Adiponectin receptor expression is elevated in colorectal carcinomas but not in gastrointestinal stromal tumors

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Abstract

Circulating adiponectin is inversely associated with colorectal carcinoma. However, adiponectin receptor expression has not been examined in normal gastrointestinal tissue, colorectal malignancies, or gastrointestinal stromal tumors (GISTs). We collected 40 colorectal carcinomas and 12 non-tumor colorectal tissue specimens from patients with colorectal cancer, as well as 45 tumor and 13 non-tumor specimens from patients with GIST. Expression and localization of adiponectin receptors (AdipoR1 and AdipoR2) were assessed using immunohistochemistry. We also confirmed expression of adiponectin receptors using rtPCR in matched normal and colorectal cancer specimens obtained from five patients. Finally, we detected adiponectin receptors and assessed adiponectin signaling in three colon cancer cell lines. Adiponectin receptor expression, assessed by either rtPCR or immunohistochemistry, was present in normal tissue and was significantly lower than in colorectal carcinomas. Among carcinomas, 95% displayed positive or strongly positive expression of AdipoR1 and 88% of AdipoR2, versus 8% and 0%, respectively, for non-tumor specimens ($P<0.0001$). AdipoR1 expression assessed by rtPCR was 1.6-fold higher in tumor than in non-tumor tissue ($P<0.05$). In addition, we found that adiponectin at physiological concentrations can activate *in vitro* intracellular signaling pathways in three colon cancer cell lines, expressing both adiponectin receptors 1 and 2. No significant differences in expression of adiponectin receptors in tumor versus non-tumor GI specimens were detected among patients with GIST. Colon cancer cell lines express adiponectin receptors, through which adiponectin activates *in vitro* intracellular signaling pathways. Adiponectin receptors are also detected in normal GI tissue and their expression is elevated in colorectal carcinomas, but not in GIST.

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Introduction

There is an accumulating evidence that adiponectin, an adipose tissue-secreted hormone with insulin-sensitizing, anti-atherogenic, and anti-diabetogenic properties (Ouchi *et al.* 1999, 2001, Yokota *et al.* 2000, Gavrila *et al.* 2003), may play a significant role in the development and/or progression of several obesity-related malignancies (Barb *et al.* 2006, Kelesidis *et al.* 2006). Epidemiologic studies have linked low adiponectin levels with increased risk of breast (Miyoshi *et al.* 2003, Mantzoros *et al.* 2004, Chen *et al.* 2006, Korner *et al.* 2007) and endometrial (Petridou *et al.* 2003, Dal Maso *et al.* 2004) cancer in women, and prostate (Freedland *et al.* 2005, Goktas *et al.* 2005, Bub *et al.* 2006, Michalakis *et al.* 2007) and colorectal (Otake *et al.* 2005, Wei *et al.* 2005) cancer

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in men. The anti-neoplastic effects of adiponectin may be either indirect, through improving insulin resistance and hyperinsulinemia or modulating neovascularization and inflammation, and/or direct, through anti-proliferative and/or pro-apoptotic actions on cancer cells (Barb *et al*. 2006, Kelesidis *et al*. 2006, Korner *et al*. 2007).

The beneficial effects of adiponectin on metabolic outcomes and carcinogenesis may be mediated through the two identified cell membrane receptors, adiponectin receptor 1 (AdipoR1) and AdipoR2 (Kadowaki & Yamauchi 2005). We have previously reported that adiponectin receptors are expressed and elevated in breast tumor tissues (Korner *et al*. 2007), and additional studies by our group and others suggest that adiponectin receptors are also expressed and may play a role in prostate cancer as well (Mistry *et al*. 2006, Michalakis *et al*. 2007). Obesity has been established as a risk factor for colorectal carcinoma in numerous prospective (Giovannucci *et al*. 1996, Martinez *et al*. 1997, Ford 1999, Moore *et al*. 2004, Pischon *et al*. 2006), case–control (Caan *et al*. 1998), and meta-analysis (Dai *et al*. 2007) studies. Additional observational research suggests that circulating adiponectin may mediate in part this association, with low adiponectin conferring an increased risk of disease (Otake *et al*. 2005, Wei *et al*. 2005). However, it remains unknown whether adiponectin receptors are expressed in the gastrointestinal tract, and expression of AdipoR1 and AdipoR2 has not been previously studied in colorectal carcinomas or gastrointestinal stromal tumors (GISTs). Given the reciprocal relationship often observed between serum levels of a ligand and tissue expression of its receptors, we hypothesized that adiponectin receptors would be elevated in colorectal carcinomas.

GIST, even as the most common of the mesenchymal tumors of the GI tract, has a low incidence rate of 15 cases per million per year (Joensuu & Kindblom 2004). Risk factors for this malignancy may include older age, male gender, or black race (Tran *et al*. 2005), but no study has demonstrated any association between adiposity and GIST. Therefore, we hypothesized that adiponectin receptor expression would be unaltered in GIST tissue relative to respective non-tumor tissue and may be considered as a negative control group for the evaluation of our hypothesis regarding the association of obesity with adiponectin receptor expression in colorectal carcinomas.

Thus, we have examined immunohistochemically the expression of AdipoR1 and AdipoR2 in colorectal carcinomas, GISTs, and non-tumor GI tissue specimens. We also confirmed using rtPCR the expression of AdipoR1 and AdipoR2 in colorectal cancer and matched normal colon tissue from five patients and assessed the effect of adiponectin, at physiological concentrations, to alter intracellular signaling pathways *in vitro*. Elevated expression of adiponectin receptors in colorectal carcinomas would be consistent with deficiency of the ligand and could in part explain and expand current understanding of the observational association between low adiponectin and risk of certain cancers including colorectal cancer, reported by observational studies.

Materials and methods

Study subjects

Available for analysis were 40 formalin-fixed paraffin-embedded colorectal adenocarcinoma tissue specimens and 12 non-tumor colorectal specimens from American male and female patients with colorectal carcinoma. Of the colorectal carcinoma specimens, 15 (37.5%) were in the sigmoid colon, 9 (22.5%) in the ascending colon, 7 (17.5%) in the rectum, 4 (10.0%) in the transverse colon, 3 (7.5%) in the cecum, and 2 (5.0%) in the descending colon. Of the colorectal cancer specimens, nine of the tumor specimens were matched to non-tumor tissues from the same patient.

We had 45 GIST specimens and 13 non-tumor stomach/small bowel specimens from American men and women with GIST. Locations of the tumor specimens were 22 (48.9%) in the stomach, 5 (11.1%) each in the jejunum and duodenum, 4 (8.9%) in the small intestine, 2 (4.4%) each in the ileum, omentum, mesentery, and rectum, and 1 (2.2%) in the abdominal cavity. All specimens were purchased in the form of tissue array slides mounted to standard silanized slides (Imgenex, San Diego, CA, USA). Of the GIST specimens, nine of the tumor specimens were matched to non-tumor tissues from the same patient.

Immunohistochemistry analysis

The 5 μm paraffin tissue sections were deparaffinized, rehydrated, microwaved for 25 min in 10 mmol/l citrate buffer, and incubated for 30 min in methanol containing 0.5% H_2O_2. After incubation in 16% normal goat serum for 1 h at room temperature, the slides were incubated for 1 h with the primary antibodies at room temperature. The primary antibodies used were the rabbit anti-human AdipoR1 (raised against amino acid residues 357–375) antiserum and the rabbit anti-human AdipoR2 (raised against amino acid residues 374–386) antiserum (both from Phoenix Pharmaceuticals Inc., Belmont, CA, USA) used at 1:500 and 1:200 dilution respectively. The secondary antibody was a biotinylated anti-rabbit antibody (1:400 dilution) and was applied for 30 min at room temperature, followed by the Vectastain Elite ABC Reagent (Vector Laboratories, Burlingame, CA, USA)

for 30 min. The POD reaction was developed with diaminobenzidine, and the slides were counterstained with hematoxylin. Intensity and distribution of positive staining was evaluated on a scale of 0 to +++ by an expert pathologist (S T-B).

Real-time PCR

Adiponectin receptors expression was studied in human-matched paired cDNA samples from normal and colon cancer tissue of five patients (Human Colon Matched cDNA Pair Panel, Clontech). cDNA (30 ng) per 50 µl reaction were amplified using TaqMan Gene Expression system (Applied Biosystems, Foster City, CA, USA), specific primers and FAM tagged probes set (Applied Biosystems) and the Standard real-time 7500 protocol (Applied Biosystems), with an initial polymerase activation step at 95 °C for 10 min and 40 cycles including a 15-s melting step at 95 °C and a 1-min annealing-elongation step at 60 °C (Afonina *et al.* 1997). TATA box-binding protein (TBP) was used as endogenous control. Similarly, adiponectin receptor expression was detected in the human colorectal cancer cell lines CaCO2, HT-29, and SW480 (ATCC, Manassas, VA, USA) with real-time PCR, after RNA extraction with Trizol (Invitrogen) and first-strand cDNA synthesis, using Superscript III (Invitrogen), according to the manufacturer protocol. The analysis of relative gene expression was based on $\Delta\Delta C_t$ values obtained from real-time PCR (Livak & Schmittgen 2001). PCR products were separated in 2% agarose gels, in 0.5×TBE, in a horizontal Mini-sub cell GT electrophoresis apparatus (Bio-Rad), under a 100 V constant voltage and visualized using Bio-Rad gel documentation system (Bio-Rad).

Western blotting

CaCO2, HT-29, and SW480 (ATCC) were cultured in DMEM, high-glucose, 10% FBS in medium (Invitrogen) and subcultured beyond 80% confluency. For immunoblotting purposes, cells were trypsinized and seeded in 12-well plates, let to adhere overnight, and subsequently starved for 24 h. Cells were treated with full-length recombinant human adiponectin (R&D systems, Minneapolis, MN, USA), AMP-activated protein kinase activator 5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR, Sigma) or recombinant IGF I (Sigma). After treatment for the indicated time, cellular proteins were collected in 1% SDS. Total protein content was determined using the BCA assay (Pierce, Holmdel, NJ, USA). Lysates containing equal amounts of protein were separated on NuPAGE Novex 4–12% acrylamide gradient Bis–Tris gels (Invitrogen), using manufacturer's 4-morpholinepropanesulfonic acid (MOPS)/SDS running buffer, as previously described (Moos *et al.* 1988). After transfer on nitrocellulose membranes using a semi-dry apparatus (Bio-Rad), blots were blocked with 2% BSA (Sigma) in TBS-T and incubated overnight with primary antibodies against the phosphorylated proteins. Strips were then washed with TBS-T, incubated with secondary anti-rabbit-HRP antibody (1:5000, Chemicon, Billerica, MA, USA), and signal was detected with an ECL system (Amersham). For normalization purposes, membranes were stripped with the Mild Reblot stripping reagent (Chemicon) and re-blotted with antibodies against the total form of kinases. All primary antibodies: anti-phospho-p44/42 MAPK (ERK1/2, Thr202/Tyr204), anti-p44/42 MAPK, anti-phospho-AMPKα (Thr172), anti-AMPKα, anti-phospho-S6 ribosomal protein (Ser235/236), and anti-S6 ribosomal protein were from Cell Signaling (Danvers, MA, USA).

Proliferation assay

CaCO2, HT-29, and SW480 (ATCC) were cultured in DMEM, high-glucose, 10% FBS medium (Invitrogen) and subcultured beyond 80% confluency. For proliferation experiments, cells were trypsinized and seeded in 96-well plates at 5×10^3 cells/well. Cells were let to adhere overnight and then treated with recombinant human adiponectin (R&D systems), ranging from 1 to 10 µg/ml, in serum-free or 1% FBS medium, for 60 and 120 h. Subsequently, medium was discarded, cells were quickly washed with PBS, and incubated with 100 µl serum-free medium and 10 µl Vybrant 3-(4,5-dimethylthiazol-2-gl)-2,5-diphenyltetrazolium bromide (MTT) solution (Invitrogen) for 2 h (Carmichael *et al.* 1987). Formazan crystals were dissolved with the addition of 100 µl of 10% SDS in 0.01 M HCl per well and absorbance was measured in an optical reader Power-Wave XS (BIOTEK, Winooski, VT, USA) at 570 nm. Experiments were performed twice in triplicates.

Statistical analysis

Descriptive characteristics of patients providing tumor and non-tumor specimens of colorectal cancer were compared using unpaired *t*-tests and χ^2-tests for continuous and categorical measures. Case characteristics are presented as proportions. Unmatched analyses of expression of adiponectin receptors were conducted using χ^2-tests. For matched analyses, difference in relative expression was assessed using non-parametric Wilcoxon rank sum tests, and pair concordance was determined using McNemar's test for matched data. The analyses of GIST were conducted using the same methods and procedures as colorectal

cancer. A fold increase was calculated for receptor expression of each paired sample and 95% confidence intervals (CI) were calculated for the group of paired samples. A level of $\alpha = 0.05$ was set to determine statistical significance. All analyses were performed using SPSS version 11.5 (SPSS Inc., Chicago, IL, USA).

Results

Descriptive and case characteristics of study subjects are presented in Tables 1 and 2. Of individuals providing colorectal specimens, 55.7% were men, and of GIST specimens, 59.2% of those providing specimens were men. The average age of colorectal sample contributors was 56.8 years, and for GIST specimens it was 57.8 years. Patients providing tumor tissue did not differ significantly with regard to age or gender in both colorectal or GIST specimen subgroups. Almost half (47.5%) of colorectal cancer cases were stage-II disease, and about three-quarters (73.9%) of GIST cases had tumors >5 cm in diameter. Of the cases, 20.0% of colorectal cancer and 6.7% of GIST cases were metastatic.

Among non-malignant tissue specimens, expression of adiponectin receptors, especially AdipoR1, may be higher in upper portions of the GI tract (stomach/small bowel) than lower portions (colorectal), with positive expression in 63.6% of upper GI versus 8.3% of lower GI for AdipoR1 ($P<0.01$) and in 23.1% vs 0.0% respectively for AdipoR2 ($P=0.07$; Fig. 1).

Colorectal cancer

Real-time PCR analysis of matched cDNA colon samples showed a significant 1.6-fold (157%) increase of adipoR1 between cancerous and normal tissue ($\mu = 1.57$, 95% CI $= (1.02, 2.12)$). However, only a trend but no significant difference between normal and tumor tissue adipoR2 expression was found ($\mu = 1.35$, 95% CI $= (0.71, 2.00)$). Using immunohistochemistry in a larger number of samples, we found that tumor tissue was significantly more likely to show positive or strong positive expression of adiponectin receptors AdipoR1 and AdipoR2 than non-tumor tissue

Table 1 Descriptive characteristics of individuals in the colon cancer study

	Tumor tissue	Non-tumor tissue
Colorectal carcinoma, *n*	40	12
Male, *n*/female, *n*	23/17	8/4
Age, year, mean (S.D.)	56.2 (10.8)	56.0 (14.0)
Stage, *n* (%)		
II	13 (32.5)	
III	19 (47.5)	
IV	8 (20.0)	
Metastatic, *n* (%)	8 (20.0)	

Table 2 Descriptive characteristics of individuals in the gastrointestinal stromal tumor (GIST) study

	Tumor tissue	Non-tumor tissue
GIST, *n*	45	13
Male, *n*/female, *n*	26/19	9/4
Age, year, mean (S.D.)	59.2 (11.5)	55.8 (12.5)
Tumor size, *n* (%)		
<5	11 (24.4)	
5 to 10	16 (35.6)	
>10	18 (40.0)	
Risk category, *n* (%)		
Low	8 (17.8)	
Intermediate	9 (20.0)	
High	28 (62.2)	
Metastatic, *n* (%)	3 (6.7)	

Individuals were classified in different risk categories for recurrence/mortality from GISTs, based on the size of the primary tumor and the histological grade: high grade and high risk group corresponds to GISTs >10 cm, intermediate risk group had GISTs ranging from 5 to 10 cm, while in the low grade and low risk group GISTs were smaller than 5 cm.

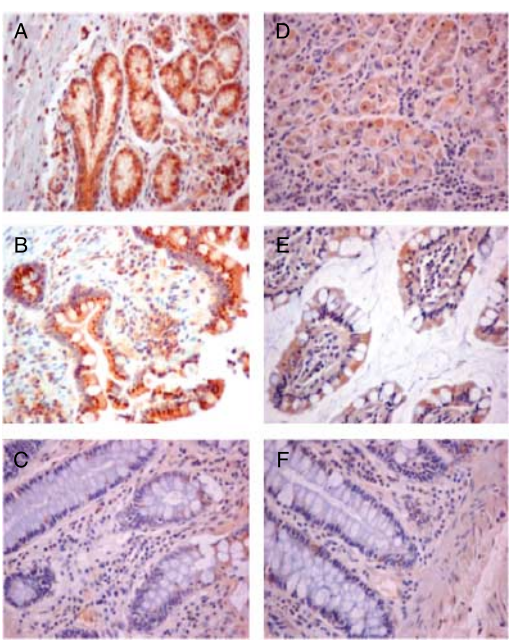

Figure 1 The expression of adiponectin receptors is higher in the proximal gastrointestinal tract. Immunohistochemical staining for AdipoR1 (A–C) and AdipoR2 (D–F) in non-malignant tissue of GI tract: stomach (A and D), small bowel (B and E), and colorectal (C and F), (400× magnification).

in colorectal specimens (Fig. 2, Table 3). Of colorectal carcinoma specimens, 95% displayed positive or strongly positive expression of AdipoR1 and 88% for AdipoR2, when compared with 8% and 0% respectively for non-tumor colorectal tissues (P for both $<$ 0.0001). For nine subjects of colorectal carcinoma cases, we had matched tumor and non-tumor tissues from the affected organ. Patients with matched colorectal specimens had a mean age of 53.3 years and seven of the nine subjects were men. Five of the matched specimens were sigmoid colon, two transverse colon, one ascending colon, and one rectum, and one patient had stage II disease, six stage III, and two stage IV. In colorectal carcinomas, tumor tissues showed significantly higher expression of AdipoR1 in seven of nine cases (78%, $P=0.02$) and AdipoR2 in six of nine cases (67%, $P=0.04$; Table 3). Results did not change when excluding the singular rectal cancer, which had marginal expression of both receptors. In addition, no significant differences in the expression of AdipoR1 and AdipoR2 were found among different stages of colorectal cancers. AdipoR1 expression was positive or strongly positive in all stages ($P=0.31$), except for two (10.5%) stage III specimens, where it was detected at marginal levels. Low AdipoR2 expression was found in 7.7, 15.8, and 12.5% of stage II, III, and IV stages samples respectively, with the rest of the samples showing positive or strongly positive staining. Similarly to AdipoR1, no association between AdipoR2 levels and colorectal cancer stage was found ($P=0.79$).

Thus, results from both unmatched and matched analyses suggest that expression of adiponectin receptors AdipoR1 and AdipoR2 is higher in colorectal carcinomas than in non-tumor colorectal tissue.

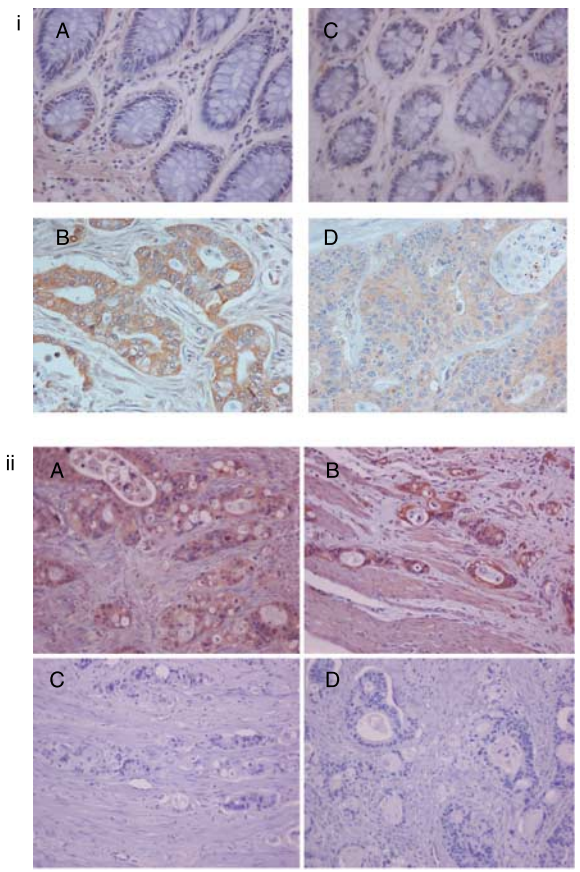

Figure 2 Adiponectin receptor expression is higher in cancerous that in normal tissue. (i) Immunohistochemical staining for AdipoR1 (A and B) and AdipoR2 (C and D) in non-tumor colon tissue (A and C) and carcinoma in ascending colon (B and D) of a 49-year-old female with T3N2M0 classification disease (400× magnification). (ii) Immunohistochemical staining for AdipoR1 and AdipoR2 in positive control of colon cancer: AdipoR1 (A); AdipoR2 (B); control pre-immune rabbit serum (C); no primary Ab (D), (400× magnification).

GIST

Tumor tissue did not show more positive or strongly positive expression of adiponectin receptors AdipoR1 and AdipoR2 than non-tumor tissue in GIST specimens (Table 4, Fig. 3). AdipoR1 was positively expressed in 47% of tumors and 64% of control tissues. Positive expression of AdipoR2 was apparent in 38% of GIST and 23% of control stomach and small bowel tissue. Differences in receptor expression between GIST and control tissue were not statistically significant ($P=0.50$ for AdipoR1, $P=0.51$ for AdipoR2).

The average age among nine GIST patients with matched tumor and non-tumor tissues from the affected organ was 62.0 years, and six of the nine subjects were men. Of the matched pairs of specimens, four were in the stomach, two each were in the small intestine and jejunum, and one in the rectum. All patients had high-risk GIST. AdipoR1 expression was similar (four of seven cases, two missing) or lower (three of seven cases) in all GISTs versus non-tumor tissues from the respective organ, and AdipoR2 expression was the same (six of nine cases) or higher (two of nine cases) in all but one (11%). There were no significant differences in adiponectin receptor expression among GIST cases ($P=0.25$ for AdipoR1 and $P=1.00$ for AdipoR2, Table 4). Excluding the one rectal GIST patient from analyses, which showed positive expression of both adiponectin receptors, did not alter the reported associations.

Thus, there appears to be no significant differences in either adiponectin receptor expression between GIST and non-tumor stomach/small bowel tissue. Furthermore, we found no significant difference in

Table 3 (A) Immunohistochemical expression of adiponectin receptor 1 (AdipoR1) and AdipoR2 in colon cancer and non-tumor tissue of colon. (B) Matched analysis of immunohistochemical expression of AdipoR1 and AdipoR2 in colon cancer and non-tumor tissues of colon

	Tumor tissue *n* (%)	Non-tumor tissue *n* (%)	*P* value
(A)			
AdipoR1	40	12	<0.0001
None or marginal	2 (5.0)	11 (91.7)	
Positive or strong positive	38 (95.0)	1 (8.3)	
AdipoR2	40	12	<0.0001
None or marginal	5 (12.5)	12 (100.0)	
Positive or strong positive	35 (87.5)	0 (0.0)	

		Tumor tissue		
	Expression	Negative or marginal	Positive or strong positive	*P* value
(B)				
AdipoR1, $n=9$	Negative or marginal	1	7	0.02
Normal tissue	Positive or strong positive	0	1	
AdipoR2, $n=9$	Negative or marginal	3	6	0.04
Normal tissue	Positive or strong positive	0	0	

P value from McNemar's test for matched pairs.

adiponectin receptor expression between tumor sizes and risk categories of GIST cases (low, intermediate, high) for either AdipoR1 or AdipoR2 (Table 5).

Adiponectin receptors are expressed and activate signaling pathway in colon cancer cell lines

Expression of both adiponectin receptors was confirmed in three different cancer cell lines: CaCO2, HT-29 and SW480 (Fig. 4), similar to the results in human colon cancer cells. AdipoR2 was expressed in lower levels in colon cancer cell lines. Treatment with adiponectin in physiological dose (10 μg/ml) for 0–40 min resulted in the activation of distinct molecular pathways, involving ERK1/2, AMPK, and S6 phosphorylation, as shown in Fig. 4. However, 60-h treatment with adiponectin (1–10 μg/ml) had no effect on cell viability when compared with the control. No promoting effect on cell proliferation was found with cell treatment up to 120 h.

Discussion

We found that adiponectin receptors AdipoR1 and AdipoR2 are expressed in normal gastrointestinal tissue, in the vast majority of colorectal carcinomas and in less than half of GIST specimens. Immunohistochemical staining of non-cancerous gastric, small bowel, and colorectal specimens indicates that adiponectin receptors are expressed in the upper portions of the GI tract more strongly than in the lower regions. Importantly, expression of adiponectin receptors was significantly higher in tumors when compared with non-tumor tissues from patients with colorectal cancer, but no similar differences were observed in specimens from GIST patients. We also detected adiponectin receptor expression in three colon cancer cell lines and found that adiponectin in physiological concentrations activates intracellular signaling pathways. These data indicate a potential for adiponectin receptors to be involved in the pathogenesis of colorectal carcinoma and to be of potential utility in the diagnosis and/or therapy for this obesity-associated carcinoma.

One of the major risk factors for colorectal carcinoma is obesity, as well as conditions leading to obesity including lack of physical activity and diets high in sugar, refined grains, and low in fiber (Potter *et al*. 1993, Chao *et al*. 2004, Cottet *et al*. 2005, Gunter & Leitzmann 2006). Similarly, individuals with insulin resistance (IR) or type 2 diabetes have up to 3 times the risk of developing colorectal carcinoma as non-diabetic individuals, and previous work has demonstrated that insulin increases proliferation and inhibits apoptosis in colorectal cancer cell lines (Gunter & Leitzmann 2006). In addition, IR induces changes in levels of IGFs, whose receptors

Table 4 (A) Immunohistochemical expression of adiponectin receptor 1 (AdipoR1) and AdipoR2 in gastrointestinal stromal tumors (GIST) and non-tumor GI tissue. (B) Matched analysis of immunohistochemical expression of AdipoR1 and AdipoR2 in GIST and GI tissue

	Tumor tissue *n* (%)	**Non-tumor tissue *n* (%)**	***P* value**
(A)			
GIST			
AdipoR1	45	11	0.50
None or marginal	24 (53.3)	4 (36.4)	
Positive or strong positive	21 (46.7)	7 (63.6)	
AdipoR2	45	13	0.51
None or marginal	28 (62.2)	10 (76.9)	
Positive or strong positive	17 (37.8)	3 (23.1)	

		Tumor tissue		
	Expression	**Negative or marginal**	**Positive or strong positive**	***P* value**
(B)				
AdipoR1, $n=7$	Negative or marginal	0	0	0.25
Normal tissue	Positive or strong positive	3	4	
AdipoR2, $n=9$	Negative or marginal	4	2	1.00
Normal tissue	Positive or strong positive	1	2	

P value from McNemar's test for matched pairs. Immunohistochemical expression of AdipoR1 was not available for $n=2$ of the 9 GIST patient non-tumor tissue samples.

are expressed in colon tissues, which further promote cellular proliferation and inhibit apoptosis (Giovannucci 2001).

Adiponectin has well-documented insulin-sensitizing effects (Ouchi *et al*. 1999, 2001, Yokota *et al*. 2000, Gavrila *et al*. 2003), which may in part explain the reduction in cancer risk associated with higher circulating levels of this adipokine. Physical activity and a Mediterranean pattern diet, which may decrease colorectal cancer risk (Trichopoulou *et al*. 2000, Chao *et al*. 2004, Cottet *et al*. 2005, Larsson *et al*. 2006), have both been associated with increased adiponectin levels (Bluher *et al*. 2006, Mantzoros *et al*. 2006). In addition, adiponectin has anti-inflammatory effects and may also play a role in suppressing neovascularization, which is required for tumor proliferation (Barb *et al*. 2006). Recently published prospective cohort (Wei *et al*. 2005) and case–control (Otake *et al*. 2005) studies have found a $>50\%$ increased risk of colorectal carcinoma in subjects with low concentrations of adiponectin. It remains uncertain, however, whether the beneficial effects of adiponectin observed in colorectal carcinoma are indirect, i.e., through changes in insulin resistance and IGF status, or whether adiponectin may act directly on the tumor cells to inhibit proliferation and/or induce apoptosis. Presence of adiponectin receptors in tumor tissues may support a direct impact of adiponectin on colorectal carcinogenesis. We clearly demonstrate not only that colon carcinoma cells express both of the classical adiponectin receptors, but also that the expression is stronger than in healthy tissue specimens from these patients. Similar to other endocrine systems, the upregulation of both adiponectin receptors in tumor cells may be a cellular response to lower circulating adiponectin levels in patients with colorectal cancer

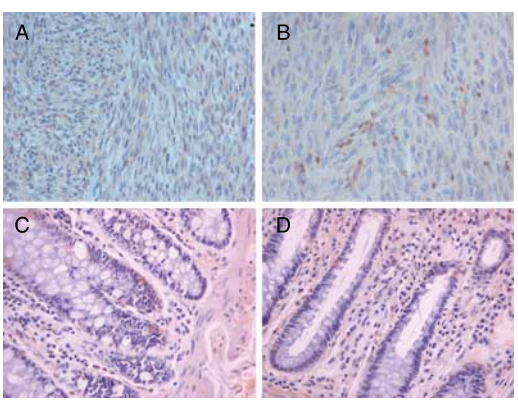

Figure 3 Adiponectin receptors present similar focal expression pattern in GIST tumors and in normal colon tissue. Immunohistochemical staining for AdipoR1 (A) and AdipoR2 (B) in gastrointestinal stromal tumor (GIST) tissue located in the rectum of a 55-year-old male with T2bN1M0 classification high-risk disease. Normal colon tissue staining for AdipoR1 (C) and AdipoR2 (D) (400× magnification).

Table 5 (A) Immunohistochemical expression of adiponectin receptor 1 (AdipoR1) and AdipoR2 in gastrointestinal stromal tumors (GIST) tumors of different tumor size. (B) Immunohistochemical expression of AdipoR1 and AdipoR2 in different risk category of GIST

	<5 CM *n* (%)	5–10 CM *n* (%)	>10 CM *n* (%)	*P* value
(A)				
GIST				
AdipoR1				0.83
None or marginal	5 (45.5)	9 (56.3)	10 (55.6)	
Positive or strong positive	6 (54.5)	7 (43.7)	8 (44.4)	
AdipoR2				0.81
None or marginal	6 (54.5)	10 (62.5)	12 (66.7)	
Positive or strong positive	5 (45.5)	6 (37.5)	6 (33.3)	
	Low *n* (%)	**Intermediate** *n* (%)	**High** *n* (%)	***P* value**
(B)				
GIST				
AdipoR1				0.82
None or marginal	4 (50.0)	4 (44.4)	13 (56.5)	
Positive or strong positive	4 (50.0)	5 (55.6)	10 (43.5)	
AdipoR2				0.22
None or marginal	4 (50.0)	8 (88.9)	15 (65.2)	
Positive or strong positive	4 (50.0)	1 (11.1)	8 (34.8)	

and/or a compensatory response of their malignant cells. In addition, we found that adiponectin stimulates the transient activation of intracellular signaling pathways in CaCO2, HT-29 and SW 480 cell lines, including ERK1/2, AMPK, and S6 ribosomal subunit phosphorylation. Recently, Kim *et al*. (2007) reported that AMPK activation could induce apoptosis in HT-29 colon cancer cells, while ERK1/2 is widely involved in the enhancement of cell proliferation and especially cell migration (Roberts & Der 2007). We have previously shown that adiponectin activated both the AMPK and ERK 1/2 signaling pathways but inhibited proliferation in the T47D breast cancer cell line (Korner *et al*. 2007). Moreover, recombinant adiponectin and adenovirus-mediated overexpression of this adipokine substantially reduced the mammary tumorigenesis of MDA-MB-231 cells in female nude mice (Wang *et al*. 2006). Adiponectin has also been found to inhibit prostate cancer cell growth (Bub *et al*. 2006). More recently, adiponectin, acting through its specific membrane receptors AdipoR1 and AdipoR2, was shown to inhibit the growth and peritoneal metastasis of gastric cancer *in vivo* (Ishikawa *et al*. 2007). Cellular proliferation was not altered in the limited time period studied herein but more detailed and longer studies are needed, especially, as adiponectin's effect on kinase activation was transient, reaching its maximum levels 20 min after its administration.

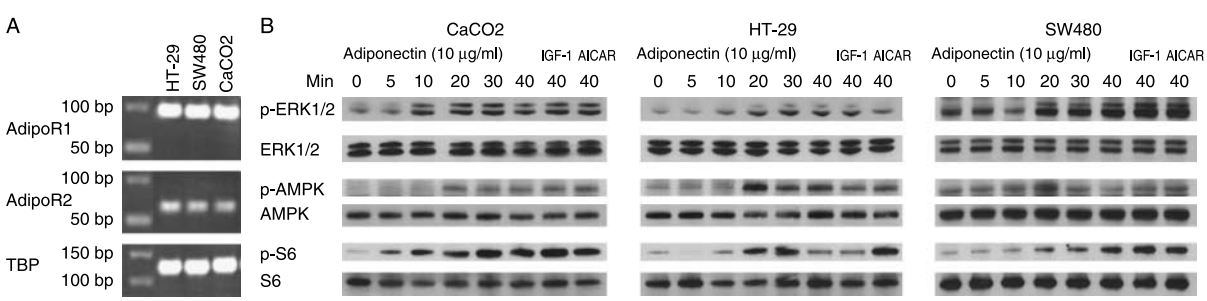

Figure 4 Adiponectin affects signaling in colon adenocarcinoma cell lines, through AdipoR1 and AdipoR2 receptors. (A) Specific expression of adiponectin receptors in HT-29, CaCO2, and SW480 cell lines was detected by RT-PCR and products were separated in 0.5×TBE 2% agarose gel. (B) Cells treatment with adiponectin (10 μg/ml) for the indicated time, in serum-free medium induced the phosphorylation of ERK1/2 and AMPK kinases and S6 ribosomal protein.

Thus, the simultaneous activation of several signaling pathways in colon cancer cells and/or the systemic insulin-sensitizing effects of adiponectin may account for the protective effect of this hormone seen in observational cohort studies. This remains to be studied by interventional studies in the future. Whether adiponectin receptor expression in colorectal tumors may also prove to have prognostic significance, similar to expression of estrogen receptors in breast cancer, remains to be studied in large prospective studies. Moreover, the increased expression of adiponectin receptors on colon cancer cells may represent a possible therapeutic target for the clinical use of adiponectin in patients with metastatic colon cancer. Further studies are necessary to confirm this potential mechanism for adiponectin to act directly on colonic epithelial cells to prevent carcinogenesis and/or to alter cancer cell growth and apoptosis.

GISTs have not previously been associated with obesity or insulin resistance. We therefore hypothesized that adiponectin receptor expression would be similar in GIST specimens and in the respective normal gastric/small bowel tissues and utilized such specimens as a negative control in our study. We found positive expression of AdipoR1 and AdipoR2 in less than half of all GIST specimens, but expression was not more pronounced than in control tissue. Because GIST is mesenchymal in origin and may not be comparable with the epithelial non-tumor GI specimens, we tested for variation in adiponectin expression among tumor specimens by low-, intermediate-, or high-risk categories and found no significant differences. The lack of strong expression of adiponectin receptors in the majority of GIST specimens, in contrast to results of this and previous studies in relation to colorectal carcinomas, does not support a major role for adiponectin in this tumor type. Additionally, when we used GIST specimens as negative controls for colorectal carcinomas, results showed that adiponectin receptor expression was significantly higher in colorectal carcinomas than in GIST. However, these variations may be due to differential expression of adiponectin receptors in the stomach or small bowel versus the colorectal region. Further studies including healthy subjects are needed to confirm these results and examine expression of adiponectin receptors in various regions of the GI tract in individuals free of any carcinoma.

In summary, we report that adiponectin receptors are expressed in normal GI tissue and that their relative expression is higher in upper parts of the GI tract. Moreover, adiponectin receptors are present in colon cancer cell lines and their activation with adiponectin in physiological concentrations results in activation of intracellular signaling pathways. Our data complement current observational evidence indicating that low circulating adiponectin concentrations may play a role in the development and/or progression of colorectal carcinomas. We have demonstrated in this study that both adiponectin receptors, AdipoR1 and AdipoR2, are expressed in nearly all colorectal carcinomas but not in non-malignant colorectal tissue and in some but not all GIST. The pathophysiological significance of the presence of adiponectin receptors as well as their potential predictive value in terms of colorectal cancer risk, relapse, and treatment outcomes need to be examined in detail in future studies.

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References

Afonina I, Zivarts M, Kutyavin I, Lukhtanov E, Gamper H & Meyer RB 1997 Efficient priming of PCR with short oligonucleotides conjugated to a minor groove binder. *Nucleic Acids Research* **25** 2657–2660.

Barb D, Pazaitou-Panayiotou K & Mantzoros CS 2006 Adiponectin: a link between obesity and cancer. *Expert Opinion on Investigational Drugs* **15** 917–931.

Bluher M, Bullen JW Jr, Lee JH, Kralisch S, Fasshauer M, Kloting N, Niebauer J, Schon MR, Williams CJ & Mantzoros CS 2006 Circulating adiponectin and expression of adiponectin receptors in human skeletal muscle: associations with metabolic parameters and insulin resistance and regulation by physical training. *Journal of Clinical Endocrinology and Metabolism* **91** 2310–2316.

Bub JD, Miyazaki T & Iwamoto Y 2006 Adiponectin as a growth inhibitor in prostate cancer cells. *Biochemical and Biophysical Research Communications* **340** 1158–1166.

Caan BJ, Coates AO, Slattery ML, Potter JD, Quesenberry CP Jr & Edwards SM 1998 Body size and the risk of colon cancer in a large case–control study. *International Journal of Obesity and Related Metabolic Disorders* **22** 178–184.

Carmichael J, Degraff WG, Gazdar AF, Minna JD & Mitchell JB 1987 Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of radiosensitivity. *Cancer Research* **47** 943–946.

Chao A, Connell CJ, Jacobs EJ, McCullough ML, Patel AV, Calle EE, Cokkinides VE & Thun MJ 2004 Amount, type, and timing of recreational physical activity in relation to

colon and rectal cancer in older adults: the Cancer Prevention Study II Nutrition Cohort. *Cancer Epidemiology, Biomarkers and Prevention* **13** 2187–2195.

Chen DC, Chung YF, Yeh YT, Chaung HC, Kuo FC, Fu OY, Chen HY, Hou MF & Yuan SS 2006 Serum adiponectin and leptin levels in Taiwanese breast cancer patients. *Cancer Letters* **237** 109–114.

Cottet V, Bonithon-Kopp C, Kronborg O, Santos L, Andreatta R, Boutron-Ruault MC & Faivre J 2005 Dietary patterns and the risk of colorectal adenoma recurrence in a European intervention trial. *European Journal of Cancer Prevention* **14** 21–29.

Dai Z, Xu YC & Niu L 2007 Obesity and colorectal cancer risk: a meta-analysis of cohort studies. *World Journal of Gastroenterology* **13** 4199–4206.

Dal Maso L, Augustin LS, Karalis A, Talamini R, Franceschi S, Trichopoulos D, Mantzoros CS & La Vecchia C 2004 Circulating adiponectin and endometrial cancer risk. *Journal of Clinical Endocrinology and Metabolism* **89** 1160–1163.

Ford ES 1999 Body mass index and colon cancer in a national sample of adult US men and women. *American Journal of Epidemiology* **150** 390–398.

Freedland SJ, Sokoll LJ, Platz EA, Mangold LA, Bruzek DJ, Mohr P, Yiu SK & Partin AW 2005 Association between serum adiponectin, and pathological stage and grade in men undergoing radical prostatectomy. *Journal of Urology* **174** 1266–1270.

Gavrila A, Chan JL, Yiannakouris N, Kontogianni M, Miller LC, Orlova C & Mantzoros CS 2003 Serum adiponectin levels are inversely associated with overall and central fat distribution but are not directly regulated by acute fasting or leptin administration in humans: cross-sectional and interventional studies. *Journal of Clinical Endocrinology and Metabolism* **88** 4823–4831.

Giovannucci E 2001 Insulin, insulin-like growth factors and colon cancer: a review of the evidence. *Journal of Nutrition* **131** 3109S–3120S.

Giovannucci E, Colditz GA, Stampfer MJ & Willett WC 1996 Physical activity, obesity, and risk of colorectal adenoma in women (United States). *Cancer Causes and Control* **7** 253–263.

Goktas S, Yilmaz MI, Caglar K, Sonmez A, Kilic S & Bedir S 2005 Prostate cancer and adiponectin. *Urology* **65** 1168–1172.

Gunter MJ & Leitzmann MF 2006 Obesity and colorectal cancer: epidemiology, mechanisms and candidate genes. *Journal of Nutritional Biochemistry* **17** 145–156.

Ishikawa M, Kitayama J, Yamauchi T, Kadowaki T, Maki T, Miyato H, Yamashita H & Nagawa H 2007 Adiponectin inhibits the growth and peritoneal metastasis of gastric cancer through its specific membrane receptors AdipoR1 and AdipoR2. *Cancer Science* **98** 1120–1127.

Joensuu H & Kindblom LG 2004 Gastrointestinal stromal tumors – a review. *Acta Orthopaedica Scandinavica* **75** 62–71.

Kadowaki T & Yamauchi T 2005 Adiponectin and adiponectin receptors. *Endocrine Reviews* **26** 439–451.

Kelesidis I, Kelesidis T & Mantzoros CS 2006 Adiponectin and cancer: a systematic review. *British Journal of Cancer* **94** 1221–1225.

Kim YM, Hwang JT, Kwak DW, Lee YK & Park OJ 2007 Involvement of AMPK signaling cascade in capsaicin-induced apoptosis of HT-29 colon cancer cells. *Annals of the New York Academy of Sciences* **1095** 496–503.

Korner A, Pazaitou-Panayiotou K, Kelesidis T, Kelesidis I, Williams CJ, Kaprara A, Bullen J, Neuwirth A, Tseleni S, Mitsiades N *et al.* 2007 Total and high-molecular-weight adiponectin in breast cancer: *in vitro* and *in vivo* studies. *Journal of Clinical Endocrinology and Metabolism* **92** 1041–1048.

Larsson SC, Rutegard J, Bergkvist L & Wolk A 2006 Physical activity, obesity, and risk of colon and rectal cancer in a cohort of Swedish men. *European Journal of Cancer* **42** 2590–2597.

Livak KJ & Schmittgen TD 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. *Methods* **25** 402–408.

Mantzoros C, Petridou E, Dessypris N, Chavelas C, Dalamaga M, Alexe DM, Papadiamantis Y, Markopoulos C, Spanos E, Chrousos G *et al.* 2004 Adiponectin and breast cancer risk. *Journal of Clinical Endocrinology and Metabolism* **89** 1102–1107.

Mantzoros CS, Williams CJ, Manson JE, Meigs JB & Hu FB 2006 Adherence to the Mediterranean dietary pattern is positively associated with plasma adiponectin concentrations in diabetic women. *American Journal of Clinical Nutrition* **84** 328–335.

Martinez ME, Giovannucci E, Spiegelman D, Hunter DJ, Willett WC & Colditz GA 1997 Leisure-time physical activity, body size, and colon cancer in women. Nurses' Health Study Research Group. *Journal of the National Cancer Institute* **89** 948–955.

Michalakis K, Williams CJ, Mitsiades N, Blakeman J, Balafouta-Tselenis S, Giannopoulos A & Mantzoros CS 2007 Serum adiponectin concentrations and tissue expression of adiponectin receptors are reduced in patients with prostate cancer: a case control study. *Cancer Epidemiology, Biomarkers and Prevention* **16** 308–313.

Mistry T, Digby JE, Chen J, Desai KM & Randeva HS 2006 The regulation of adiponectin receptors in human prostate cancer cell lines. *Biochemical and Biophysical Research Communications* **348** 832–838.

Miyoshi Y, Funahashi T, Kihara S, Taguchi T, Tamaki Y, Matsuzawa Y & Noguchi S 2003 Association of serum adiponectin levels with breast cancer risk. *Clinical Cancer Research* **9** 5699–5704.

Moore LL, Bradlee ML, Singer MR, Splansky GL, Proctor MH, Ellison RC & Kreger BE 2004 BMI and waist circumference as predictors of lifetime colon cancer risk in Framingham Study adults. *International Journal of Obesity and Related Metabolic Disorders* **28** 559–567.

Moos M Jr, Nguyen NY & Liu TY 1988 Reproducible high yield sequencing of proteins electrophoretically separated and transferred to an inert support. *Journal of Biological Chemistry* **263** 6005–6008.

Otake S, Takeda H, Suzuki Y, Fukui T, Watanabe S, Ishihama K, Saito T, Togashi H, Nakamura T, Matsuzawa Y *et al.* 2005 Association of visceral fat accumulation and plasma adiponectin with colorectal adenoma: evidence for participation of insulin resistance. *Clinical Cancer Research* **11** 3642–3646.

Ouchi N, Kihara S, Arita Y, Maeda K, Kuriyama H, Okamoto Y, Hotta K, Nishida M, Takahashi M, Nakamura T *et al.* 1999 Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin. *Circulation* **100** 2473–2476.

Ouchi N, Kihara S, Arita Y, Nishida M, Matsuyama A, Okamoto Y, Ishigami M, Kuriyama H, Kishida K, Nishizawa H *et al.* 2001 Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class A scavenger receptor expression in human monocyte-derived macrophages. *Circulation* **103** 1057–1063.

Petridou E, Mantzoros C, Dessypris N, Koukoulomatis P, Addy C, Voulgaris Z, Chrousos G & Trichopoulos D 2003 Plasma adiponectin concentrations in relation to endometrial cancer: a case–control study in Greece. *Journal of Clinical Endocrinology and Metabolism* **88** 993–997.

Pischon T, Lahmann PH, Boeing H, Friedenreich C, Norat T, Tjonneland A, Halkjaer J, Overvad K, Clavel-Chapelon F, Boutron-Ruault MC *et al.* 2006 Body size and risk of colon and rectal cancer in the European prospective investigation into cancer and nutrition (EPIC). *Journal of the National Cancer Institute* **98** 920–931.

Potter JD, Slattery ML, Bostick RM & Gapstur SM 1993 Colon cancer: a review of the epidemiology. *Epidemiologic Reviews* **15** 499–545.

Roberts PJ & Der CJ 2007 Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene* **26** 3291–3310.

Tran T, Davila JA & El Serag HB 2005 The epidemiology of malignant gastrointestinal stromal tumors: an analysis of 1458 cases from 1992 to 2000. *American Journal of Gastroenterology* **100** 162–168.

Trichopoulou A, Lagiou P, Kuper H & Trichopoulos D 2000 Cancer and Mediterranean dietary traditions. *Cancer Epidemiology, Biomarkers and Prevention* **9** 869–873.

Wang Y, Lam JB, Lam KS, Liu J, Lam MC, Hoo RL, Wu D, Cooper GJ & Xu A 2006 Adiponectin modulates the glycogen synthase kinase-3beta/beta-catenin signaling pathway and attenuates mammary tumorigenesis of MDA-MB-231 cells in nude mice. *Cancer Research* **66** 11462–11470.

Wei EK, Giovannucci E, Fuchs CS, Willett WC & Mantzoros CS 2005 Low plasma adiponectin levels and risk of colorectal cancer in men: a prospective study. *Journal of the National Cancer Institute* **97** 1688–1694.

Yokota T, Oritani K, Takahashi I, Ishikawa J, Matsuyama A, Ouchi N, Kihara S, Funahashi T, Tenner AJ, Tomiyama Y *et al.* 2000 Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages. *Blood* **96** 1723–1732.