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Resistin Effect on HCT-116 Colorectal Cancer Cells Proliferation and Telomerase Expression

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Background: The plasma concentration of resistin, an adipokine, and its expression are increased significantly in patients with colorectal cancer that suggests its possible role in cancer progression.
Objectives: The aim of this study was to investigate resistin presence and its effect on cell proliferation and telomerase gene expression in colorectal cancer cell line (HCT-116).
Materials and Methods: Resistin and telomerase gene expression in cells were investigated by RT-PCR at mRNA level and the results were confirmed by ELISA method at protein level. The HCT-116 cells were stimulated with resistin. XTT assay was performed to examine cell proliferation. Telomerase gene expression was assessed to increase our knowledge about the possible underlying mechanism on resistin treated cells. The data were obtained from ELISA method, XTT assay, living cells number, and expression level of gene. The statistical analysis was performed with SPSS version15.
Results: Resistin was expressed in neither cell lysate nor supernatant. Colorectal cancer cells proliferation was induced by resistin after 24 and 48 hours in a dose-dependent manner (mean ± SD, 0.82 ± 0.0095; $P < 0.05$). Human telomerase reverse transcriptase (h TERT) gene expression was increased after treating cells with resistin.
Conclusions: According to our results, HCT-116 cell is not the main source of resistin in colorectal cancer and high-level plasma concentrations of resistin in colorectal cancer might be due to the increased inflammatory cells in pro-inflammatory state of cancer. Interestingly, resistin promoted colorectal cancer growth via enhancing telomerase expression in a paracrine manner.

*Keywords:*Resistin; Telomerase; TERT protein, human; Colorectal Neoplasms

1. Background

Obesity is associated with numerous diseases including cardiovascular diseases, diabetes mellitus, hypertension, and cancer. The association of colorectal cancer (CRC) with obesity and adipose tissue hormones such as resistin was reported previously. High levels of resistin in CRC might be related to its overproduction by adipose tissue or cancerous cells. If cancerous cells produce resistin, it might have a protective response against cancer or promote the cancer (1-10). CRC is the third most common malignant tumor in the world; it is estimated that 1.23 million people have CRC, which is equal to 9.7% of the total cancers). Moreover, it is the third most common cause of cancer death worldwide after lung and prostate cancers among men and after lung and breast cancers among women. Many epidemiological studies have shown the association between abdominal obesity and increased CRC risk, which seems to be stronger in obese men than obese women (11-13). Many studies have been assessed adipocytokines (including leptin, adiponectin, tumor necrosis factor-α, resistin, visfatin, and interleukin-6), which are active polypeptides mainly produced from adipose tissue particularly visceral abdominal fat, due to their association with malignant tumors such as CRC. Adipokines secretion might be altered with increasing body mass index (BMI) and accumulation of adipose tissue, which might increase the risk of cancer (14). Leptin has been shown to regulate proliferation and apoptosis of CRC cell lines through the PI3K/Akt/mTOR signaling pathway; however, adiponectin inhibits CRC growth by the AMPK/mTOR pathway (15, 16). While there are some

Implication for health policy/practice/research/medical education:
Several researchers have reported the association of obesity and cancer, however, the underlying molecular mechanism is not clear yet. The results of this study showed that adipose tissue might mediate this association via adipokines secretion (adipose tissue hormones). It can justify further studies that assess anti-adipokines medications in cancer treatment.

evidences that partly reveal the role of adiponectin and leptin in colorectal carcinogenesis, resistin effect on CRC cell progression and the underlying mechanism are still unclear. Resistin, a member of cysteine-rich proteins family called "Resistin-like molecules" (RELMs), has been shown to play an important regulatory role in glucose homeostasis, insulin sensitivity, and inflammatory diseases (17, 18). Recently resistin has been found to be associated with the risk of CRC, as its serum level in patients with CRC was higher than controls and gradually increased with cancer progression (19-22). A previous study showed that resistin was significantly expressed heterogeneously in CRC tissues (19).

2. Objectives

In the present study, we investigated the presence of resistin in CRC cells and its effect on cancer cell proliferation and expression of telomerase gene, which has a key role in cellular immortality and tumorigenesis and is activated in many malignancies, especially in CRC (23-26). We aimed to clarify the possible molecular link between high resistin level and increased CRC risk.

3. Materials and Methods

3.1. Cell Culture, Reagents, and Treatments

Proposal of this ex vivo experimental study was approved in the ethical committee of Research Institute for Endocrine Sciences (ERC-294-1390-07-11), Shahid Beheshti University of Medical Sciences. Human HCT-116 colorectal epithelial cancer cells were obtained from Pasture institute cell bank (IRI). High glucose DMEM medium (Invitrogen, USA), 10% fetal bovine serum, penicillin/streptomycin were purchased from Biochem GmbH (Biochem, Germany, http://www.biochem.de/). The cells were seeded at the concentration of 5×10^3 cells/well in 96-well culture plates and allowed to attach in an incubator overnight. The complete serum was then replaced with serum-free medium for 24 h to allow for cell cycle synchronization. The medium was then replaced with serum-free medium containing different doses of human recombinant resistin protein (Syd Labs, Japan) and cells were incubated for 24 and 48h for cell proliferation assay. For gene expression assay, 7×10^5 cells in 25-T culture flasks were seeded and incubated overnight. The total medium was then replaced with serum-free medium for 24h and 48h to avoid any disturbing factor for resistin detection by ELISA.

3.2. Cell Preparation and ELISA

Supernatant of HCT-116 cells was obtained from 24 and 48 h serum-free cell flasks. One mL cell lysis buffer1X (containing 20 mM Tris-HCl (pH 7.5), 150 mMol NaCl, one mMol Na2EDTA, one mMol EGTA, 1% Triton, 2.5 mMol Sodium pyrophosphate, one mMol beta-glycerophosphate, 1mMol Na3VO4, 1 μg/mL leupeptin), and 10μL Protease Inhibitor Cocktail (Gold biotechnology, USA) were added following the cell harvesting by trypsin-EDTA and centrifuging. Thereafter, cells were vortexed and centrifuged for five minutes at 4°C. Resistin presence in cell lysate and supernatants was investigated by human resistin ELISA kit (Koma Biotech, Korea ELISA kit, sensitivity 0.01 ng/mL, inter and intra-assay coefficient of variation < 10%).

3.3. Cell Proliferation Assay

Growing cells were seeded in 96-well culture plates and incubated with various concentrations of rh-Resistin for 24 and 48 h. Cell number and viability were assessed using a hemocytometer and trypan blue staining. Cell proliferation was analyzed using the XTT assay kit (Biotium, USA). Briefly, 50μL activated XTT was added per well and the cells were incubated for 4h, 37˚C, 5% CO2. Formazan production by viable cells was assessed at 510 nm with a 96-well plate reader Sunrise (TECAN, Austria).

3.4. RNA Extraction, cDNA Synthesis and RT-PCR

Total RNA was isolated from the cells using RNX plus solution (CinnaGen INC, IRI). Briefly, after trypsin/EDTA harvesting, centrifugation at 1000g for 10 min at 4°C, 1 mL ice cold guanidine/phenol solution (RNX-Plus) was added to a 2 mL tube containing cell pellet and incubated at RT, for 5 min. Then, 200μL of chloroform was added and shacked rigorously for 15s and incubated on ice for 5 min and centrifuged (12000 g, 15 min). The aqueous phase was transferred to a sterile RNase-free tube. The total RNA was precipitated by adding 0.5 mL isopropyl alcohol and incubating for 15 min at RT. The pellet including total RNA was washed using 75% ethanol and centrifuged at 7500g for 8 min. After the ethanol was dried, the RNA pellet was dissolved in DEPC treated water. RNA yield and purity were assessed by spectrophotometer. To confirm the absence of RNA degradation, RNA electrophoresis was performed on a 1.5% agarose gel containing ethidium bromide. Total RNA (3 μg) was subjected to reverse transcription by the first strand cDNA synthesis kit (Fermentas, Lithuania).

To study the resistin expression, the RT reaction aliquot was amplified under gradient conditions in a total volume of 50μL using forward primer 5´-TGGAAGAAGCCATCAATGAGAGG-3´ and reverse primer 5´-CGCACTGGCAGTGACATGTG-3´. GAPDH, as a housekeeping gene was amplified by using forward 5´-CAAGGTCATCCATGACAACTTTG-3´ and reverse 5´-GTCCACCACCCTGTTGCTGTAG-3´.

3.5. Real-Time PCR

The cDNA of treated cells was amplified by RT-PCR using Maxima SYBR Green/ROXqpcr Master Mix (Fermentas, Vilnius, Lithuania) and the Rotor-GeneTM 6000 system (Corbett Research, Australia) with the following primers: forward 5´-CCGCCTGAGCTGTACTTTGT-3´, reverse 5´-CAG-

GTGAGCCACGAACTGT-3′ for hTERT gene and forward 5′-CAAGGTCATCCATGACAACTTTG-3′ and reverse primer 5′-GTCCACCACCCTGTTGCTGTAG-3′ for GAPDH primer as housekeeping gene. Real-time PCR reactions (25μL total volume containing 5pmol primer, 12.5μL SYBR green master mix, and 2.5μL cDNA) were performed with the following condition: initial denaturation at 95°C for 10 min, denaturation at 95°C for 15s, annealing at 60°C for 30s and extension at 72°C for 30s for about 40 cycles. Finally, amplicons were assessed by melting curve analysis of 70°C to 95°C. The quality of real-time PCR reactions was controlled by running standard samples as triplicate.

To examine the possible molecular link between resistin and HCT-116 cell proliferation induction, telomerase (hTERT) gene expression was investigated. Therefore, HCT-116 cells were treated with 10 ng/mL resistin for 6, 12, and 24 h. Then Real-time PCR was performed after RNA extraction and cDNA synthesis. HCT-116 cells were incubated in serum-free medium supplemented with various concentrations of recombinant human resistin in the pathophysiologic range of resistin level for 24 and 48 h to evaluate the resistin effect on CRC cell proliferation. Then XTT assay was performed to analyze the cell proliferation.

3.6. Statistical Analysis

Differences among groups were analyzed using one-way ANOVA with Dennett's multiple comparison tests. Student t-test was performed for comparison between the two groups. The statistical analysis was performed using SPSS version15.

4. Results

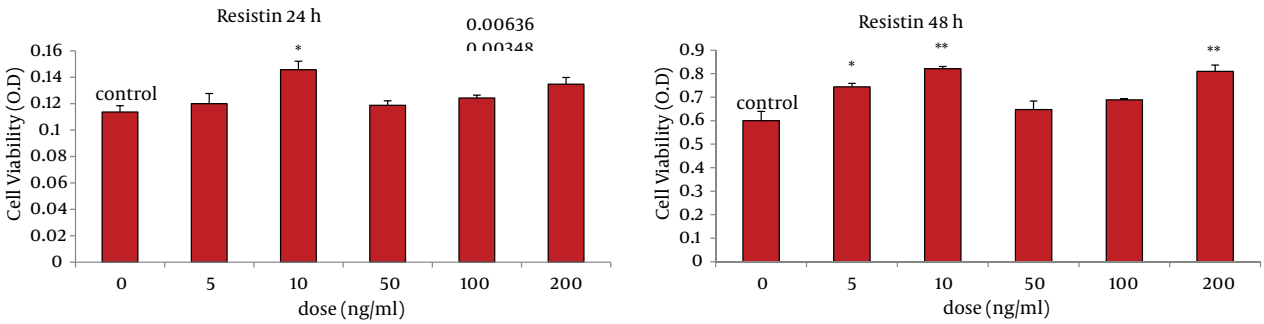
To explore resistin protein in HCT-116 cell line, ELISA was employed that could detect any resistin protein in neither cell lysate nor supernatant. Accordingly, resistin mRNA in the colorectal cell line was not detectable by RT-PCR. The results of incubation of HCT-116 with different amounts of resistin and its effect on cell proliferation and viability are presented in Table 1. Viability of HCT-116 cells treated with 10 ng/mL of resistin after 24 h and 5, 10 and 200 ng/mL after 48 h was enhanced in a dose-dependent manner, in which the most effective dosage was 10 ng/mL (Figure 1).

Viable HCT-16 cells counting were performed after plating and treatment with resistin in the same manner of XTT assay to confirm the resistin effect on CRC cell proliferation. Control and resistin treated cells were compared regarding the number of live cells under serum-free conditions using a trypan blue exclusion assay. As shown in Figure 2, live cells number was higher in resistin treated wells than control wells. The data from both methods showed an increase in growth of resistin-treated HCT-116 cells in comparison to control HCT-116 cells. The results of HCT-116 cells treatment with 10 ng/mL resistin in different times, on telomerase expression Showed increase telomerase gene expression that telomerase gene was expressed significantly more in resistin-treated HCT-116 cells than control cells after 24 h (Figure 3).

Table 1. Results of Resistin Treatment on Cells for 24 and 48 Hours

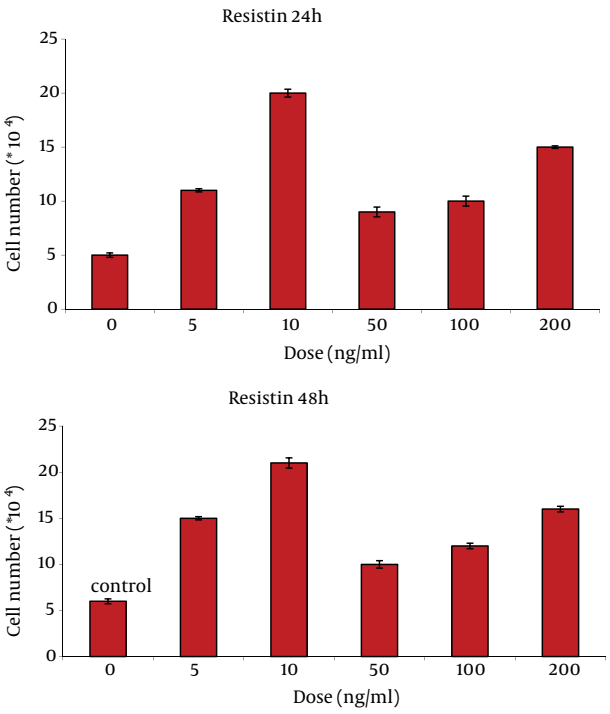
Resistin 24h XTT Results						
dose	0	5	10	50	100	200
mean ± SD	0.11±0.0048	0.12±0.0076	0.15 ± 0.0064	0.12 ± 0.0035	0.12 ± 0.0020	0.13 ± 0.0052
P value			<0.05			
Resistin 48h XTT Results						
dose	0	5	10	50	100	200
mean ± SD	0.60 ± 0.0400	0.74 ± 0.0145	0.82 ± 0.0095	0.65 ± 0.0365	0.69 ± 0.0025	0.81 ± 0.0275
P value		< 0.05	< 0.01			<0.01

Figure 1. Resistin Induces Colorectal Cancer Cell Proliferation



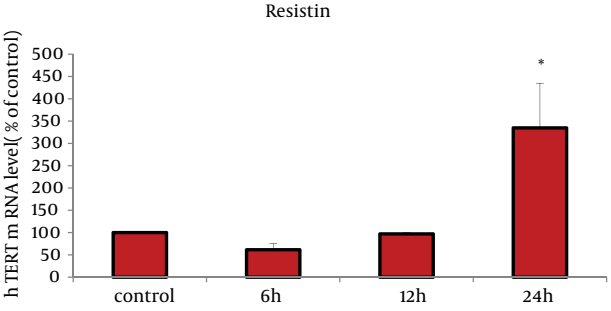
We performed XTT assay to evaluate the resistin effect on HCT-116 cell proliferation, after triplicate cell plating, treatment, and incubation. All values are expressed as the mean ± SEM of three wells per group. *P < 0.05; **P < 0.01 for resistin vs. control.

Figure 2. Resistin Effect on the Number of Colorectal Cancer Cell



Cell counting was performed after triplicate plating and treating cells with the same concentration of resistin in XTT assay. The most effective dose of resistin on cell viability of HCT-116 was 10 ng/mL. Data are shown as the mean ± SEM.

Figure 3. Resistin Effect on hTERT mRNA in Colorectal Cancer Cell Line



HCT-116 cells were plated, treated with resistin at 10 ng/mL, and incubated for 6, 12, and 24 h to examine telomerase gene expression by real-time PCR. Three independent experiments were performed. Data are represented as the mean ± SEM.

5. Discussion

Although increase of adipokines levels in obesity is closely associated with many malignancies including CRC (27-30), a recent study has revealed that resistin plasma level was elevated in patients with CRC regardless of BMI. Thus, resistin has been suggested to be a good biomarker for colorectal malignant potential and stage progression (10). Although there are a few evidences concerning this adipokine expression in CRC, its main source of secretion in patients with CRC is still unknown. A previous immunohistochemistry study on CRC tissue has shown that expression of resistin was increased in the cancer tissue as a heterogeneously diffuse weak pattern. The CD68+ cells, identified as macrophages found predominantly in cancer tissues, seem to secrete resistin adipokine. No association between resistin level in cancer tissue and plasma levels or clinical characteristics such as Dukes' stage, location, and gender has been reported (31). In the present study, we assessed the CRC cell line ability to express resistin mRNA and protein. The results showed that CRC cell HCT-116 was not the source of resistin production in CRC. Interestingly, it has been reported that while resistin protein synthesis is restricted to adipocytes in rodents, peripheral blood mononuclear cells (PBMCs), macrophages, and bone marrow cells are the main origins of resistin production in humans, while human adipocytes can express resistin only moderately (32, 33). Moreover, resistin, also called FIZZ3 (found in inflammatory zone), has been known as a pro-inflammatory factor in many chronic inflammatory and autoimmune diseases including atherosclerosis, inflammatory bowel disease, chronic pancreatitis, systemic lupus erythematous (SLE), renal disease, rheumatoid arthritis, and cancers (17, 34-38).

Preceding in vitro study demonstrated that stimulating PBMCs with endotoxin or pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α enhanced resistin mRNA expression (32). On the other hand, association between resistin plasma level, regardless of BMI, and CRP (C - reactive protein) as an inflammatory phase protein factor in inflammatory related disorders including cancers, especially CRC, has been widely reported (19, 39, 40). While resistin expression in prostate cancer tissue and cell lines are detectable (41), our result showed that HCT-116 CRC cell had no potential in resistin synthesis. Taken together, it seems reasonable to consider monocytes and other inflammatory cells, but not the CRC cells, as the major sources for high resistin expression in CRC tissue and increased plasma levels due to chronic low-grade inflammatory state in CRC. Since the resistin receptor is still not identified, its molecular pathway on cells is not clear and deserves more investigations. Resistin has been suggested to link obesity and type II diabetes mellitus. Previous studies have indicated the role of resistin in the regulation of glucose homeostasis as it reduces insulin sensitivity in adipocytes, skeletal muscles, and hepatocytes by impairing insulin-mediated glucose transport (34, 42). In human studies, resistin circulating levels varies from unchanged to increase in obesity or type II diabetes mellitus. In a cross-sectional study, the mean of serum resistin levels in obese patients was significantly higher in obese subjects than in slim ones (24.59 ng/mL vs.12.83 ng/mL). Moreover, resistin levels were increased significantly in patients with type II diabetes mellitus in comparison with subjects without diabetic mellitus (24.7 ng/mL vs.15

ng/mL) (43-45). In a population study, the resistin serum level was 4.5 ng/mL in patients with CRC in contrast to 3.1 ng/mL in control healthy patients (21). Therefore, in the present study, 5ng/mL to 200ng/mL concentrations was used for covering pathophysiological range of resistin.

Recently resistin has been suggested to owe cancer-promoting effect; hence, we attempted to assess its proliferative ability on CRC cell line HCT-116. The result of XTT assay showed that resistin could increase CRC cells proliferation at most effective dose of 10 ng/mL, which is in conformity with serum resistin level in patients with CRC. In agreement with our findings, there are a few studies showing resistin stimulatory effect on prostate cancer and smooth muscle cell proliferation, and its inductive role on human endothelial cell proliferation and migration via ERK1/2, p38 and PI3K signaling pathways (41, 46, 47). However, it has been reported that resistin induces rat insulinoma cell RINm5F apoptosis (35). All these results suggest that although resistin has a promoting role on cell proliferation, especially on cancer cells, its function is cell type-specific.

Telomerase, a ribonucleoprotein complex, synthesizes DNA onto the ends of chromosomes, providing the genomic integrity and stability. There are two essential genes constitute the cellular RNA-containing enzyme complex, TERC and TERT. Interestingly, only hTERT is sufficient to activate telomerase and bypass senescence, which led to cell immortalization. Therefore, in many cancers including CRC, telomerase is unregulated or reactivated and is considered as a potential target for treatment. A high level of telomerase activity has been suggested as the prognostic indicator in patients with CRC regardless of disease stage and the Dukes' classification (48, 49). In previous studies, leptin, as an adipokine related to obesity, has been demonstrated to upregulate telomerase activity and hTERT mRNA in MCF-7 breast cancer cells (50). Furthermore, leptin has been reported as a modulator of telomerase reverse transcriptase capable of prompting hepatocellular carcinoma development (51), while there is little evidence about resistin effect on telomerase expression. Therefore, we evaluated hTERT mRNA level in HCT-116 cells after treating with resistin for 6, 12, and 24 h and observed that this adipokine could enhance hTERT expression after 24 h. This result suggests that enhancement of telomerase gene expression might be the molecular linkage between resistin and CRC cell progression and malignant phenotype. However, more in vivo investigations are necessary to confirm the resistin role in CRC.

In conclusion, our data revealed that CRC cell line HCT-116 does not have the potential for resistin secretion, while monocytes and other inflammatory cells in cancerous tissues are the major sources of elevated resistin plasma level in patients with CRC. For the first time, our study showed that resistin can affect CRC proliferation predominantly in a paracrine and probably slightly in an endocrine (produced by adipocytes) manner. Moreover, it contributes to CRC development by elevating the telomerase gene expression in cancer cells and promotes them into phenotypes that are more aggressive. Therefore, resistin can be considered not only as a CRC biomarker, but also as a target molecule in treatment of patients with CRC. Ex vivo study for encountering a define cell line and controlling conditions is a powerful method for data production; however, there are many cell lines related to CRC. The limitation of this study was to focus on a common cell line in this area and we suggest repeating these experiments on other related cell lines.

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Authors' Contribution

Sara Ghaemmaghami, prepared the manuscript. Mehdi Hedayati and Seyed Mojtaba Mohaddes designed the study and supervised all the steps. Masumeh Gorgian Mohammadi and Amin Barkhordari assisted in laboratory assessment.

Financial Disclosure

The authors did not have any actual or potential conflict of interest for the work submitted.

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