Reduced expression of the *AdipoR1* gene is correlated with venous invasion in colorectal cancer

NAOTO YAMAMOTO[1], TAKASHI OSHIMA[1], KAZUE YOSHIHARA[1], TSUTOMU SATO[1], ROPPEI YAMADA[1], SHOICHI FUJII[1], YASUHIKO NAGANO[1], MANABU SHIOZAWA[2], MAKOTO AKAIKE[2], NOBUYUKI WADA[3], YASUSHI RINO[3], CHIKARA KUNISAKI[1], MUNETAKA MASUDA[3], KATSUAKI TANAKA[1] and TOSHIO IMADA[4]

[1]Gastroenterological Center, Yokohama City University Medical Center, Kanagawa-ken 232-0024; [2]Department of Surgery, Kanagawa Cancer Center, Kanagawa-ken 241-0815; [3]Department of Surgery, Yokohama City University; [4]Yokohama City University, Kanagawa-ken 236-0004, Japan

Received November 14, 2008; Accepted May 7, 2009

DOI: 10.3892/mmr_00000136

Abstract. Serum adiponectin concentrations are negatively correlated with body fat percentage and with the risk of colorectal cancer. However, few studies have examined the relationship between adiponectin receptor expression and colorectal cancer. We measured the expression levels of the *AdipoR1* and *AdipoR2* genes by quantitative real-time reverse-transcription polymerase chain reaction in 202 paired specimens of cancer tissue and adjacent normal mucosa obtained from patients with colorectal cancer. To evaluate the clinical significance of *AdipoR1* and *AdipoR2*, correlations between the expression of these genes and clinicopathological features were examined. Both genes were expressed in colorectal cancer and in adjacent normal mucosa. The expression levels of the genes were significantly higher in cancer tissue than in normal mucosa (P<0.0001). Reduced expression of the *AdipoR1* gene was correlated with venous invasion, but not with any other clinicopathological feature examined. Our findings suggest that reduced expression of the *AdipoR1* gene may be a useful predictor of venous invasion.

Introduction

Obesity has been identified as a risk factor for colorectal and several other types of cancer (1-3). Adiponectin, a hormone that is secreted exclusively by adipocytes, has potent insulin-sensitizing effects. Serum adiponectin concentrations are negatively correlated with body fat percentage (4,5). Hypoadiponectinemia is associated with insulin resistance and hyperinsulinemia (4). Serum adiponectin concentrations are negatively correlated with the risk of colorectal cancer (5).

Two types of adiponectin receptors (AdipoR1 and AdipoR2) have been cloned (6). In mice, AdipoR1 is expressed in various organs, including skeletal muscle, the lungs and the spleen, whereas AdipoR2 is predominantly expressed in the liver (6). In humans, AdipoR1 and AdipoR2 are expressed in pancreatic islet cells, macrophages, adipocytes and vascular smooth muscle, as well as in human breast epithelial and breast cancer cells (7-10). AdipoR1 is a receptor for globular adiponectin, whereas AdipoR2 is a receptor for full-length adiponectin (11). AdipoR1 and R2 are thought to mediate increased AMP kinase activity and peroxisome proliferator activated receptor (PPAR)-α ligand activity, fatty acid oxidation and glucose uptake by adiponectin (11).

A recent study showed that adiponectin inhibits the growth of gastric cancer and the development of peritoneal metastasis *in vivo* through AdipoR1 and AdipoR2 (12). Adiponectin also induces tumor vessel apoptosis and stimulates endothelial cell growth and angiogenesis (13,14).

In this study, we measured the expression levels of the *AdipoR1* and *AdipoR2* genes in 202 paired specimens of cancer tissue and adjacent normal mucosa obtained from patients with colorectal cancer. To evaluate the clinical significance of AdipoR1 and AdipoR2, we examined the correlation between the expression of these genes and clinicopathological features.

Materials and methods

Patients and samples. Surgical specimens of cancer tissue and adjacent normal mucosa obtained from 202 patients with untreated colorectal carcinoma were studied. The patients underwent surgery at the Gastroenterological Center of Yokohama City University Medical Center and at Kanagawa Cancer Center between 2002 and 2006. Informed consent was obtained from each patient. The ethics committees of Yokohama City University Medical Center and Kanagawa Cancer Center approved the protocol before initiation of the study. All tissue samples were embedded in OCT compound

Correspondence to: Dr Naoto Yamamoto, Gastroenterological Center, Yokohama City University Medical Center, 4-57 Urafune-cho, Minami-ku, Yokohama-shi, Kanagawa-ken 232-0024, Japan
E-mail: naoto-y@urahp.yokohama-cu.ac.jp

Key words: adiponectin, *AdipoR1*, *AdipoR2*, colorectal cancer, venous invasion

Table I. PCR primers and conditions.

Gene	Primer	Annealing temperature (˚C)	Product size (bp)
AdipoR1	5'-CCAGGAAGAAGAGGAGGAG-3' 5'-ATGTAGCAGATAGTCGTTGTC-3'	60.0	166
AdipoR2	5'-TTCTTTCTTCTCCCTTTCTCTC-3' 5'-CTCCCTCCCTCCCTTACC-3'	57.4	199
ß-actin	5'-AGTTGCGTTACACCCTTTCTTGAC-3' 5'-GCTCGCTCCAACCGACTGC-3'	60.0	171

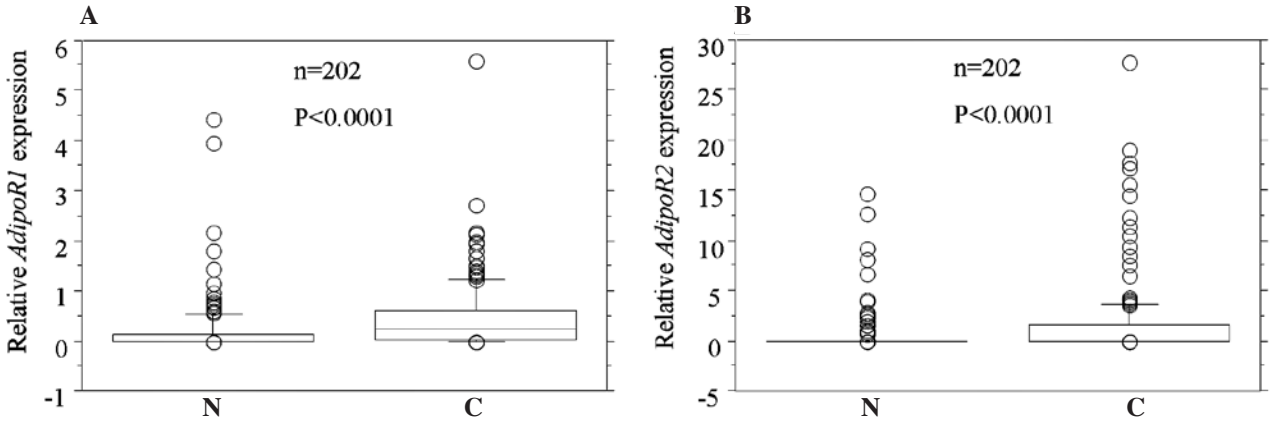

Figure 1. Comparison of *AdipoR1* (A) and *AdipoR2* (B) gene expression levels in colorectal cancer tissue (C) and adjacent normal mucosa (N). *AdipoR1* and *AdipoR2* gene expression levels were higher in cancer than in adjacent normal mucosa (P<0.0001, P<0.0001, respectively).

(Sakura Finetechnical Co., Ltd.; Tokyo) and were immediately stored at -80˚C until use. No patient had any other malignancies. The specimens were stained with hematoxylin and eosin and examined histopathologically. Sections that consisted of >80% carcinoma were used to prepare total RNA.

Quantitative real-time reverse-transcription polymerase chain reaction. Total RNA isolated from colorectal cancer and adjacent normal mucosa was prepared using Trizol (Gibco, Life Tech, Gaitherburg, MD). Complementary DNA (cDNA) was synthesized from 2 μg of total RNA using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). After synthesis, the cDNA was diluted 1:4 with water and stored at -20˚C until use. Quantitative real-time PCR was performed with an iQ SYBR Green Supermix Kit (Bio-Rad Laboratories). PCR reactions were carried out in a total volume of 15 μl containing cDNA derived from 75 ng of RNA, 0.27 μM of each primer, 7.5 μl of iQ SYBR Green Supermix containing dATP, dCTP, dGTP and dTTP at concentrations of 400 μM each, and 50 U/ml of iTag DNA polymerase. The PCR consisted of 10 min at 94˚C, annealing for 30 sec at an appropriate temperature (Table I), and a primer extension for 1 min at 72˚C followed by 72˚C for 10 min. PCR primer sequences of *AdipoR1*, *AdipoR2* and *ß-actin*, used as an internal control, are shown in Table I.

Immunohistochemical analysis of AdipoR1. Sections (4-μm) were prepared from paraffin blocks, deparaffinized, rehydrated, microwaved for 15 min in 10 mmol/l citrate buffer (pH 6.0), and incubated for 10 min in Peroxidase Blocking Reagent (Dako, Glostrup, Denmark). After incubation in 5% skim milk for 10 min at room temperature, the slides were incubated for 1 h with the primary antibodies at room temperature. The primary antibody used was rabbit anti-human AdipoR1 (raised against amino acid residues 357-375) antiserum (Phoenix Pharmaceuticals Inc., Belmont, CA, USA) at a dilution of 1:500. The secondary antibody was peroxidase-labeled polymer, EnVision+ rabbit (Dako), applied for 60 min at room temperature. The color reaction was developed with 3,3'-diaminobenzidine (Dako), and the slides were counterstained with hematoxylin.

Statistical analysis. Gene expression levels of colorectal cancer were compared with those of the normal adjacent mucosa using the Wilcoxon test. Relationships between gene expression and potential explanatory variables, including age, gender, tumor size, histological type, depth of invasion, lymph node metastasis, location, lymphatic invasion, venous invasion and liver metastasis, were evaluated using the χ^2 test. Associations between variables were assessed using the Mann-Whitney U test. All statistical analysis was performed using Statview J 5.0 software

Table II. Relationship between expression of the *AdipoR1* or *AdipoR2* gene and clinicopathological features.

	AdipoR1 expression			*AdipoR2* expression		
Variables/categories	Low (n=101)	High (n=101)	P-value	Low (n=101)	High (n=101)	P-value
Age	66.0±10.7	65.3±10.9		66.9±11.0	64.5±10.6	
Gender						
Male	49	61	0.090	51	59	0.258
Female	52	40		50	42	
Location						
Colon	54	55	0.888	55	54	0.888
Rectum	47	46		46	47	
Size						
≤5 cm	56	56	1.000	53	59	0.396
>5 cm	45	45		48	42	
Histological type						
Well differentiated	31	28	0.774	27	32	0.724
Moderately differentiated	55	60		59	56	
Poorly differentiated	15	13		15	13	
Depth of invasion						
Tl	10	7	0.123	8	9	0.154
T2	38	55		39	54	
T3	46	34		47	33	
T4	7	5		7	5	
Lymph node metastasis						
Absent	44	49	0.480	49	44	0.480
Present	57	52		52	57	
Lymphatic invasion						
Absent	63	69	0.442	66	66	1.000
Present	38	32		35	35	
Venous invasion						
Absent	27	48	0.002	33	42	0.190
Present	74	53		68	59	
Liver metastasis						
Absent	71	69	0.760	69	71	0.760
Present	30	32		32	30	

(Abacus, CA). Two-sided P-values were calculated, and a P-value <0.05 was considered to indicate statistical significance.

Results

Comparison of AdipoR1 and AdipoR2 mRNA expression in colorectal cancer tissue and adjacent normal mucosa. *AdipoR1* and *AdipoR2* gene expression levels were higher in cancer than in adjacent normal mucosa (P<0.0001 and <0.0001, respectively) (Fig. 1).

Relationship between AdipoR1 and AdipoR2 gene expression and clinicopathological features. The expression levels of the *AdipoR1* and *AdipoR2* genes were categorized as low or high according to their median values. The relationships between the expression of these genes and clinicopathological features were then examined. Reduced expression of *AdipoR1* was correlated with venous invasion (P=0.002). *AdipoR1* and *AdipoR2* expression levels were unrelated to age, gender, tumor location, tumor size, histological type, depth of invasion, lymph node metastasis, lymphatic invasion and liver metastasis (Table II).

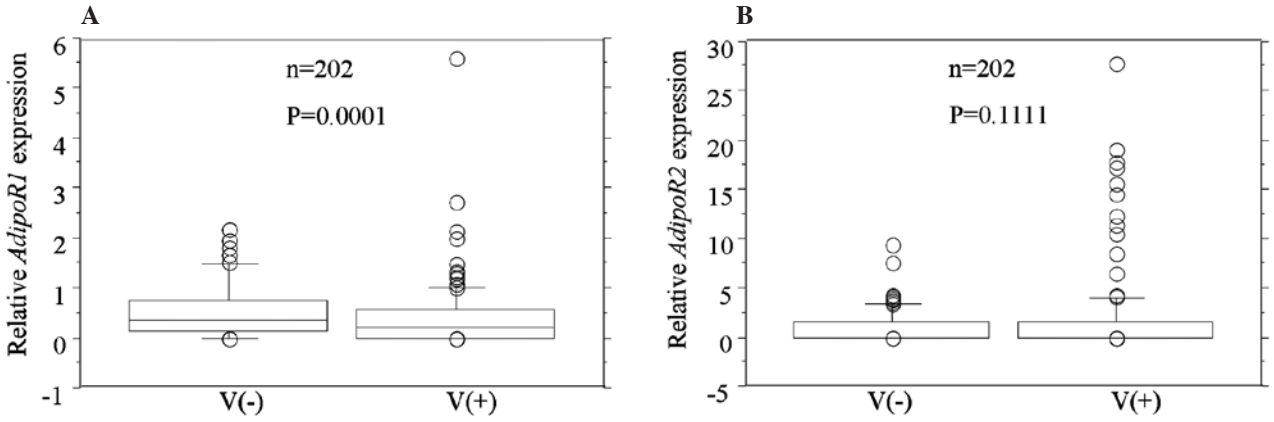

Figure 2. Association of *AdipoR1* (A) and *AdipoR2* (B) gene expression levels with venous invasion in 202 patients with colorectal cancer. Box boundaries, the 25th and 75th percentiles of the observed values; capped bars, the 10th and 90th percentiles; solid line, median. P-values were calculated by the Mann-Whitney U test. *AdipoR1* gene expression was higher in the absence V(-) than in the presence V(+) of venous invasion (P=0.0001).

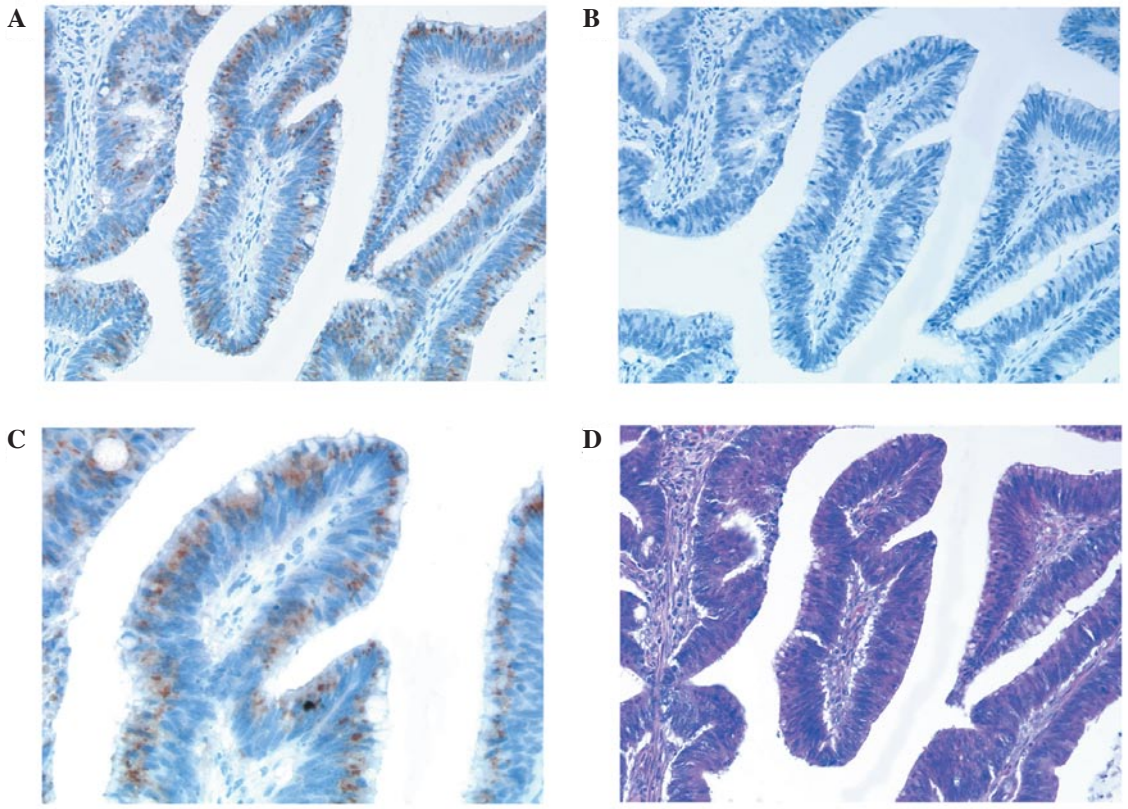

Figure 3. Immunostaining of AdipoR1 in human colorectal cancer tissue. Immunohistochemical staining of AdipoR1 in surgically resected colorectal cancer tissues (A, x200; C, x400). Negative control (B, x200). Hematoxylin and eosin staining (D, x200). AdipoR1 was positively detected in the cytoplasm of cancer cells expressing high levels of *AdipoR1* mRNA.

Association of AdipoR1 and AdipoR2 gene expression with venous invasion in patients with colorectal cancer. AdipoR1 gene expression levels were higher in the absence than in the presence of venous invasion (P=0.0001) (Fig. 2).

Immunohistochemical analysis of AdipoR1. Upon immunohistochemical analysis, tumor tissue expressing high levels of *AdipoR1* mRNA showed positive expression of adiponectin receptor AdipoR1 (Fig. 3).

Discussion

Although many studies have examined the relationship between adiponectin and cancer (5,15-17), the relationship between adiponectin receptors and cancer remains largely unexplored (18). Takahata *et al* (10) suggested that adiponectin modulates the growth of cancer cells directly through AdipoR1 and AdipoR2. In this study, we examined the expression levels of the *AdipoR1* and *AdipoR2* genes and

the relationships between these levels and clinicopathological variables.

First, we compared the relative expression of the *AdipoR1* and *AdipoR2* genes in colorectal cancer tissue and adjacent normal mucosa. Several studies have previously assessed the expression of AdipoR1 and AdipoR2 in cancer tissue. Takahata *et al* (10) examined the expression of the *AdipoR1* and *AdipoR2* genes in four breast cancer cell lines. *AdipoR1* and *AdipoR2* gene expression was found in all cancer cell lines and in normal breast epithelial cells. More recently, Williams *et al* (19) studied the expression of AdipoR1 and AdipoR2 in 40 colorectal carcinomas and 12 non-tumor colorectal tissue specimens from patients with colorectal cancer. AdipoR1 was expressed in 95% of the cancer tissue specimens and 8% of the non-tumor colorectal tissue specimens. AdipoR2 was expressed in 88% of the cancer tissue specimens and 0% of the non-tumor tissue specimens. In the present study, we found that the *AdipoR1* and *AdipoR2* genes were expressed in colorectal cancer tissue, as well as in normal colorectal mucosa.

We went on to examine the correlation between *AdipoR1* and *AdipoR2* gene expression and clinicopathological features. Several studies have investigated correlations between serum adiponectin concentrations and venous invasion by cancer cells (20,21), To our knowledge, however, no previous study has examined the correlation between the expression of adiponectin receptors and venous invasion. Our study showed that reduced expression of the *AdipoR1* gene was correlated with venous invasion in colorectal cancer. These results are partially consistent with the findings of previous studies. Horiguchi *et al* (22) found no significant association between total adiponectin and venous invasion in renal cell carcinoma. In another study performed by the same group, the serum concentration of leptin, another adipokine, was significantly higher in patients with renal cell carcinoma who had venous invasion than in those without (20). On the other hand, Ishikawa *et al* (21) reported that venous invasion and lymph node metastasis tended to be more frequent in gastric cancer patients with low serum adiponectin concentrations than in those with high adiponectin concentrations. Their results suggested that adiponectin may prevent tumor invasion or metastasis.

In conclusion, our results show that reduced expression of the *AdipoR1* gene is correlated with venous invasion in colorectal cancer. Our findings suggest that reduced expression of *AdipoR1* may be a useful predictor of venous invasion.

References

1. Wolk A, Gridley G, Svensson M, *et al*: A prospective study of obesity and cancer risk (Sweden). Cancer Causes Control 12: 13-21, 2001.
2. Giovannucci E, Ascherio A, Rimm EB, Colditz GA, Stampfer MJ and Willett WC: Physical activity, obesity, and risk for colon cancer and adenoma in men. Ann Intern Med 122: 327-334, 1995.
3. Moghaddam AA, Woodward M and Huxley R: Obesity and risk of colorectal cancer: a meta-analysis of 31 studies with 70,000 events. Cancer Epidemiol Biomarkers Prev 16: 2533-2547, 2007.
4. Trujillo ME and Scherer PE: Adiponectin – journey from an adipocyte secretory protein to biomarker of the metabolic syndrome. J Intern Med 257: 167-175, 2005.
5. Wei EK, Giovannucci E, Fuchs CS, Willett WC and Mantzoros CS: Low plasma adiponectin levels and risk of colorectal cancer in men: a prospective study. J Natl Cancer Inst 97: 1688-1694, 2005.
6. Yamauchi T, Kamon J, Ito Y, *et al*: Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. Nature 423: 762-769, 2003.
7. Kharroubi I, Rasschaert J, Eizirik DL and Cnop M: Expression of adiponectin receptors in pancreatic beta cells. Biochem Biophys Res Commun 312: 1118-1122, 2003.
8. Chinetti G, Zawadski C, Fruchart JC and Staels B: Expression of adiponectin receptors in human macrophages and regulation by agonists of the nuclear receptors PPARalpha, PPARgamma, and LXR. Biochem Biophys Res Commun 314: 151-158, 2004.
9. Berner HS, Lyngstadaas SP, Spahr A, *et al*: Adiponectin and its receptors are expressed in bone-forming cells. Bone 35: 842-849, 2004.
10. Takahata C, Miyoshi Y, Irahara N, Taguchi T, Tamaki Y and Noguchi S: Demonstration of adiponectin receptors 1 and 2 mRNA expression in human breast cancer cells. Cancer Lett 250: 229-236, 2007.
11. Kadowaki T and Yamauchi T: Adiponectin and adiponectin receptors. Endocr Rev 26: 439-451, 2005.
12. Ishikawa M, Kitayama J, Yamauchi T, *et al*: Adiponectin inhibits the growth and peritoneal metastasis of gastric cancer through its specific membrane receptors AdipoR1 and AdipoR2. Cancer Sci 98: 1120-1127, 2007.
13. Brakenhielm E, Veitonmaki N, Cao R, *et al*: Adiponectin-induced antiangiogenesis and antitumor activity involve caspase-mediated endothelial cell apoptosis. Proc Natl Acad Sci USA 101: 2476-2481, 2004.
14. Ouchi N, Kobayashi H, Kihara S, *et al*: Adiponectin stimulates angiogenesis by promoting cross-talk between AMP-activated protein kinase and Akt signaling in endothelial cells. J Biol Chem 279: 1304-1309, 2004.
15. Miyoshi Y, Funahashi T, Kihara S, *et al*: Association of serum adiponectin levels with breast cancer risk. Clin Cancer Res 9: 5699-5704, 2003.
16. Barb D, Pazaitou-Panayiotou K and Mantzoros CS: Adiponectin: a link between obesity and cancer. Expert Opin Investig Drugs 15: 917-931, 2006.
17. Housa D, Housova J, Vernerova Z and Haluzik M: Adipocytokines and cancer. Physiol Res 55: 233-244, 2006.
18. Mistry T, Digby JE, Chen J, Desai KM and Randeva HS: The regulation of adiponectin receptors in human prostate cancer cell lines. Biochem Biophys Res Commun 348: 832-838, 2006.
19. Williams CJ, Mitsiades N, Sozopoulos E, *et al*: Adiponectin receptor expression is elevated in colorectal carcinomas but not in gastrointestinal stromal tumors. Endocr Relat Cancer 15: 289-299, 2008.
20. Horiguchi A, Sumitomo M, Asakuma J, *et al*: Increased serum leptin levels and over expression of leptin receptors are associated with the invasion and progression of renal cell carcinoma. J Urol 176: 1631-1635, 2006.
21. Ishikawa M, Kitayama J, Kazama S, Hiramatsu T, Hatano K and Nagawa H: Plasma adiponectin and gastric cancer. Clin Cancer Res 11: 466-472, 2005.
22. Horiguchi A, Ito K, Sumitomo M, Kimura F, Asano T and Hayakawa M: Decreased serum adiponectin levels in patients with metastatic renal cell carcinoma. Jpn J Clin Oncol 38: 106-111, 2008.