# Association of leptin, visfatin, apelin, resistin and adiponectin with clear cell renal cell carcinoma

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**Abstract.** Although an association between obesity and the occurrence of renal cell carcinoma (RCC) has been identified, the mechanism by which obesity functions to increase this risk of cancer remains unclear. Leptin, visfatin, apelin, resistin and adiponectin are peptide hormones secreted by adipocytes; it is considered that these may affect RCC development by exerting effects on proliferation, cell growth and inflammation. The aim of the present study was to investigate the association between the aforementioned adipokine genes and clear cell RCC (CC-RCC). The GSE6344 dataset was downloaded from the Gene Expression Omnibus database, and the relative expression levels of the adipokine genes were analyzed. To verify the results of the mRNA microarray, 77 paired samples of CC-RCC and corresponding adjacent normal tissue were allocated into two groups. The extraction of total RNA was conducted, and the mRNA expression of adipokine genes was analyzed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The data from the GSE6344 dataset indicated that the expression of visfatin and apelin was upregulated ($P<0.0001$ and $P<0.01$, respectively), and adiponectin was downregulated ($P<0.001$) in the CC-RCC tissues compared with the adjacent normal tissues. The data from RT-qPCR demonstrated that visfatin and resistin gene expression was increased ($P<0.01$ and $P<0.05$, respectively) in the CC-RCC tissues. Furthermore, the mRNA expression level of leptin and adiponectin in the adjacent normal tissue was higher than those in the cancer tissue ($P<0.01$). The current study verifies that visfatin and adiponectin are associated with an increased risk of CC-RCC, which presents further insights into the molecular mechanisms of CC-RCC tumorigenesis.

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## Introduction

Renal cell carcinoma (RCC) is one of the most lethal types of cancer within the urinary system, and up to one-third of patients with RCC already present with primary metastases at the time of diagnosis (1). RCC is categorized into four major subtypes according to the Heidelberg classification system (2): i) Clear cell RCC (CC-RCC); ii) papillary RCC: iii) chromophobe RCC; and iv) renal oncocytoma. CC-RCC is the most prevalent subtype of RCC, accounting for ~80% of cases (3). Specific molecular mechanisms have been identified in RCC tumorigenesis, including von Hipple-Lindau gene mutation (4). Somatic mutation of this gene has been associated with the development of ~60% of sporadic CC-RCC (5). However, no current theory is able to explain all cases of CC-RCC development. To understand the pathogenesis of CC-RCC in detail, further research is warranted.

A number of epidemiological studies have connected the occurrence of renal cancer to obesity (6-8). A specific association between obesity and RCC has been established, however, the mechanism by which obesity increases the risk of cancer remains unclear (9). Recently, certain obesity-associated biomarkers have been identified and are considered as a possible link between the two features (10). Among these biomarkers, the adipokines are of particular note. Adipokines, including adiponectin, leptin, resistin, visfatin and apelin, are peptide hormones secreted primarily by adipose tissue and are associated with metabolic syndrome (11). Studies have identified that adipokines affect various pro-neoplastic mechanisms, including inflammation, cell growth and proliferation (12,13). Studies have also demonstrated that adipokines are promising predictors of risk and progression in various types of cancer (14,15). Such evidence suggests that adipokines contribute to the initiation of carcinogenesis and tumor progression.

Microarray technology provides a wide range of information regarding molecules that have been associated with disease pathogenesis, and subsequently aids the elucidation of the underlying molecular mechanisms of disease. To gain insights into the pathogenesis of CC-RCC, several gene expression

Table I. Sequences of primers used for reverse transcription-quantitative polymerase chain reaction analysis of the adipokine genes and the GAPDH gene.

| Gene | Orientation | Primer sequence (5'-3') |
|---|---|---|
| GAPDH | Forward | CAACTTTGGTATCGTGGAAGGACTC |
| | Reverse | AGG GATGATGTTCTGGAGAGCC |
| Leptin | Forward | CGTTAAGGGAAGGAACTCTGG |
| | Reverse | TGGCTTAGAGGAGTCAGGGA |
| Resistin | Forward | GTGTGCCGGATTTGGTTAGC |
| | Reverse | AGGAGGAGGAGACAGAGAGC |
| Visfatin | Forward | CTTCGGTTCTGGTGGAGGTT |
| | Reverse | ATCGGCCCTTTTTGGACCTT |
| Apelin | Forward | GCTGACAGTTCGCCCTTACT |
| | Reverse | ATATGTGGGCATGGGGACAC |
| Adiponectin | Forward | ATGGCCCCTGCACTACTCTA |
| | Reverse | CAGGGATGAGTTCGGCACTT |

profiling studies have been conducted analyzing differences between tumor and normal tissue (16,17). Such studies have identified a number of differentially-expressed genes, including the adipokine genes. In the present study, to investigate the association between adipokine genes and the molecular mechanisms of RCC, gene expression profiles of 10 CC-RCC and 10 adjacent normal tissue controls were downloaded from the Gene Expression Omnibus (GEO) database, and the relevant adipokine gene data was analyzed. To verify the results of mRNA microarray, the expression of various adipokine genes were analyzed in 77 CC-RCC patients using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The present study aimed to identify the underlying mechanisms of obesity that may result in an increased risk of CC-RCC.

## Materials and methods

*Patients*. In the current study, a total of 77 patients were enrolled, who had been histologically diagnosed with CC-RCC between December 2005 and September 2012. The patients consisted of 14 females and 63 males, with ages ranging from 17 to 85 years (mean ± standard deviation, 56.22±12.27 years).

All protocols were approved by the ethics committee of Wuxi People's Hospital Affiliated to Nanjing Medical University (Wuxi, China) prior to the initiation of the study, and the protocols conformed to the ethical guidelines of the 1975 Helsinki Declaration. Informed consent was obtained from each patient prior to surgery.

*RNA extraction*. Tumor tissue samples and corresponding adjacent normal tissues were obtained from resection surgical specimens, and were immediately dissociated into single cells using the Medimachine system (BD Biosciences, Franklin Lakes, NJ, USA). The cells were then dissolved in 1 ml Trizol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and stored at -80˚C. Total cellular RNA was extracted using the Trizol reagent following the manufacturer's protocols. The quantity and purity of RNA were determined with the Beckman DU-640 Spectrophotometer (Beckman Coulter, Inc., Brea, CA, USA).

*Reverse transcription reaction*. Total RNA (2 $\mu$g) and 200 ng random primer (Sangon Biotech Co., Ltd., Shanghai, China) were added to 15 $\mu$l diethylpyrocarbonate (DEPC) treated with $H_2O$; this was subsequently incubated at 65˚C for 5 min and then cooled rapidly on ice. Following this, 2.0 $\mu$l DEPC $H_2O$, 5.0 $\mu$l 5X RT buffer (containing 375 mM/l KCl, 250 mM/l Tris-HCl, 3 mM/l $MgCl_2$ and 50 mM/l dithiothreitol), 1.25 $\mu$l 10 mM/l deoxynucleoside triphosphate (dNTP; Sangon Biotech Co., Ltd.), 0.75 $\mu$l 40 U/$\mu$l RNAsin (Sangon Biotech Co., Ltd.) and 1.0$\mu$l 200 U/$\mu$l MMLV Reverse Transcriptase (Promega Corporation, Madison, WI, USA) were added, centrifuged at 1,000 x *g* for 30 sec at 4˚C and incubated at 37˚C for 60 min, and finally stored at -20˚C.

*Gene expression analysis using RT-qPCR*. RT-qPCR was performed using the LightCycler® 480 Real-Time PCR system (Roche Diagnostics, Basel, Switzerland). The primers utilized during PCR are presented in Table I. PCR was performed using a final volume of 20 $\mu$l, which contained 2 $\mu$l 25 mM/l $MgCl_2$, 10 mM/$\mu$l sense and antisense primers (1.0 $\mu$l each), 0.4 $\mu$l 10 mmol/l dNTP, 1.0 $\mu$l EvaGreen® dye (Biotium Inc., Hayward, CA, USA), 2.0 $\mu$l 5X PCR buffer (Promega Corporation), 2.0 $\mu$l complementary DNA, 0.5 units Taq DNA Polymerase buffer (Promega Corporation) and up to 20 $\mu$l $H_2O$. Following initial denaturation at 94˚C for 2 min, a total of 40 cycles were performed. Each cycle consisted of denaturation at 94˚C for 5 sec, annealing at 58˚C for 20 sec and elongation at 72˚C for 20 sec, followed by a single fluorescence measurement. Melting curve analyses of the amplified products were performed to confirm the specificity of qPCR assay. Samples were normalized using the housekeeping gene GAPDH.

*Affymetrix microarray analysis*. The gene expression profile of GSE6344 was downloaded from the National Center for Biotechnology Information GEO database, which is based on the Affymetrix Human Genome U133A Array platform (Affymetrix, Inc., Santa Clara, CA, USA). A total of 20 samples, including 10 CC-RCC and 10 adjacent normal tissue specimens, were available for GSE6344 microarray

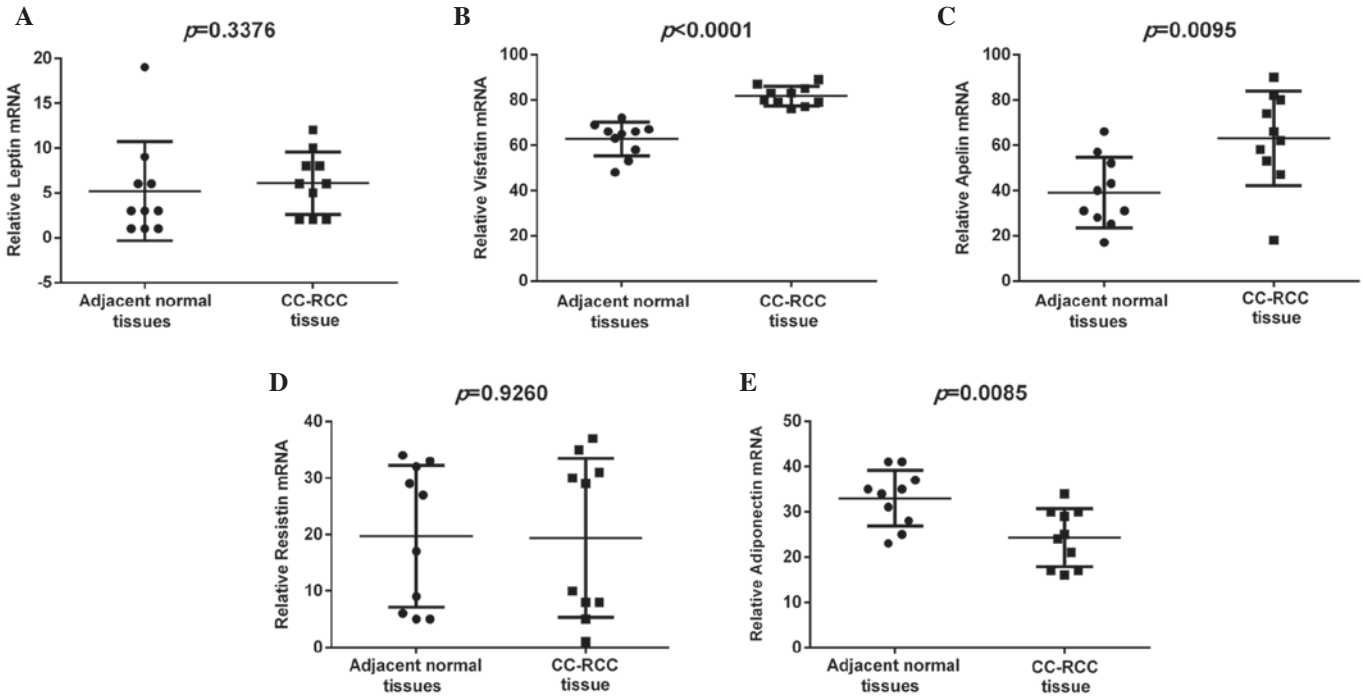


Figure 1. Analysis of (A) leptin, (B) visfatin, (C) apelin, (D) resistin and (E) adiponectin mRNA expression profiles in CC-RCC and adjacent normal tissues in the GSE6344 dataset. CC-RCC, clear cell renal cell carcinoma.

analysis. The original mRNA expression profile was standardized using rank value, and the relative expression levels of adipokine genes were analyzed.

*Statistical analysis*. Changes in adipokine genes expression between CC-RCC and adjacent normal tissues were determined by the comparative ΔΔCq method (18), using GAPDH as an internal reference. ΔΔCq = ΔCq (CC-RCC group) - ΔCq (normal group) for RNA samples. Quantification cycle (Cq) is defined as an index of the number of cycles required for the fluorescent signal to cross the threshold. ΔCq represents the difference in Cq values derived from the target gene (in each sample assayed) and the GAPDH gene, while ΔΔCq represents the difference between the paired samples. The n-fold differential ratio was expressed as $2^{-\Delta\Delta Cq}$. Comparisons between the samples (CC-RCC vs. adjacent normal tissue) were performed using a one-way analysis of variance. All statistical data were analyzed using SPSS software v15.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was used to indicate a statistically significant difference. As compared with the adjacent normal tissue, genes in the CC-RCC tissue with statistically significant differences at P<0.05 and a fold change of ≥1.5 were considered as upregulated, whereas those with P<0.05 with a fold change of ≤0.75 were considered as downregulated. Comparisons between the samples (CC-RCC vs. adjacent normal tissue) in the GSE6344 dataset were performed using a non-parametric Mann-Whitney U test.

## Results

*Expression of leptin, visfatin, apelin, resistin and adiponectin mRNA in CC-RCC with Affymetrix microarray analysis*. Analysis of leptin, visfatin, apelin, resistin and adiponectin relative expression was performed between the CC-RCC and adjacent normal tissues from the GSE6344 microarray dataset (Fig. 1). The GSE6344 dataset demonstrated that the expression of visfatin and apelin was upregulated (P<0.0001 and P<0.01, respectively), whilst adiponectin was downregulated (P<0.001) in the CC-RCC tissues compared with the adjacent normal tissues, relative to GAPDH. However, the expression of leptin and resistin exhibited no significant difference between the CC-RCC and adjacent normal tissues (P>0.05).

*Expression of leptin, visfatin, apelin, resistin and adiponectin mRNA in CC-RCC and adjacent normal tissue samples with RT-qPCR*. RT-qPCR was used to confirm the results of gene microarray for the selected adipokine genes. Table II presents the mRNA expression of leptin, visfatin, apelin, resistin and adiponectin between the CC-RCC and adjacent normal tissues. The data demonstrated that visfatin and resistin gene expression was upregulated in the CC-RCC tissues (P<0.01 and P<0.05, respectively). However, the mRNA expression level of leptin and adiponectin in adjacent normal tissue was higher than that in the cancer tissue (P<0.01). The data also indicated that the expression of apelin was not significantly different between the CC-RCC and adjacent normal tissues (P>0.05).

## Discussion

Although obesity is recognized as a potent risk factor for RCC development (19), the mechanism through which it functions to increase RCC risk is unclear. Recently, adipokines have been thoroughly investigated as novel biomarkers that may indicate cancer risk. Adipokines, derived from adipose tissue, serve roles in lipid and glucose metabolism, regulation of energy balance and inflammatory processes (20). Adipokines are understood to be involved in certain obesity-associated forms of cancer, and serve a key role in tumorigenesis and

Table II. mRNA expression profiles of adipokine in 77 patients with CC-RCC, acquired through reverse transcription-quantitative polymerase chain reaction.

| Gene | CC-RCC tissue, mean ± SD | Adjacent normal tissue, mean ± SD | $2^{-\Delta\Delta Cq}$ |
|---|---|---|---|
| Leptin[a] | 30.61±1.62 | 30.63±2.46 | 0.37 |
| Resistin[b] | 30.76±2.05 | 32.90±1.31 | 1.62 |
| Visfatin[a] | 22.73±0.88 | 25.24±1.07 | 2.09 |
| Ampelin | 28.31±1.61 | 30.02±2.02 | 1.20 |
| Adiponectin[a] | 31.57±1.99 | 31.55±2.64 | 0.37 |
| GAPDH | 20.48±1.18 | 21.92±0.80 | |

All results are expressed as the mean ± SD of Cq. [a]P<0.01 and [b]P<0.05, CC-RCC vs. adjacent normal tissues. CC-RCC, clear cell renal cell carcinoma; SD, standard deviation.

prognosis (21,22). To the best of our knowledge, the current study is the first to identify a difference in visfatin and apelin expression levels in CC-RCC and control tissues. Furthermore, several studies have reported a difference in leptin, resistin and adiponectin serum levels in CC-RCC tissues (23,24). However, the physiological role of leptin and adiponectin in CC-RCC remains controversial. Therefore, in the present study, the mRNA expression variation of visfatin, resistin, apelin, leptin and adiponectin was investigated in CC-RCC.

Leptin is a multifunctional peptide hormone that regulates energy expenditure (25), promotes proliferation (26) and angiogenesis (27), and inhibits cell apoptosis (28). Studies have demonstrated that leptin levels are higher in lung cancer (15), breast cancer (29) and RCC (30), whilst other studies have reported that serum leptin levels are inversely associated with RCC risk (23) or are not significantly associated with RCC (24,31). These contradictory results, and the serum level features, suggest that further investigation is required. The microarray analysis from the present study demonstrated that the expression of leptin exhibited no significant difference between the CC-RCC and adjacent normal tissues. However, it was also identified that the mRNA expression level of leptin in the CC-RCC tissues was significantly lower than that in the adjacent normal tissues with RT-qPCR.

Visfatin is an adipokine associated with obesity and glucose regulation. Visfatin was originally identified as a cytokine and was termed pre-B cell-enhancing factor, also known as Nampt (32). The adipokine possesses nicotinamide adenine dinucleotide biosynthetic activity and regulates mammalian cell growth and apoptosis (33); it is also known to promote novel blood vessel formation and migration (34). The function of visfatin in carcinogenesis and as a chemotherapeutic target has gained the increasing attention of researchers. Studies have reported that the expression of visfatin is correlated with various types of cancer, including breast (35), colorectal (36) and gastric (37) cancer. However, to the best of our knowledge, the association between visfatin and CC-RCC has not been confirmed until now. In the present study, the GSE6344 dataset demonstrated that the expression of visfatin was upregulated in the CC-RCC tissues compared to the adjacent normal tissues. The RT-qPCR data of 77 CC-RCC patients also indicated that visfatin gene expression was upregulated.

Apelin, a recently described adipokine, is a mitogenic factor expressed in endothelial cells (38). Apelin is involved in the process of cell proliferation (39) and stimulates cancer angiogenesis (40). A case-control study observed that serum apelin levels increased significantly in patients with gastro-esophageal cancer (41). However, there is no epidemiological data regarding the association between apelin and CC-RCC risk. The GSE6344 dataset demonstrates that the expression of apelin was upregulated in the CC-RCC tissues compared to the adjacent normal tissues. However, the RT-qPCR data indicated that the expression of apelin was not significantly different between the CC-RCC and adjacent normal tissues (P>0.05).

Resistin is a member of the recently identified family of cysteine-rich proteins; it is the primary product of macrophages that infiltrates into adipose tissue (42), and has been associated with adiposity, inflammation and insulin resistance (43). Resistin serum concentrations have been observed to be significantly higher in patients with cancer than in controls (41,44). However, one study has reported that circulating levels of resistin are not associated with RCC (24). In the present study, the GSE6344 dataset demonstrated that the expression of resistin exhibited no significant difference between the CC-RCC and adjacent normal tissues. However, the RT-qPCR data indicated that the mRNA expression level of resistin in the CC-RCC tissues was significantly higher than that in the adjacent normal tissues.

Adiponectin is a circulating adipokine secreted by mature adipocytes. The circulating level of adiponectin is inversely associated with insulin resistance and obesity (45). Abundant evidence indicates that adiponectin suppresses the growth of cancer cells and reduces the risk of cancer (14,46). Studies have reported that serum adiponectin levels are inversely associated with RCC, and breast and colon cancer (47,48). While the preponderance of evidence suggests that an inverse association exists between adiponectin and malignancy, another study reported that higher levels of serum adiponectin were associated with RCC risk, specifically among African-American males (31). Enhanced adiponectin serum concentrations appear to indicate inflammatory status and advanced stages of malignancy (49,50). However, all aforementioned studies were confined to studying adiponectin levels within the circulatory system. In the present study, the RT-qPCR data indicated that

the mRNA expression level of adiponectin in the CC-RCC tissues was significantly lower than that of the adjacent normal tissues, which was consistent with the GSE6344 dataset.

Microarray technology provides a wide range of information regarding molecules that are associated with disease pathogenesis. However, the high cost of detection limits the application in larger samples, which may lead to reduced sensitivity. RT-qPCR is the gold standard for verification of microarray data. In the present study, the data regarding visfatin and adiponectin is consistent between microarray and RT-qPCR. However, the data concerning leptin, resistin and apelin from RT-qPCR cannot verify the results from the GSE6344 microarray dataset. This may be attributed to the small sample size of the GSE6344 microarray. Furthermore, it was demonstrated in another study that the expression level of leptin varies in different ethnicities (31), which may lead to the contradictory results.

In conclusion, such findings suggest that visfatin and resistin are high-risk factors for the development of CC-RCC. By contrast, leptin and adiponectin are inversely associated with CC-RCC risk. The present study may provide novel information concerning the role of adipokines in CC-RCC risk. Due to the relatively small number of patients and contradictory results from microarray data, further studies are required to investigate the etiological significance of adipokine levels in CC-RCC risk.

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