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Adiponectin deficiency enhances colorectal carcinogenesis and liver tumor formation induced by azoxymethane in mice

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Abstract

AIM: To investigate the causal relationship between hypoadiponectinemia and colorectal carcinogenesis in *in vivo* experimental model, and to determine the contribution of adiponectin deficiency to colorectal cancer development and proliferation.

METHODS: We examined the influence of adiponectin deficiency on colorectal carcinogenesis induced by the administration of azoxymethane (AOM) (7.5 mg/kg, intraperitoneal injection once a week for 8 wk), by using adiponectin-knockout (KO) mice.

RESULTS: At 53 wk after the first AOM treatment, KO mice developed larger and histologically more progressive colorectal tumors with greater frequency compared with wild-type (WT) mice, although the tumor incidence was not different between WT and KO mice. KO mice showed increased cell proliferation of colorectal tumor cells, which correlated with the expression levels of cyclooxygenase-2 (COX-2) in the colorectal tumors. In addition, KO mice showed higher incidence and frequency of liver tumors after AOM treatment. Thirteen percent of WT mice developed liver tumors, and these WT mice had only a single tumor. In contrast, 50% of KO mice developed liver tumors, and 58% of these KO mice had multiple tumors.

CONCLUSION: Adiponectin deficiency enhances colorectal carcinogenesis and liver tumor formation induced by AOM in mice. This study strongly suggests that hypoadiponectinemia could be involved in the pathogenesis for colorectal cancer and liver tumor in human subjects.

Key words: Adiponectin; Colorectal carcinogenesis; Azoxymethane; Cell proliferation; Cyclooxygenase-2; Liver tumor formation

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INTRODUCTION

Obesity is a global medical problem because of its association with the development of various disorders such as diabetes, cardiovascular diseases, and several

cancers[1,2]. Recent studies have indicated that obesity is a significant risk factor for colorectal cancer[3], but the relationship between obesity and colorectal carcinogenesis is poorly understood at the molecular level.

Adipose tissue is currently recognized as an important endocrine organ that secretes various bioactive substances, conceptualized as adipokines or adipocytokines[1,2,4,5]. We and others identified adiponectin as an adipose-specific secretory factor[6-8]. Adiponectin exists abundantly in the circulation[9], and its plasma levels are reduced in obesity, type-2 diabetes, and coronary artery disease[9-11]. Recently, adiponectin has attracted much attention because of its potential role in the development and progression of various obesity-related malignancies[12]. Several clinical studies have shown that plasma adiponectin level is inversely associated with the risk of obesity-related cancers, including uterine, breast, gastric, and prostate cancers[13-16]. It has been reported that treatment with recombinant adiponectin significantly suppresses the growth of several types of cancer cells in cultured cells and/or in xenograft models by inhibiting cell proliferation[17-20].

Recent clinical studies have shown that low plasma adiponectin levels is an independent risk factor for colorectal cancer and its precursory adenoma[21-23]. Moreover, colorectal adenomas in patients with low plasma adiponectin levels tend to be larger and histologically more progressive[23]. However, the causal relationship between low plasma adiponectin levels and colorectal carcinogenesis has not been fully elucidated in *in vivo* experimental model.

Azoxymethane (AOM) is a well-characterized colon carcinogen, and AOM-induced colorectal cancer in rodents is similar to human colorectal cancer with respect to morphology, proliferation characteristics and involvement of gene mutation[24,25]. The present study was designed to explore the mechanisms of hypoadiponectinemia and colorectal carcinogenesis. For this purpose, we used AOM to induce colorectal cancer in adiponectin-knockout (KO) mice.

MATERIALS AND METHODS

Mice and experimental procedures

The animal care and use procedures were approved by the Animal Care Committee of Osaka Medical Center for Cancer and Cardiovascular Diseases. The generation of KO mice has been described previously[26]. We mated wild-type (WT) littermate mice produced by backcrossing to the C57BL/6J strain for five generations and used their offspring as WT controls in this study. Mice were maintained on a 12-h light/dark cycle with free access to drinking water and a standard diet. We injected 10-wk-old male mice with AOM (Sigma Chemical Co., St. Louis, MO) at a dose of 7.5 mg/kg body weight intraperitoneally once a week for 8 wk, and control mice received equal volume of saline injection (WT + saline, $n = 9$; WT + AOM, $n = 23$; KO + saline, $n = 13$; and KO + AOM, $n = 24$). The mice were sacrificed 53 wk after the first AOM injection, and the colons and small intestines were removed immediately. The harvested specimens were opened longitudinally, and the number and size of tumors were recorded. Using calipers, we measured the length (L), width (W), and depth (D) of each intestinal tumor and calculated the tumor volume using the formula $V = L \times W \times D \times \pi/6$, as described previously[27]. We also noted the development of liver tumors.

Histopathology and immunohistochemistry

Tumors were fixed in 10% buffered formalin, embedded in paraffin blocks, and sectioned at 3.0-μm thickness. Some sections of the colon tumors were subjected to hematoxylin and eosin (HE) staining for histopathology, and others were used for immunohistochemistry. proliferating cell nuclear antigen (PCNA) was visualized by staining with rat anti-mouse PCNA monoclonal antibody (Dakocytomation, Glostrup, Denmark). To determine the PCNA labeling index, we selected five representative PCNA-positive fields in each section, counted more than 200 tumor cells in each field, and then calculated the percentage of PCNA-positive cells. cyclooxygenase-2 (COX-2) was visualized by staining with rabbit anti-mouse COX-2 polyclonal antibody (Cayman, Ann Arbor, MI). We observed immunoreactive COX-2 expression in the periluminal stromal cells and epithelium of the colorectal tumors. Therefore, we evaluated COX-2 expression; both the intensity of immunoreactivity and the percentages of positively-stained areas in relation to the circumference of the tumor. We graded COX-2 expression of each immunostained section on a 0 to 4+ scale; no immunoreactivity (0), weak immunoreactivity and 1% to 25% positive regions (1+), mild immunoreactivity and 26% to 50% positive regions (2+), moderate immunoreactivity and 51% to 75% positive regions (3+), strong immunoreactivity and 76% to 100% positive regions (4+). We regarded a case displaying very weak immunoreactivity, or less than 1% positive regions, as negative. Sections of the liver tumors were subjected to HE staining for histopathology.

Statistical analysis

Results are expressed as mean ± SE. Statistical analyses of data were performed using the Student's t-test, the chi-square test, Fisher's exact probability test, Wilcoxon rank sum test, or the Spearman's rank correlation. Statistical significance was defined as $P < 0.05$.

RESULTS

Enhanced colorectal carcinogenesis induced by AOM in KO mice

We treated WT and KO mice with AOM at a dose of 7.5 mg/kg or with saline vehicle once a week for 8 wk. At 53 wk after the first AOM treatment, the mice were sacrificed to evaluate the development of colorectal tumors. Body weight changes did not differ between WT and KO

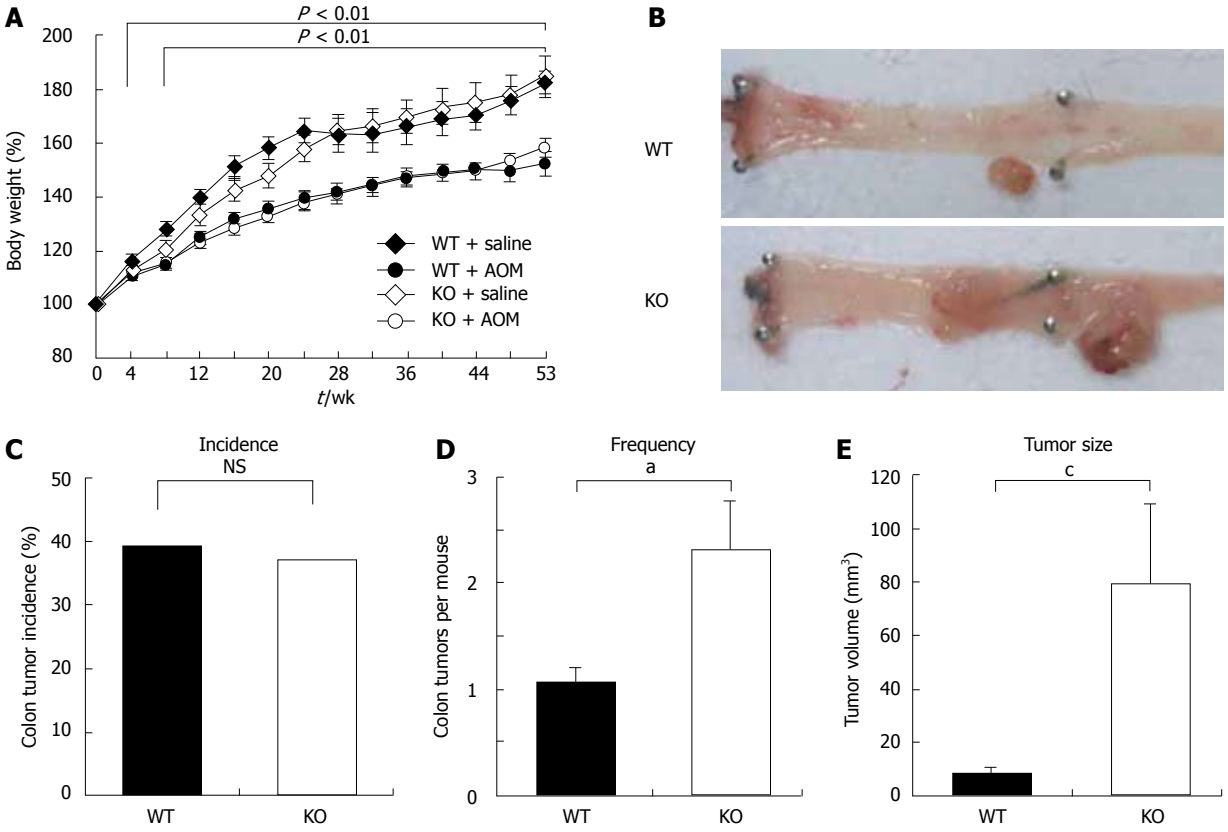

Figure 1 Enhanced AOM-induced colorectal carcinogenesis in KO mice. A: Changes in percentage of body weight. $P < 0.01$, between WT + saline ($n = 9$) and WT + AOM ($n = 23$). $P < 0.001$, between KO + saline ($n = 13$) and KO + AOM ($n = 24$); B: Representative pictures of the colorectal tumors arising in WT and KO mice after AOM treatment; C: Tumor incidence is expressed as the ratio of mice with tumor/total number of mice (NS: Not statistically significant; χ^2 test); D: Tumor frequency. WT, $n = 9$; KO, $n = 9$ ($^aP < 0.05$, Student's *t*-test); E: Tumor size. WT, $n = 9$; KO, $n = 19$ ($^cP < 0.05$, Student's *t*-test). Results are presented as mean ± SE.

mice after AOM treatment, although AOM treatment resulted in a significant reduction in body weight gain of both WT and KO mice (16.4% *vs* 14.4% reduction at sacrifice, Figure 1A). We observed no colorectal tumors in either WT or KO mice treated with only saline (data not shown). Figure 1B shows representative pictures of the colorectal tumors arising in WT and KO mice treated with AOM. There was no difference in the incidence of colorectal tumor formation between WT and KO mice (39.1% *vs* 37.5%, Figure 1C). However, the number of colorectal tumors per mouse was significantly greater in KO mice (2.33 ± 0.47, range: 1-4) than in WT mice (1.11 ± 0.11, 1-2) ($P < 0.05$; Figure 1D), and the average volume of colorectal tumors was markedly larger in KO mice (79.2 ± 29.2 mm^3) than in WT mice (9.02 ± 1.46 mm^3) ($P < 0.05$; Figure 1E). Histological analysis revealed that KO mice developed more progressive tumors in the colon than WT mice (Figure 2). We observed one adenoma (1 of 9, 11%), three carcinomas *in situ*, as assessed by the findings of high-grade dysplasia (3 of 9, 33%) and five adenocarcinomas (5 of 9, 56%) of the colorectal tumors arising in WT mice (Figure 2A). In contrast, of the colorectal tumors in KO mice examined, all tumors (14 of 14) were classified as adenocarcinomas (2 well- and 12 moderately-differentiated adenocarcinomas) (Figure 2B). These findings suggest that adiponectin deficiency enhances AOM-induced colorectal carcinogenesis. One KO mouse developed a small intestinal tumor, classified as a moderately-differentiated adenocarcinoma, in the duodenum (data not shown), whereas none of WT mice developed small intestinal tumors.

High expression of PCNA and COX-2 in colorectal tumors of KO mice

To characterize the influence of adiponectin deficiency on colorectal tumor growth, we evaluated cell proliferation of colorectal tumor cells using the PCNA labeling index assessed by immunohistochemistry. Figure 3A shows representative sections of PCNA-labeled nuclei in colorectal tumors from WT and KO mice. Expression of PCNA was identified by cell nuclei that stained brown to PCNA. For quantitative analysis, we determined the PCNA labeling index by calculating the percentage of PCNA-positive cells. The PCNA-labeling index of KO mice (68.5% ± 2.3%) was greater than that of WT mice (44.8% ± 6.3%) ($P < 0.01$; Figure 3B), suggesting that adiponectin deficiency increased cell proliferation of colorectal tumor cells. To investigate the mechanisms by which adiponectin deficiency increased cell proliferation, we first examined the expression of cyclin-dependent kinase inhibitors (CDKIs), $p21^{CIP}$ and $p27^{KIP}$, in colorectal tumors by immunohistochemistry. However, WT and KO mice showed absence or only focal positivity of both proteins in the colorectal tumors, and there was no significant difference (data not shown).

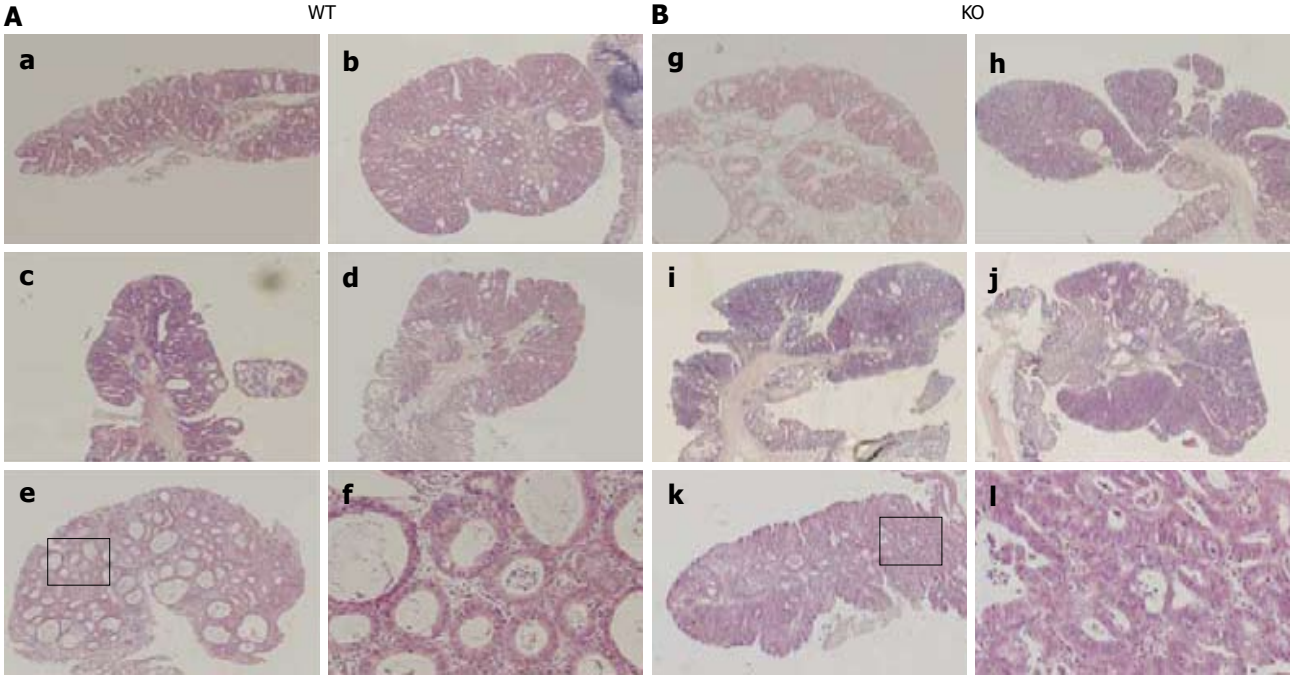

Figure 2 Histological analysis of colorectal tumors induced by AOM. Representative HE-stained sections of colon tumors in WT mice (A): a: Adenoma; b, c, e: Carcinomas *in situ*; d: Adenocarcinoma; f: Boxed area in e is shown at a higher magnification. Representative HE-stained sections of colorectal tumors in KO mice (B). All tumors in KO mice showed features of adenocarcinoma: l: Boxed area in k is shown at a higher magnification. Original magnification: × 20 for b, c, d, h, i, j; × 40 for a, e, g, k; and × 200 for f and l.

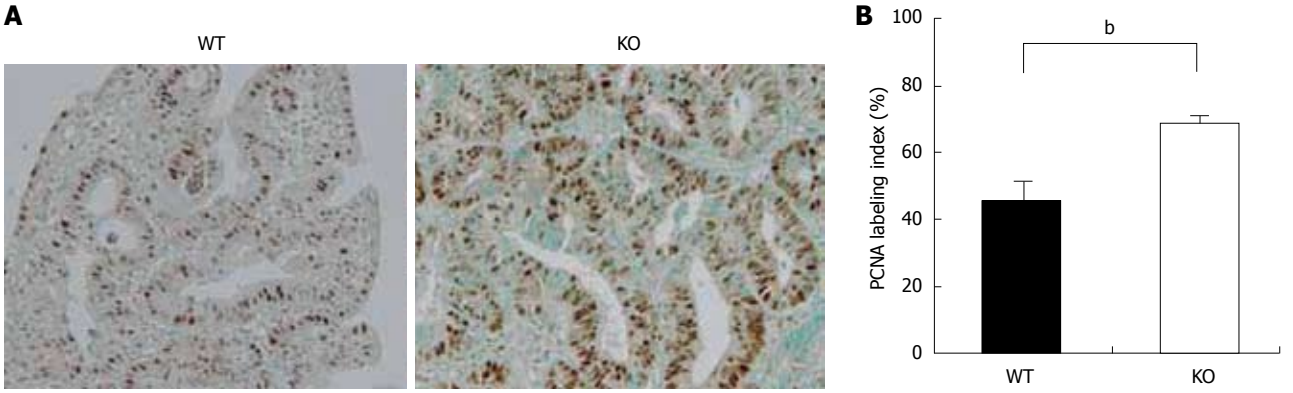

Figure 3 PCNA overexpression in colorectal tumor cells of KO mice. Immunohistochemical staining for PCNA in colorectal tumors using rat anti-mouse PCNA monoclonal antibody. Cells with strongly stained nuclei were considered positive for PCNA. A: Representative section of a colorectal tumor in a WT mouse and a KO mouse (× 200); B: PCNA labeling index in colorectal tumors. WT mice (n = 9), KO mice (n = 11) ($^bP < 0.01$, Student's t-test). Results are mean ± SE.

Next, we examined the expression of COX-2, a well-established pathogenic factor in colorectal carcinogenesis[28,29], by immunohistochemistry. Figure 4 depicts representative sections stained for COX-2. We found no COX-2 positive staining in normal colonic tissues of WT and KO mice. In the colorectal tumor tissues, we observed immunoreactive COX-2 expression in the epithelium and periluminal stromal cells, predominantly in the myofibroblasts. We detected very weak COX-2 staining in 22% (2 of 9) and only local COX-2 staining in 56% (5 of 9) of the colorectal tumors in WT mice (Figure 4A), whereas colorectal tumors in KO mice showed higher levels of COX-2 staining (Figure 4B). In 36% (4 of 11) of the colorectal tumors in KO mice, we detected marked COX-2 staining almost all over the surface of the tumor. Statistical analysis revealed that the expression of COX-2 in colorectal tumors of KO mice was higher than in WT mice (Figure 4C, $P < 0.05$; Wilcoxon rank sum test). Moreover, we found a significant correlation ($r = 0.89$, $P < 0.001$; Spearman's rank correlation) between the PCNA-labeling index and COX-2 expression level (Figure 4D). These results suggest that adiponectin deficiency may promote proliferation of colorectal tumor cells, which may be associated with overexpression of COX-2 in the periluminal stromal cells.

Enhanced liver tumor formation induced by AOM in KO mice

AOM is primarily metabolized by the liver and induces DNA damage in both the colon and the liver[30]. While AOM can induce tumor formation in the liver[31,32], tu-

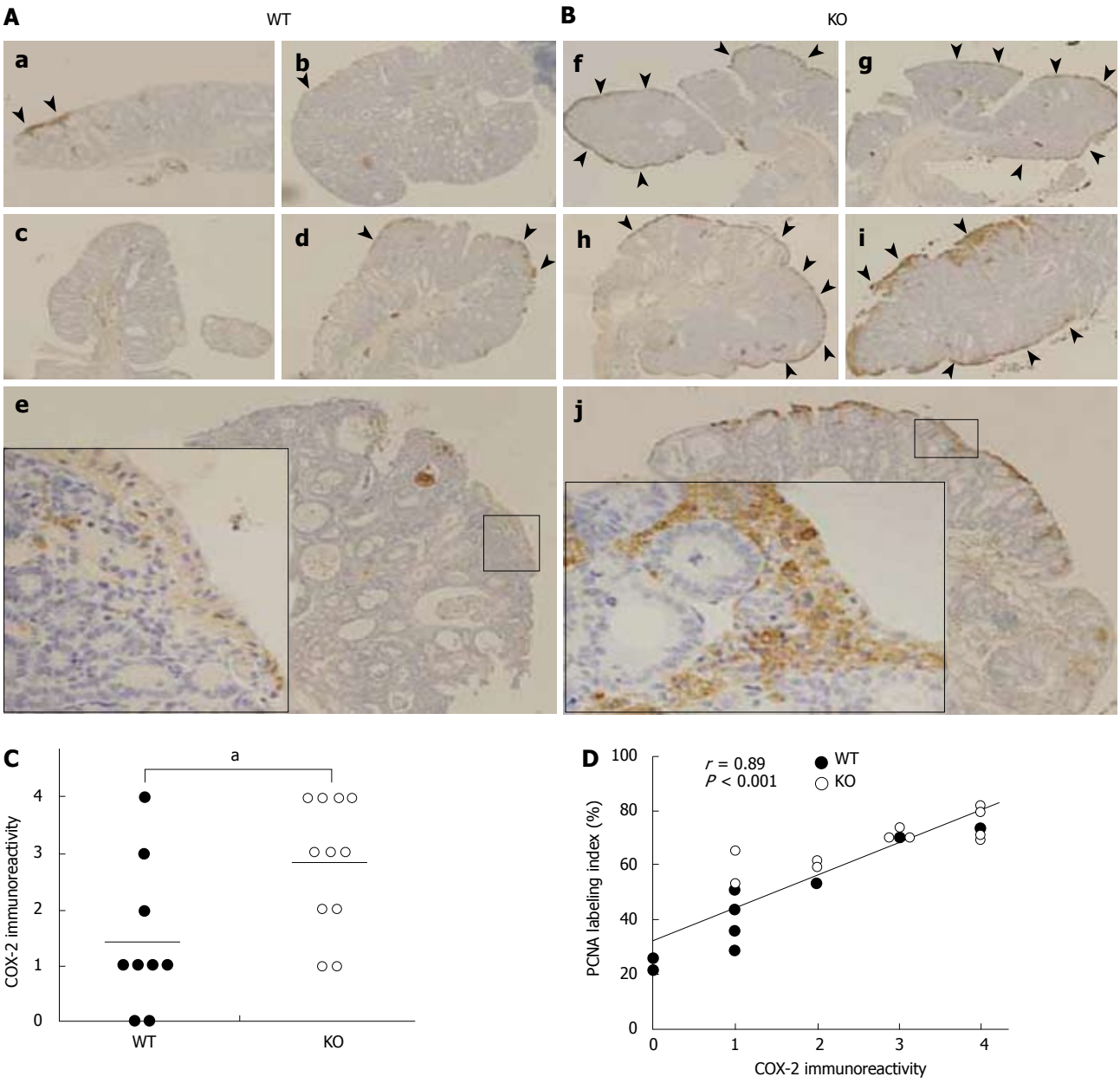

Figure 4 COX-2 overexpression in colorectal tumors of KO mice. Immunohistochemical staining for COX-2 in colorectal tumors using rabbit anti-mouse COX-2 polyclonal antibody. Immunoreactive COX-2 expression is observed in the epithelium and periluminal stromal cells of the tumors (Arrowheads). A: Representative sections of colorectal tumors in WT mice: e: Boxed area is shown at a higher magnification; B: Representative sections of colorectal tumors in KO mice: j: Boxed area is shown at a higher magnification. Original magnification: × 20 for b, c, d, f, g, h; × 40 for a, e, i, j; × 200 for the boxed area in e and j. C: Statistical analysis of immunoreactive COX-2 expression in colorectal tumors. COX-2 expression was higher in colorectal tumors of KO mice (n = 11) compared with WT mice (n = 9; $^{a}P < 0.05$, Wilcoxon rank sum test). Horizontal lines: mean value of COX-2 immunoreactivity in colorectal tumors of WT or KO mice. D: Significant correlation between COX-2 immunoreactivity and PCNA-labeling index in colorectal tumors (r = 0.89, $P < 0.001$, Spearman's rank correlation). WT mice (n = 9), KO mice (n = 11).

mors are reported to be formed almost exclusively in the colon[30]. Consistent with the previous observation[32], the incidence of liver tumor formation after AOM treatment was only 13% (3 of 23) in WT mice (Figure 5A), and all three WT mice had only a single tumor (Figure 5B). In contrast, the incidence of liver tumor formation was 50% (12 of 24) in KO mice (Figure 5A); 5 mice with a single tumor (42%), 2 mice with two tumors (16%), and 5 mice with more than three tumors (42%) (Figure 5B). Figure 5C and D show representative HE-stained sections of the liver tumors in KO mice. The majority of the liver tumors were identified as hepatocellular neoplastic nodules (Figure 5C), and one was a hepatocellular carcinoma (Figure 5D). We also observed that WT mice developed 2 hepatocellular neoplastic nodules and one hepatocellular carcinoma (data not shown). These results suggest that adiponectin deficiency may promote AOM-induced liver tumor formation.

DISCUSSION

Recent epidemiological studies have shown an inverse association between plasma adiponectin levels and the risk of colorectal cancer and its precursor adenoma[21-23]. In the present study, KO mice developed larger and more advanced colorectal tumors compared with WT

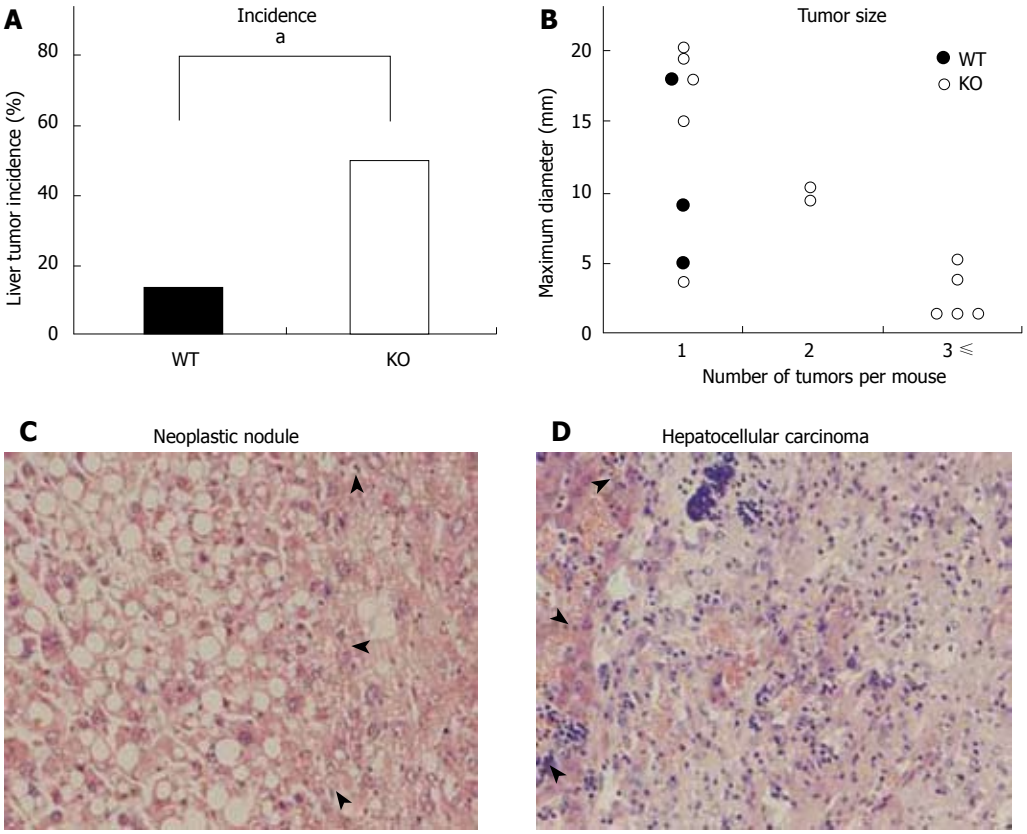

Figure 5 Enhanced AOM-induced liver tumor formations in KO mice. A: Tumor incidence is expressed as the ratio of mice with tumor/total number of mice ($^aP < 0.05$; Fisher's exact probability test); B: Tumor size is evaluated by its maximum diameter. Mice were divided into three groups according to the number of tumors per mouse. Maximum diameter of the tumors is plotted individually in each group. WT mice ($n = 3$), KO mice ($n = 12$). C and D: Representative HE-stained sections liver tumors in KO mice (× 200). Arrowheads indicate liver tumors. C: Neoplastic nodule; D: Hepatocellular carcinoma.

mice, although there was no difference in the tumor incidence between WT and KO mice. These findings suggest that adiponectin deficiency may influence colorectal tumor growth. Indeed, we found that cell proliferation of the colorectal tumor cells was significantly increased in KO mice compared with WT mice, as evaluated by the PCNA labeling index. The anti-proliferative effect of adiponectin on several types of cancer cells has been well documented in cultured cells and/or xenograft models[17-20]. Here we examined whether adiponectin deficiency directly influences colorectal tumor cells. AMP-activated protein kinase (AMPK), which is traditionally thought of as a regulator of cellular energy balance[33], has been reported to suppress cell proliferation in a variety of cell types including several cancer cells[34,35]. These effects of AMPK may be mediated partly through the cell cycle regulation by augmenting the p53-p21 axis[34,35]. Since adiponectin is known to stimulate AMPK[36], the molecular pathways regulated by AMPK could be the potential mechanisms through which adiponectin regulates carcinogenesis[12]. However, immunohistochemical analysis of the CDKIs such as $p21^{CIP}$ and $p27^{KIP}$, as downstream targets of AMPK, showed a negative or only focal immunopositivity of these CDKIs in colorectal tumors of both WT and KO mice, and there was no significant difference between the two strains (data not shown). Further studies are required to elucidate the involvement of this AMPK-CDKIs pathway in the anti-proliferative effect of endogenous adiponectin on colorectal tumors.

Next, to investigate other mechanisms by which adiponectin deficiency promoted colorectal tumor growth in KO mice, we determined the levels of COX-2 expression in the colorectal tumor tissues by immunohistochemistry. COX-2, the inducible enzyme involved in prostaglandins production, plays a crucial role in colorectal carcinogenesis[28,29]. It has been shown that inactivation of COX-2, by disruption of the COX-2 gene or the use of selective COX-2 inhibitors, markedly reduces intestinal tumor formation in the *Apc*$^{\Delta 716}$ mice[29]. Treatment with selective COX-2 inhibitors also suppressed AOM-induced colorectal carcinogenesis in rodents[37]. Moreover, clinical trials of selective COX-2 inhibitors reported a successful reduction in intestinal tumors of patients with familial adenomatous polyposis[38] and sporadic colorectal adenomas[39,40]. Here, we found that adiponectin deficiency enhanced COX-2 expression in the epithelium and periluminal stromal cells of the colorectal tumors, predominantly in the myofibroblasts. We also found that adiponectin deficiency promoted tumor cell proliferation in proportion to COX-2 expression levels. Intestinal myofibroblasts, a family of α-smooth muscle actin-positive fibroblast-like cells, play a pivotal role in the carcinogenic process[41]. The tumor cell-derived cytokines such as transforming growth factor-β provoke the transdifferentiation of fibroblasts

into myofibroblasts, which promote adjacent tumor cell proliferation through paracrine secretion of various mediators including cytokines, chemokines, growth factors, and extracellular matrix molecules[41]. Moreover, it has been shown that COX-2-expressing myofibroblasts synthesize and release prostaglandin E_2 into the tumor microenvironment, which is reported to promote epithelial cell proliferation[42]. Based on these reports, the influence of adiponectin deficiency on cell proliferation of colorectal tumor cells observed in this study could be explained, at least in part, by COX-2 overexpression in the stromal myofibroblasts.

KO mice treated with AOM had greater incidence and frequency of liver tumors, compared with WT mice. AOM is metabolically activated in the liver mainly by cytochrome P450 2E1 (CYP2E1)[43], and can induce tumor formation also in the liver[31]. The increased hepatic CYP2E1 level is considered as one possible mechanism that ethanol treatment may enhance DNA adduct formation by AOM metabolites and may potentiate dysplasia of the liver[44]. In a mouse model of nonalcoholic steatohepatitis induced by choline-deficient *L*-amino acid-defined diet, KO mice showed increased expression of hepatic CYP2E1, which might lead to the progression of liver tumor formation through the enhancement of oxidative stress[45]. On the basis of these reports, adiponectin deficiency might increase hepatic CYP2E1 activity, due to AOM exposure and, therefore, contribute to enhanced liver tumor formation. Further studies are required to clarify these points.

In conclusion, adiponectin deficiency in mice was directly associated with enhanced colorectal carcinogenesis and liver tumor formation induced by AOM. This is the first evidence that adiponectin deficiency a more caused severer and increased frequent carcinogenesis in *in vivo* model. Our results strongly suggest that hypoadiponectinemia could be involved in the pathogenesis for colorectal cancer and liver tumor in human subjects.

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COMMENTS

Background

Adiponectin has attracted much attention because of its potential role in the development and progression of obesity-related malignancies. It has been reported that hypoadiponectinemia is an independent risk factor for colorectal cancer and its precursory adenoma.

Research frontiers

The anti-proliferative effect of adiponectin on several types of cancer cells has been well documented in cultured cells and/or xenograft models. The molecular pathways regulated by AMP-activated protein kinase (AMPK) have been reported to be the potential mechanisms through which adiponectin regulate carcinogenesis.

Innovations and breakthroughs

This study represented the causal relationship between hypoadiponectinemia and colorectal carcinogenesis in an *in vivo* model. Adiponectin deficient (KO) mice developed more advanced colorectal tumors, and showed enhanced liver tumor formation after azoxymethane (AOM) treatment.

Applications

This study demonstrated overexpression of cyclooxygenase-2 (COX-2) in the stromal myofiroblasts of the colorectal tumors in knockout (KO) mice. COX-2 overexpression might be a potential mechanism through which adiponectin deficiency was involved in the colorectal carcinogenesis.

Terminology

Adiponectin is one of the major adipocytokines, and its plasma levels are reduced in obesity, type-2 diabetes, and coronary artery disease.

Peer review

The present paper shows highest growth and progression of AOM-induced colon cancer in KO mice, with respect to wild type (WT) control mice. The results are of certain interest. Nevertheless, some points need to be further clarified and considered in depth to increase the interest of the paper.

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