c-Jun.[22 23] However, the involvement of the JNK/c-Jun pathway in colon carcinogenesis under a HFD condition has not been previously reported.

Therefore, in this study, we investigated the activity of the insulin signalling pathway and the functional role of the JNK/c-Jun pathway in colorectal carcinogenesis and epithelial cell proliferation in the presence of hyperinsulinaemia induced by a HFD. This novel mechanism may explain the involvement of the JNK/c-Jun pathway in the effect of dietary fat intake on colon carcinogenesis.

MATERIALS AND METHODS

Animals and diets

C57Bl/6J mice were purchased from CLEA Japan (Tokyo, Japan). The animals were fed either a normal diet (ND) or a HFD until the end of the study. The compositions of the ND (MF; Oriental Yeast Co., Tokyo, Japan) and the HFD (High Fat Diet 32; CLEA Japan, Tokyo, Japan) have been previously described.[12] Three to five mice were housed per metallic cage, with sterilised softwood chips used as bedding, in a barrier-sustained animal room air-conditioned at 24 (SD 2)°C and 55% humidity under a 12 h light/dark cycle.

Analysis of aberrant crypt foci

Six-week-old male mice were divided into a ND group and a HFD group. Mice in each group were given two weekly intraperitoneal injections of 10 mg/kg of azoxymethane (AOM) (Sigma, St. Louis, Missouri, USA) and were killed at 6 weeks following the initiation of AOM injection (fig 1A). The entire colon was removed, gently flushed with saline to remove any faecal contents, opened longitudinally, and fixed in 10% neutralised formalin; the numbers of ACF and aberrant crypts

Table 1 Blood plasma levels of various metabolites in mice fed a normal diet or a high-fat diet

	Normal diet		High-fat diet	
	SP600125 (−)	SP600125 (+)	SP600125 (−)	SP600125 (+)
Glucose (mg/dl)	81.80 (2.20)	76.50 (2.93)	180.00 (9.63)**	143.00 (10.54)†
Insulin (ng/ml)	1.69 (0.32)	1.96 (0.44)	3.38 (0.52)*	1.54 (0.50)†
Triglycerides (mg/dl)	57.43 (5.21)	55.00 (6.21)	77.68 (10.31)	61.66 (5.81)
Cholesterol (mg/dl)	87.86 (5.96)	77.85 (2.94)	120.14 (5.62)*	92.97 (5.43)††
TNFα (pg/ml)	41.49 (7.19)	32.62 (0.64)	36.18 (0.75)	35.29 (2.03)

Data represent the mean (SEM) of 6–9 mice/group.
*p<0.05, **p<0.01 compared with mice fed a normal diet.
†p<0.05, ††p<0.01 compared with mice fed a HFD treated (−) with SP600125.
TNFα, tumour necrosis factor α

(ACs) were then counted as described previously.[24] To facilitate the counting, the colons were stained with 0.2% methylene blue solution and were observed using stereomicroscopy.

Assay for assessing the proliferative activity of colonic epithelial cells

We evaluated the 5-bromodeoxyuridine (BrdU) labelling index to determine the proliferative activity of the colonic epithelial cells. BrdU (BD Biosciences, New Jersey, USA) was diluted in phosphate-buffered saline at 1 mg/ml and was administered intraperitoneally at a dose of 50 mg/kg, 1 h before the mice were killed. The immunohistochemical detection of BrdU was performed using a commercial kit (BD Biosciences). The BrdU labelling index was expressed as the ratio of the number of positively stained nuclei to the total number of nuclei counted in the crypts of the colon. The criteria for crypt selection included the presence of a clearly visible and continuous cell column on each side of the crypt. Twenty crypts were evaluated in each mouse.

Plasma lipid levels and insulin resistance

The levels of plasma triglycerides, cholesterol, insulin, insulin-like growth factor-1 (IGF-1), tumour necrosis factor α (TNFα) and blood glucose were measured using a WAKO enzyme-linked immunosorbent assay (ELISA) kit (Wako Pure Chemical, Osaka, Japan) (n = 10 from each group). We measured the plasma concentrations of triglycerides and cholesterol according to the manufacturer's instructions.

Immunoblotting

The extracted protein was separated using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham, London, UK). The membranes were probed with primary antibodies specific for phospho-JNK, JNK, phospho-extracellular signal regulated kinase (ERK), ERK, phospho-p38 MAPK, p38 MAPK, phospho-c-Jun, c-Jun, phospho-insulin receptor substrate-1 (IRS-1) (Ser307), IRS-1, phospho-Akt (Ser473), Akt, phospho-mammalian target of rapamycin (mTOR), mTOR, phospho-p70 ribosomal S6 kinase (S6K), S6K (Cell Signalling Technology, Danvers, Massachusetts, USA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Trevigen, Gaithersburg, Maryland, USA). Horseradish-peroxidase-conjugated secondary antibodies and the ECL detection kit (Amersham) were used for the detection of specific proteins.

Gene expression analysis

Total RNA was extracted from the colonic epithelium using the RNeasy Mini Kit (Qiagen, Hilden, Germany). For real-time reverse transcription polymerase chain reaction, total RNA was reverse-transcribed into cDNA and amplified using real-time quantitative polymerase chain reaction using the ABI PRISM 7700 System (Applied Biosystems, Foster City California, USA). Probes and primer pairs specific for *cyclin D1* and *β-actin* were purchased from Applied Biosystems. The concentrations of the target genes were determined using the competitive computed tomography method and the values were normalised to the internal control.

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays were performed according to a previously described method.[25] Briefly, nuclear extracts from colonic tissue were prepared and gel shift assays using an AP-1 consensus oligonucleotide (Promega, Madison, Wisconin, USA) were performed. Samples were separated using 4% polyacrylamide gel electrophoresis (PAGE), and the gels were dried and exposed to radiograph film.

Effect of the JNK inhibitor SP600125 on the induction of ACF formation

The JNK inhibitor anthra[1,9-*cd*]pyrazol-6(2*H*)-one (SP600125) was purchased from Calbiochem (San Diego, Calfornia, USA). C57Bl/6J mice (6 weeks old) were intraperitoneally injected with SP600125 (10 or 50 mg/kg) or vehicle (5% dimethyl sulfoxide (DMSO), 20% Cremophor EL, 75% saline) daily until the end of the experiment. The mice were fed the ND or the HFD and received AOM injections according to the ACF protocol.

Statistical analysis

Statistical analyses of the number of ACF, the BrdU labelling index, and the blood test results were conducted using the Mann–Whitney U test. Other statistical analyses were performed using the Student t test. Values of $p<0.05$ were regarded as denoting statistical significance.

RESULTS

Enhanced formation of aberrant crypt foci and increase in the cell proliferative activity in mice fed a high-fat diet

To examine the effect of a HFD on the promotion of colonic epithelial cell proliferation, the formation of chemically induced ACF (defined as clusters of ACs) was examined in the colon specimens as a marker of early stage colorectal carcinogenesis.[26 27] The proliferative activity of the colonic epithelial cells was also determined using the BrdU labelling index. The ACF experimental protocol is shown in fig 1A. The numbers of ACF and ACs were significantly higher in mice fed a HFD than in those fed a ND (fig 1C). The macroscopic characteristics of the ACF in the ND and HFD groups are shown in fig 1D; no morphological differences in the ACF were observed between the ND and the HFD groups. Furthermore, a significant increase

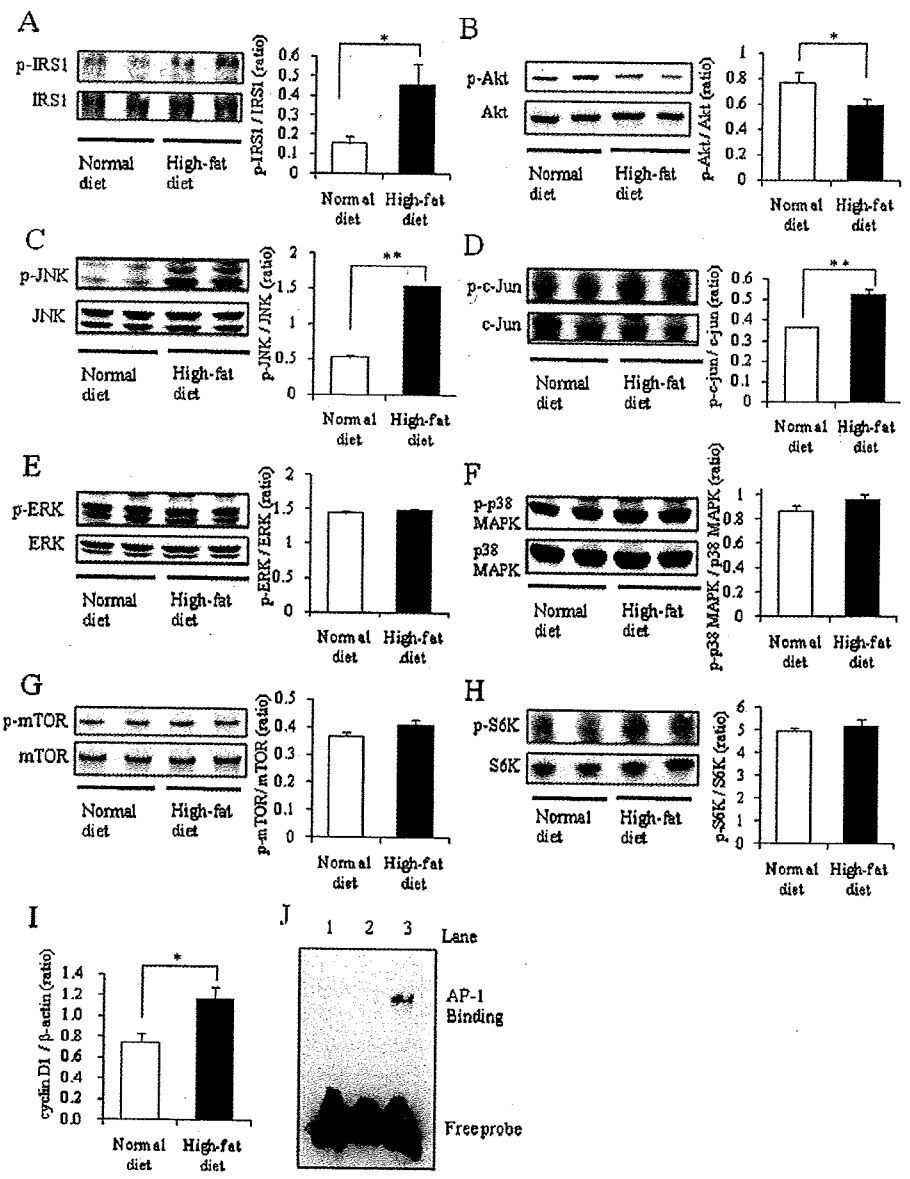

Figure 2 JNK activity and the signalling pathways for both insulin resistance and cell proliferation. A western blot analysis for phosphorylated and total IRS-1 (A), Akt (B), JNK (C), c-Jun (D), ERK (E), p38 MAPK (F), mTOR (G) and S6K (H) in colon specimens from mice fed a normal diet (n = 5) and mice fed a high-fat diet (n = 5). Representative western blotting images are shown. Right panel: graphs showing the ratios of the phosphorylated protein level to the total protein level. (I) Real time reverse transcription polymerase chain reaction analysis for the gene expression of *cyclin D1* in colon specimens from mice fed a normal diet (n = 6) and mice fed a high-fat diet (n = 6). Each column represents the mean with the SEM; *p<0.05, **p<0.01. (J) Electromobility shift assay autoradiogram shows the marked induction of AP-1 DNA binding under high-fat diet conditions. Lane 1, free probe alone (no nuclear extracts); lane 2, normal diet; lane 3, high-fat diet. ERK, extra-cellular signal-related kinase; IRS-1, insulin receptor substrate-1; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; S6K, p70 ribosmal S6 kinase.

in the BrdU index was observed in the HFD group, compared with in the ND group (fig 1E,F). These results indicate that the proliferative activity of the colonic epithelial cells was promoted by the HFD, resulting in an increase in the number of ACF.

The body weights were significantly higher in the HFD group than in the ND group after 1 week of treatment (that is, at 7 weeks of age), and this difference was maintained until the end of the study (fig 1B). We next investigated the blood plasma levels of various metabolites in the ND and HFD groups. The levels of fasting plasma glucose, insulin, TNFα, lipids and IGF-1 were measured and are shown in table 1 and supplementary fig 1. Compared with the ND group, the HFD group had significantly higher fasting plasma glucose, insulin cholesterol and IGF-1 levels. Meanwhile, the plasma TNFα and triglyceride levels were not significantly different between the two groups.

The JNK pathway is activated in the colonic epithelium of mice fed a high-fat diet

To clarify the mechanisms underlying the enhanced proliferative activity of the colonic epithelial cells in the HFD group, we investigated the expression levels of various potential target proteins in colon specimens prepared from the ND and HFD groups. Because the plasma insulin level was significantly increased in the HFD group, we first speculated that an insulin signalling pathway downstream of the insulin receptor, such as the PI3K/Akt signalling pathway, might be involved in the promotion of colonic epithelial cell proliferation in the presence of a HFD. Akt plays an important role in a variety of biological processes including cell survival, cell growth, and oncogenesis[28] and is activated by insulin via the phosphorylation of IRS-1.[29] Surprisingly, the results of a western blot analysis revealed that the amount of phosphorylated Akt was lower in the HFD group than in the ND group (fig 2B). However, a significant increase in the level of phosphorylated IRS-1 was observed in the HFD group, compared with in the ND group (fig 2A). These results imply the existence of insulin resistance in the colonic epithelium. The phosphorylation of IRS-1 has emerged as a key event in insulin resistance.[30] Indeed, a large number of protein kinases have been shown to cause the phosphorylation of IRS-1, including JNK,[31] ERK,[32] mTOR,[33] and S6K.[34] Therefore,

we next examined the expression levels of these proteins. No differences in the protein levels of phosphorylated ERK, p38 MAPK, mTOR and S6K were observed between the HFD and ND groups (fig 2E–H). On the other hand, significant increases in the levels of phosphorylated JNK and c-Jun were observed in the HFD group, compared with in the ND group (fig 2C,D). The expressions of JNK and c-Jun in the colonic epithelium were confirmed using a western blot analysis and an immunohistochemical analysis (supplementary fig 2). Some studies have reported that Akt suppresses the JNK pathway.[55 56] Moreover, the mRNA level of cyclin D1 was significantly higher in the HFD group than in the ND group (fig 2I). The JNK/c-Jun pathway is a critical component of the proliferative response and induces G0 to G1 cell cycle progression in many cell types;[57] furthermore, cyclin D1, which is a regulator of the G1 to S phase transition, has emerged as an important target for the JNK/c-Jun pathway in driving proliferation.[55] We next directed our attention to the AP-1 transcription factor, which is the target of the JNK signalling pathway and, as mentioned earlier, is an important transcription factor involved in oncogenic transformation and cell proliferation. Our data clearly indicated an impressive increase in the activation of AP-1 in the HFD group (fig 2J), indicating that the JNK/c-Jun pathway was activated in the colonic epithelia of these animals.

Inhibition of JNK suppresses colonic epithelial cell proliferation in mice fed a high-fat diet

To confirm the direct involvement of the JNK pathway in the promotion of colonic epithelial cell proliferation in the presence of a HFD, we used a specific JNK inhibitor, SP600125, in the ACF experiment. ACF formation and the BrdU labelling index were significantly suppressed by SP600125 in the HFD group in a dose dependent manner, but no effect was observed in the ND group (fig 3B–D). Furthermore, the JNK inhibitor attenuated the increase in the protein levels of phosphorylated c-Jun in the colons of mice in the HFD group (fig 3A). These results indicated that the activation of the JNK pathway played important roles in the increase in epithelial cell proliferation observed in the mice fed a HFD and might play an important role in promoting colonic epithelial cell proliferation in mice fed a HFD. The fasting plasma glucose, insulin and cholesterol

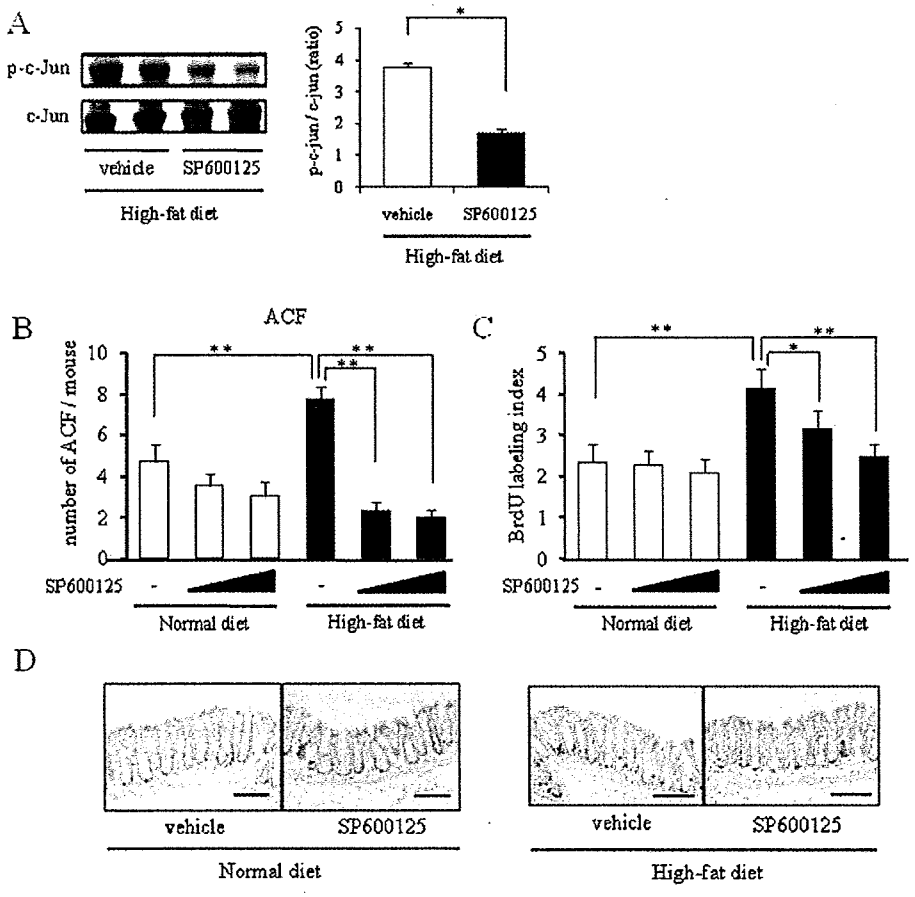

Figure 3 Suppression of colonic epithelial cell hyper-proliferation by JNK inhibitor in the presence of a high-fat diet. Mice (6 weeks old) were fed a normal diet or a high-fat diet and were injected intraperitoneally with the JNK inhibitor, SP600125 (10 or 50 mg/kg) or with the vehicle only daily until the end of the experiment. The mice in each group were also given two weekly intraperitoneal injections of 10 mg/kg of AOM. (A) Western blot analysis of phosphorylated and total c-Jun in colon specimens from mice treated (+) or not treated (−) with SP600125. Right panel: graphs showing the ratios of the phosphorylated protein levels to the total protein levels. Each column represents the mean with the SEM of 6 mice/group; *$p<0.05$, compared with mice treated (−) with SP600125. (B) Average numbers of ACF in the mice fed a normal diet and the mice fed a high-fat diet treated (+) or not treated (−) with SP600125. Each column represents the mean with the SEM of 6 mice/group; **$p<0.01$. (C) The average BrdU labelling index decreased in mice fed a high-fat diet in a dose dependent manner, but not in mice fed a normal diet. Each column represents the mean with the SEM of 6 mice/group; *$p<0.05$, **$p<0.01$. (D) Representative immunohistochemical staining for BrdU in each group. Scale bar: 100 μm. ACF, aberrant crypt foci; AOM, azoxymethane; BrdU, 5-bromodeoxyuridine; JNK, c-Jun N-terminal kinase.

levels were significantly reduced by SP600125 in the HFD group, but no effect was observed in the ND group (table 1). The plasma TNFα and triglyceride levels after treatment with SP600125 were similar between the two groups.

DISCUSSION

Previous studies have provided evidence of an association between dietary fat intake and an increased risk of colorectal carcinogenesis,[2 4] but the molecular mechanisms underlying the promotion of colorectal carcinogenesis by a HFD remain unclear. In the present study, we showed a significant enhancement in the formation of ACF and an increase in the proliferative activity of the colonic epithelial cells in the HFD group, compared with in the ND group. Additionally, we demonstrated the activation of the JNK/c-Jun pathway and the inactivation of Akt in colonic epithelial cells in the HFD group. Furthermore, a JNK specific inhibitor significantly suppressed the increase in epithelial cell proliferation only in the HFD group, suggesting that JNK/c-Jun may play an important role in promoting colorectal carcinogenesis and epithelial cell proliferation in the presence of a HFD.

Animal models exhibiting high-fat-induced hyperinsulinaemia provide a unique opportunity to explore the potential molecular mechanisms underlying the promotion of colorectal carcinogenesis and epithelial cell proliferation by a HFD. Insulin is now known to be an important growth factor for colonic epithelial cells,[7 8] and the insulin receptor has been detected in normal colorectal epithelium and cancer tissue.[38] The insulin-signal transduction pathway can be mediated by the activation of IRS-1/PI3K/Akt signalling and is involved in the regulation of gene expression and mitogenicity. Therefore, we first speculated that PI3K/Akt activation might be involved in the promotion of colonic cell proliferation in the presence of a HFD because the plasma insulin level was elevated in the HFD group. However, our results surprisingly showed that the Akt activity was decreased in the HFD group compared with in the ND group, whereas a significant increase in the level of phosphorylated IRS-1 was observed in the HFD group, compared with in the ND group. These results suggested that, in vivo, HFD induced insulin resistance in the colonic epithelium, causing the inhibition of PI3K/Akt signalling (supplementary fig 3). These molecular mechanisms of insulin resistance have been studied in the liver and adipose tissues of various models of diabetes,[13] with results that are compatible with the presently reported results. Thus, we next examined signalling pathways involved in both insulin resistance and proliferation, including the JNK pathway.

We observed an increased in JNK/c-Jun activity in colonic epithelial cells from the HFD group. Therefore, we investigated the effect of SP600125, a specific JNK inhibitor, on the colonic epithelium to determine the direct involvement of JNK activation in colon carcinogenesis and epithelial proliferation under a HFD condition. The inhibition of JNK activation reportedly improves insulin resistance.[39] Our results indeed demonstrated that SP600125 attenuated the blood glucose and plasma insulin levels, suggesting that this inhibitor might suppress colonic epithelial cell proliferation in the presence of a HFD via some indirect effects, such as the amelioration of insulin resistance. However, a western blot analysis for colonic epithelial protein in the HFD group clearly showed the attenuation of JNK activity after treatment with SP600125. Furthermore, the increase in ACF formation and the BrdU labelling index were distinctly restored by SP600125 in the HFD. Therefore, these results imply that insulin resistance and the promotion of colonic epithelial proliferation in the presence of a HFD occur via independent mechanisms. Our data strongly suggest that a HFD promotes the JNK/c-Jun pathway, resulting in the promotion of cell proliferative activity and, consequently, the promotion of colon carcinogenesis.

We also investigated the activation of the mTOR/S6K pathway in colonic epithelial cells to elucidate the involvement of this pathway in cell proliferation in the presence of a HFD. The important role of mTOR in mammalian cells is related to its control of mRNA translation. The targets for mTOR signalling are proteins involved in the control of the translational machinery; these targets include S6K, which regulates the initiation and elongation phases of translation.[40] Regarding upstream control, mTOR is regulated by signalling pathways linked to several oncoproteins or tumour suppressors.[41] Recently, the mTOR/S6K pathway, which is similar to the JNK pathway, has emerged as a critical signalling pathway in the development of insulin resistance.[42] Although the excess nutrient levels associated with an obese state can lead to the activation of mTOR/S6K and the desensitisation of insulin signalling,[42] we found no differences in the colonic epithelial protein levels of phosphorylated mTOR and S6K between the HFD and ND groups in the present study. Furthermore, we previously reported that treatment with rapamycin, an mTOR specific inhibitor, did not reduce the BrdU labelling index or ACF formation in mice fed a HFD.[12] These results indicate that the activation of the mTOR pathway may not play an important role in the increase in colonic epithelial cell proliferation in the presence of a HFD condition (supplementary fig 4).

Adipocytokines, such as free fatty acid and tumour necrosis factor α, have been reported to be potent JNK activators,[15 43] although the molecular pathways involved in their action remain unclear. Several studies have demonstrated that transforming growth factor β (TGFβ) regulates the JNK signalling pathway.[44] TGFβs are known to act as inhibitors of cell proliferation;[45] recently, however, the elevated expression of TGFβs has been suggested to be responsible for oncogenesis.[46] Raju *et al*[47] showed that TGFβs were upregulated in colonic tumours and a select subset of ACF and that dietary fat modulated TGFβ expression in colonic tumours and mucosae. In the present study, however, the expression levels of TGFβs in the colonic epithelium were not elevated in the HFD group (data not shown). Further studies are warranted to investigate whether the JNK signalling pathway via TGFβ might play an important role in colon carcinogenesis and epithelial proliferation under a HFD condition.

This study presents a novel mechanism explaining the involvement of the JNK pathway in the effect of dietary fat intake on colorectal carcinogenesis and epithelial cell proliferation. Importantly, JNK activation was associated with the promotion of colonic cell proliferative activity only in the presence of a HFD, but not in the presence of a ND. At present, an elevated plasma insulin level is thought to possibly enhance the proliferative state through the activation of an insulin signalling pathway downstream of the insulin receptor, such as the PI3K/Akt signalling pathway, in the colon.[10] Currently, however, in vivo mechanistic evidence has confirmed this hypothesis to be insufficient.[48] Our in vivo results suggested that a HFD induced insulin resistance and an abnormally elevated level of JNK activity in the colonic epithelial and that the activated JNK pathway promoted colorectal epithelial cell proliferation. This study therefore demonstrated that JNK activation is one of several possible mechanisms underlying the promotion of colonic cell proliferative activity and the early

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stage of colon carcinogenesis in the presence of a HFD. We propose that JNK/c-Jun may be a novel therapeutic target for the prevention of colorectal cancer in obese populations consuming a HFD. In the future, continued investigations are needed to elucidate the JNK activators and the precise role of JNK activation in the process of colorectal carcinogenesis under the HFD conditions.

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Association of visceral fat accumulation and plasma adiponectin with rectal dysplastic aberrant crypt foci in a clinical population

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The association between obesity and the risk of colorectal cancer (CRC) cannot be easily evaluated because CRC itself is associated with a gradual loss of bodyweight. Aberrant crypt foci (ACF) can be classified as dysplastic ACF or non-dysplastic ACF by magnifying colonoscopy, and dysplastic ACF are thought to be a biomarker of CRC. Ninety-four participants who underwent colonoscopy at Yokohama City University Hospital, Japan, were enrolled in the current study. We detected 557 ACF, including 67 dysplastic ACF (12.0%). Univariate regression analysis was conducted to determine correlations between the number of dysplastic ACF and various potential risk factors, including patient age, waist circumference, body mass index, visceral fat area (VFA), and plasma adiponectin level. The results of multiple regression analysis revealed that the number of dysplastic ACF correlated with age (correlation coefficient $r = 0.212$, $P = 0.0383$) and plasma adiponectin level ($r = -0.201$, $P = 0.0371$), even after adjustments for sex, waist circumference, body mass index, and VFA. Our univariate correlation analysis data showed a significant correlation with the number of dysplastic ACF with VFA ($r = 0.238$, $P = 0.0209$), no correlation with subcutaneous fat area, and an inverse correlation with the plasma level of adiponectin ($r = -0.258$, $P = 0.0118$). Thus, our results suggest that aging and visceral fat accumulation could correlate moderately with colorectal carcinogenesis. The novelty of our study lies in the finding that visceral fat accumulation and a low plasma adiponectin level may promote colorectal carcinogenesis; therefore, these obesity-related parameters may serve as novel targets for CRC prevention. (*Cancer Sci* 2009; 100: 29–32)

Obesity and its associated visceral fat accumulation have been reported to be linked to an elevated risk of cardiovascular disease, diabetes mellitus, and mortality, and these complications are rapidly becoming significant problems.[1,2] Visceral adipose tissue is not only fat storage tissue, but also a metabolically active organ secreting many adipocytokines, such as adiponectin.[3] Obesity is reportedly an important risk factor for CRC.[4] CRC has high mortality and morbidity rates, and its prevalence has been increasing.[5,6] The precise risk factors for CRC remain unclear, although a family history and several dietary and lifestyle factors have been proposed to be involved.[7]

The association between obesity and the risk of CRC cannot be easily evaluated because of the confounding effect of bodyweight loss with CRC. Therefore, we sought to identify a biomarker for risk assessment and monitoring of CRC. ACF, which were first discovered in mice treated with azoxymethane,[8] have been clearly shown to be precursor lesions of CRC, and are now established as a biomarker of the risk of CRC in azoxymethane-treated mice and rats.[9] In humans, ACF can be classified as dysplastic or non-dysplastic through the use of magnifying colonoscopy.[10] ACF have not been firmly established to be precursors of CRC; however, dysplastic ACF could possibly serve as a biomarker of the risk of CRC. Previous studies have reported that individuals with CRC have more ACF than those without CRC, therefore dysplastic ACF represent potential clinical precursors of CRC and colorectal adenoma.[11–14] Recently, an association was suggested to exist between obesity and the risk of CRC.[15,16] However, the relationship between obesity and ACF remains unclear. Therefore, the current study in a clinical population aimed to investigate the relationship between various obesity-associated parameters and rectal dysplastic ACF.

Patients and Methods

Study population. We prospectively evaluated 94 subjects recruited from the population of healthy individuals who underwent colonoscopy at Yokohama City University Hospital, Japan. The exclusion criteria included: presence of contraindications to colonoscopy; current or past non-steroidal anti-inflammatory drug use including aspirin; family history of CRC; or history of adenoma, carcinoma, familial adenomatous polyposis, inflammatory bowel disease, or radiation colitis. Subjects with a history of colectomy, gastrectomy, or colorectal polypectomy, and those treated with daily insulin self-injection or sulfonylurea for diabetes mellitus, were also excluded. In order to investigate the influence of obesity on colorectal carcinogenesis, patients with colorectal adenoma or carcinoma at the time of colonoscopy were also excluded from the study. Written informed consent was obtained from all subjects prior to their participation. The study protocol was approved by the Yokohama City University Hospital Ethics Committee.

Collection and analysis of blood samples for adiponectin level. Blood samples were obtained in the morning on the day of colonoscopy after overnight fasting. Plasma adiponectin levels were measured by enzyme-linked immunosorbent assay of the total forms of human adiponectin (SRL Co., Tokyo, Japan).

Magnifying colonoscopy for identification of ACF. Participants' bowel preparation for the colonoscopy was carried out using

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Abbreviations: ACF, aberrant crypt foci; BMI, body mass index; CRC, colorectal cancer; CT, computed tomography; SFA, subcutaneous fat area; TFA, total fat area; VFA, visceral fat area.

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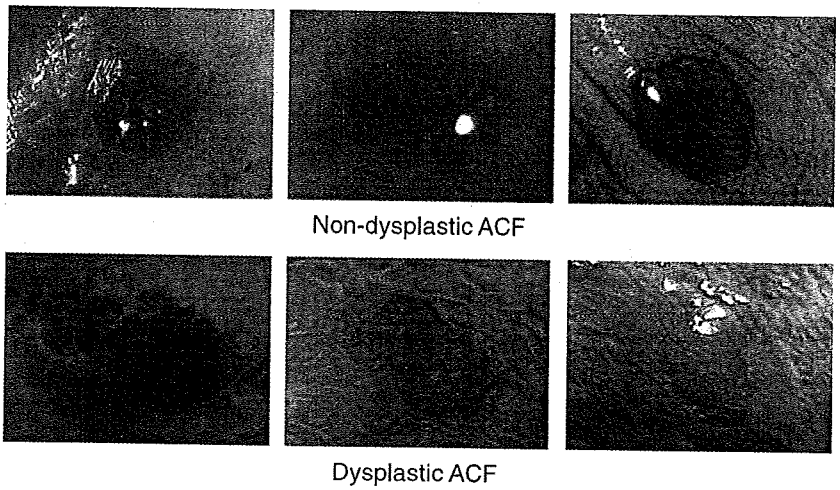

Fig. 1. Typical features of non-dysplastic and dysplastic aberrant crypt foci (ACF) on magnifying colonoscopy after methylene blue staining.

polyethylene glycol solution. A Fujinon EC-490ZW5/M colonoscope was used for the magnifying colonoscopy (Fujinon Toshiba ES Systems, Tokyo, Japan). Total colonoscopy was carried out before imaging of rectal ACF. Subsequently, 0.25% methylene blue was applied to the mucosa with a spray catheter. Aberrant crypts were distinguished from normal crypts by their deeper staining and larger diameter, and the number of ACF in the rectum was counted. This counting was conducted in the lower rectal region, extending from the middle Houston valve to the dentate line, based on the results of a previous study.[10] All ACF were recorded photographically and evaluated by two independent observers who were unaware of the subjects' clinical histories.

Criteria used for endoscopic diagnosis. ACF were defined as lesions in which the crypts were more darkly stained with methylene blue than normal crypts and had larger diameters, often with oval or slit-like lumens and a thicker epithelial lining.[17–20] 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 normal surrounding crypts. Non-dysplastic ACF were classified as hyperplastic or non-hyperplastic.[10]

Measurement of VFA and SFA. BMI was calculated using the following equation: bodyweight (kg)/(height [m]^2). Intra-abdominal adipose tissue was assessed, as described previously by measuring the VFA, SFA, TFA, and waist circumference from CT images at the level of the umbilicus.[4,10] All CT scans were carried out with the subjects in the supine position. The borders of the intra-abdominal cavity were outlined on the CT images, and the VFA was quantified using Fat Scan software (N2 System Corporation, Kobe, Japan).

Statistical analysis. We examined the associations between the risk factors for CRC and the number of dysplastic ACF. All data were expressed as mean ± SD, unless otherwise indicated. The relationships between the number of dysplastic ACF and relevant covariates were examined by univariate regression analysis, and standardized correlation coefficients were determined using Stat View software (SAS Institute, Cary, NC, USA). Multiple regression analysis was carried out to assess the relationship between the number of dysplastic ACF and potentially associated variables, and to determine the standardized correlation coefficients. The dependent variable was the number of dysplastic ACF, and the independent variables were age, sex, VFA, and plasma adiponectin level. Waist circumference and BMI were excluded from this analysis because these factors have a high correlation with VFA. P-values < 0.05 were considered to denote statistical significance.

Results

Colonoscopic features of ACF. A total of 557 ACF, including 67 dysplastic ACF, were counted by magnifying colonoscopy in the 94 patients. The aberrant crypts were larger, thicker, and more darkly stained than the normal crypts. Dysplastic ACF and non-dysplastic ACF accounted for 12.0% (67 of 557) and 88.0% (490 of 557) of the total, respectively. The number of subjects with dysplastic ACF was 34, and the number with non-dysplastic ACF was 76. In the lesions detected by magnifying colonoscopy, the size (i.e. median number of crypts ± SD) per ACF was 15.1 ± 10.4 and per dysplastic ACF was 8.5 ± 11.8. The average number of composition crypts per ACF was 93.2 ± 124.3 and per dysplastic ACF was 16.3 ± 26.2. In the 94 patients, the mean total number of ACF (non-dysplastic and dysplastic) per patient was 5.92 ± 6.50, and the mean number of dysplastic ACF per patient was 0.71 ± 1.16. The typical colonoscopic features of dysplastic and non-dysplastic ACF are shown in Figure 1.

Patient characteristics. The clinical characteristics of the study participants are shown in Table 1. The mean age was 65.1 ± 10.8 years, and there were 48 men and 46 women. The mean waist circumference, BMI, TFA, VFA, SFA, and plasma adiponectin level were 86.3 ± 10.0 cm, 23.3 ± 3.1 kg/m^2, 200.8 ± 91.4 cm^2, 83.9 ± 50.1 cm^2, 116.7 ± 60.4 cm^2, and 11.0 ± 5.6 μg/mL, respectively.

Univariate regression analysis: Correlations between risk factors for CRC and the number of dysplastic ACF. Age correlated significantly with the number of dysplastic ACF, as shown in Table 2 ($r = 0.232$, $P = 0.0242$). Sex showed no correlation with the number of dysplastic ACF. All of the obesity parameters, except SFA ($r = -0.001$, $P = 0.9979$), correlated significantly with the number of dysplastic ACF, as follows: waist circumference ($r = 0.225$, $P = 0.0293$), BMI ($r = 0.307$, $P = 0.0325$), and VFA ($r = 0.238$, $P = 0.0209$). The plasma level of adiponectin showed a significant inverse correlation with the number of dysplastic ACF ($r = -0.258$, $P = 0.0118$). Age was the only parameter that correlated significantly with the number of non-dysplastic ACF ($r = 0.218$, $P = 0.0336$), which were much more abundant than dysplastic ACF in the study subjects.

Multiple regression analysis: Correlations between risk factors for CRC and the number of dysplastic ACF. The results of the multiple regression analysis are shown in Table 3. After adjustments for sex, waist circumference, BMI, and VFA, the parameters of age and plasma adiponectin level still correlated significantly with the number of dysplastic ACF ($P = 0.0383$ and $P = 0.0371$, respectively).

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Table 1. Clinical characteristics of study participants

Characteristic	Overall	Subjects with non-dysplastic ACF	Subjects with dysplastic ACF
Number	94	76	34
Age (years)	65.1 ± 10.8	66.3 ± 10.1	66.2 ± 8.1
Sex (male : female)	48:46	43:33	21:13
Waist circumference (cm)	86.3 ± 10.0	86.0 ± 10.5	88.4 ± 11.2
Body mass index (kg/m^2)	23.3 ± 3.1	23.3 ± 3.2	24.2 ± 3.0
Total fat area (cm^2)	200.8 ± 91.4	199.5 ± 95.7	222.0 ± 96.0
Visceral fat area (cm^2)	83.9 ± 50.1	86.3 ± 51.6	103.6 ± 52.6
Ssubcutaneous fat area (cm^2)	116.7 ± 60.4	112.9 ± 60.8	117.8 ± 58.4
Plasma adiponectin (μg/mL)	11.0 ± 5.6	11.3 ± 5.8	9.4 ± 4.3

Data are expressed as mean ± SD. ACF, aberrant crypt foci.

Table 2. Univariate correlation analysis: Correlations between the number of non-dysplastic or dysplastic aberrant crypt foci (ACF) and the risk factors for colorectal cancer

Risk factor	Non-dysplastic ACF		Dysplastic ACF	
	r	*P*	*r*	*P*
Age	0.218	0.0336*	0.232	0.0242*
Sex	0.109	0.2928	0.087	0.4069
Waist circumference	0.076	0.4651	0.225	0.0293*
Body mass index	0.169	0.1011	0.307	0.0325*
Total fat area	0.126	0.2257	0.135	0.1941
Visceral fat area	0.137	0.1868	0.238	0.0209*
Subcutaneous fat area	0.078	0.4560	−0.001	0.9979
Plasma adiponectin	−0.019	0.8538	−0.258	0.0118*

Age, waist circumference, body mass index, visceral fat area, and plasma adiponectin level correlated with the number of dysplastic ACF.
*$P < 0.05$.

Table 3. Multiple regression analysis: Correlations between the number of dysplastic aberrant crypt foci and the risk factors for colorectal cancer

Risk factor	Correlation coefficient	*P*
Age	0.212	0.0383*
Sex	0.038	0.7141
Waist circumference	−0.152	0.4508
Body mass index	0.249	0.1618
Visceral fat area	0.089	0.5807
Plasma adiponectin	−0.201	0.0371*

R^2 for the entire model = 0.368.
After adjustments for sex, waist circumference, body mass index, and visceral fat area, the parameters of age and plasma adiponectin level still correlated with the number of dysplastic aberrant crypt foci.
*$P < 0.05$.

Discussion

In the present study a total of 557 ACF were counted in the 94 patients, and we demonstrated a significant correlation between the number of dysplastic ACF and the VFA, and a significant inverse correlation between the number of dysplastic ACF and the plasma adiponectin level. Age was also associated with the number of ACF, that is, the number of dysplastic and non-dysplastic ACF increased with age. CRC is thought to progress through several morphological stages, from the formation of polyps to the onset of malignant change.[21] Genetic alterations, including mutations in the *K-ras*, *p53*, and *APC* genes, have been reported to be associated with the disease progression.[22] The *K-ras* mutation has also been reported in human ACF.[23] Therefore, the increased risk of ACF formation with age may be influenced mainly by these genetic alterations. Sex showed no correlation with the number of dysplastic ACF in the present study; however, the incidence of CRC is lower in women than in men.[24,25] It has been suggested that the initiation of dysplastic ACF is comparable in men and women, but thereafter tumor progression differs because visceral fat accumulation is higher in men than woman. This visceral fat accumulation may affect tumor progression.

Waist circumference has often been suggested to be associated with VFA. Consistent with this suggestion, our data showed that both waist circumference and VFA were associated with the number of dysplastic ACF. Recent reports have suggested that obesity may be associated with a high risk of CRC.[4] Several studies have shown that increased BMI is associated with an increased risk of CRC.[26] The importance of the size of ACF has been reported,[27] and the correlation between size, measured as the median number of crypts per both non-dysplastic ACF and dysplastic ACF, and risk factors was analyzed. The correlation between the median number of crypts per ACF and any risk factors had almost the same result as the number of ACF (data not shown). Our data showed a direct correlation between the VFA and the number of dysplastic ACF, and an inverse correlation between the plasma adiponectin level and the number of dysplastic ACF (Table 2). A previous study showed that the *K-ras* gene was mutated in 50–60% of patients with dysplastic ACF,[10] thus genetic alterations were already underway. Visceral fat correlated with dysplastic ACF in the current study, and another study showed that increased visceral adiposity was a significant predictor of lower rates of disease-free survival in patients with resectable colorectal cancer,[28] suggesting that visceral fat plays an important role in colorectal carcinogenesis and progression. Visceral fat tissue is known to be an endocrine organ that secretes adiponectin, which has an inverse relationship with obesity and visceral fat.[29] We carried out multiple regression analysis to assess whether plasma adiponectin may be a risk factor for dysplastic ACF growth, independent of the effects of obesity. If dysplastic ACF are a biomarker of the risk of colorectal adenoma and CRC, then some factors associated with the risk of CRC may also influence the number of dysplastic ACF. Very little is known about the factors that initiate or promote the growth of dysplastic ACF in humans. Our results suggest that plasma adiponectin levels are inversely associated with the number of ACF, and that visceral fat may be associated directly with ACF and thus could be a risk factor for the early stage of colorectal carcinogenesis.

There are many reports on the existence of relationships between the risk of CRC and exercise, energy use, glycemic index, and food choices and dietary constituents.[30–32] These factors affect each other, therefore it is difficult to evaluate the relationship between any one factor and the risk of CRC. Obesity

is thought to result from many of these factors. It is also thought that aging, visceral fat, and adiponectin are important in CRC carcinogenesis. Further investigation is needed to elucidate the mechanisms that affect these relationships and the impact on the development of CRC.

The novelty of our study lies in our use of dysplastic ACF as a biomarker for risk of CRC to show that visceral fat accumulation and low plasma adiponectin level may affect colorectal carcinogenesis. Further studies should be conducted to clarify the role that visceral fat accumulation and reduced plasma adiponectin play in dysplastic ACF growth and whether these obesity-related parameters may serve as novel targets for CRC prevention.

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