

Rosiglitazone suppresses gastric carcinogenesis by up-regulating HCaRG expression

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Abstract. Our previous study demonstrated that PPAR γ ligand rosiglitazone prevents gastric carcinogenesis in rats induced by chemical carcinogen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). In this study, we attempted to identify novel anti-cancer mechanisms of rosiglitazone. By examining the gene expression profiles of MNNG-induced and rosiglitazone-treated gastric cancer with Uniset Rat I Bioarray microarray, we identified a gene that showed prominent responses in the rosiglitazone-treated group. The hypertension-related, calcium-regulated gene (HCaRG) was significantly up-regulated in rat gastric carcinoma of the rosiglitazone-treated group when compared with the MNNG group. We further examined HCaRG expression in human gastric cancer and found that the expression of HCaRG was down-regulated in human gastric cancerous tissue. Rosiglitazone markedly induced the expression of HCaRG in the AGS cell line. The up-regulation of HCaRG may be one of the mechanisms underlying the chemopreventive effect of rosiglitazone in gastric cancer.

Introduction

Peroxisome proliferator-activated receptors (PPARs) are a family of nuclear receptors comprising of the isoforms PPAR α , PPAR γ and PPAR β (1). In particular, PPAR γ has been extensively evaluated as a target for cancer therapy in preclinical models (2). PPAR γ is activated by its ligands which are divided into natural and synthesized. The thiazolidinedione (TZD) family is one of the synthetic PPAR γ agonists which include rosiglitazone, pioglitazone and

troglitazone. Rosiglitazone binds PPAR γ with a higher affinity than troglitazone or pioglitazone. Recent studies found that TZDs have significant anti-cancer effects on various human malignant tumor cells *in vitro* including lung, gastric, breast, liver, colorectal and pancreatic cancer types (3-9). Increasing evidence shows that PPAR γ ligands exert their anti-neoplastic effect by inducing cancer cell growth arrest, differentiation, apoptosis, or by inhibiting neovascularization of cancer, and suppressing tumor migration (10-12). PPAR γ ligands also regulate the expression of cytokines, adhesion molecules, and other signal transductional molecules such as NF- κ B (2,13). PPAR γ has strong anti-inflammatory and antiangiogenic effects, extending the repertoire of potential targets of PPAR γ ligands beyond autonomous cell mechanisms of cancer (10). In this regard, we have previously shown that the carriage of polymorphism in the PPAR γ gene increases susceptibility to gastric cancer development in *H. pylori*-infected individuals (14).

Our recent study showed that rosiglitazone prevents gastric carcinogenesis in rats induced by MNNG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) (15). In this study, we used oligonucleotide microarray to determine the gene expression profiles of gastric cancer in rats treated with rosiglitazone. Among the genes up-regulated by rosiglitazone, HCaRG (hypertension-related, calcium-regulated gene) was found to have markedly increased in rosiglitazone-treated cancer. HCaRG is a novel calcium-regulated gene and its expression generally decreases in tumors and cancer cell lines (16). We further determined the effects of the PPAR γ ligand on HCaRG expression in human gastric cancer.

Materials and methods

Animals. We used the rat gastric cancer model induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) as described previously (17). Briefly, six-week-old male Wistar rats, ~50 g in weight, were obtained from the Laboratory Animal Centre of the Sun Yat-Sen University. The rats were kept at 21°C, humidity 50% and with a 12-h light-dark cycle. Rats had free access to regular chow pellets and drinking water. There was one acclimatization week prior to starting this experiment. The study protocol was approved by the animal ethics committee

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of the Sun Yat-Sen University and the experiments were performed in compliance with local regulations.

MNNG-induced gastric cancer. MNNG (Fluka, Germany) at 100 $\mu\text{g/ml}$ was prepared in distilled water three times per week. The MNNG solution was protected from light and given *ad libitum* to rats through drinking water. In addition to MNNG, 1 ml of 10% sodium chloride was given to rats weekly by oral gavage in the first six weeks to enhance the development of gastric cancer. Rats were either treated with MNNG alone or in combination with rosiglitazone at 0.8 mg/kg/d by oral gavage for a total of 40 weeks. At the end of the 40-week rosiglitazone treatment, the two groups of rats were sacrificed and gastric tissues were snap-frozen in liquid nitrogen for further analysis.

Gene microarray analysis. Three pairs of rat gastric tissues (tumor and adjacent normal) in the MNNG group and two pairs of rat gastric tissues (tumor and adjacent normal) in the rosiglitazone-treated group were available for gene microarray analysis. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The CodeLink cDNA expression assay kit (Amersham Biosciences, Piscataway, NJ, USA) was used. Reverse transcription, second-strand cDNA synthesis and biotin-labeled amplification of RNA were performed according to standard protocol. Using UniSet Rat I Bioarray (Amersham), the tissues were hybridized, washed, and scanned according to the manufacturer's standard protocols. Images were captured on an Axon GenePix scanner (Arlington, TX, USA) using CodeLink expression scanning software and were analyzed using CodeLink expression analysis software. The data were analyzed using the significance analysis of microarrays (SAM) software and fold-difference filtering.

Quantitative real-time RT-PCR. Microarray results were confirmed by quantitative RT-PCR analysis to measure HCaRG mRNA expression levels in a different set of rat gastric cancer tissues. Six pairs of gastric cancer tissues in the MNNG group and 5 pairs in the rosiglitazone group were tested.

After RNA isolation, cDNA was synthesized from 2 μg of total RNA with oligo (dT)₁₈ primer and MMLV reverse transcriptase (Promega, Madison, WI, USA). Quantitative RT-PCR was performed by using LightCycler 1.5 (Roche, IN, USA) with LightCycler FastStart DNA Master^{PLUS} Hybridization Probes (Roche). The sequences of the primers are: rat HCaRG (forward) 5'-GAAACTTGCTAGTCGGG-3' and (reverse) 5'-ACCGGAAGTAAGACAC-3' and rat β -actin (forward) 5'-TACTGCCCTGGCTCCTAGCA-3' and (reverse) 5'-TGGACAGTGAGGCCAGGATAG-3'. Amplified fragment sizes were 314 and 101 bp for HCaRG and β -actin, respectively. Expression levels of HCaRG were normalized to rat β -actin levels.

Human gastric cancer samples. Sixteen surgically excised gastric cancer tissues were obtained. The mean age of these patients was 63 years and there were 10 males. As a control, normal gastric tissues were obtained by endoscopic biopsy from 15 dyspeptic patients (mean age 45 years; 8 males) who

were found to have no abnormality on upper gastrointestinal endoscopy. Written informed consent was obtained prior to tissue collection and the study was approved by the Human Ethics Committees of the University of Magdeburg, Germany and the Joint CUHK-NTE Clinical Research Ethics Committee of Hong Kong. Gastric tissue samples were snap-frozen in liquid nitrogen for subsequent RNA extraction or 10% buffered formalin for immunohistochemistry.

Immunohistochemistry. Formalin-fixed gastric tissues were dehydrated through graded concentrations of ethanol, embedded in paraffin, and sectioned. Sections (5 μm) were mounted on slides, cleared, hydrated, and treated with a buffered blocking solution (3% bovine serum albumin) for 15 min. Sections were then incubated with primary antibody against HCaRG (rabbit polyclonal; Proteintech Group Inc., Chicago, IL, USA) at a dilution of 1:100 at 4°C overnight. Sections were washed with PBS and co-incubated with secondary antibody (anti-rabbit IgG, 1:500 in PBS, v/v; Sigma, Spain) at room temperature for 1 h. Thereafter, sections were washed with Tris-HCl 0.05 M, pH 7.66 and incubated with a 3,3'-diaminobenzidine solution in the dark at room temperature for 10 min. Sections were washed with Tris-HCl and stained with haematoxylin according to standard protocols.

Cell cultures. The PPAR- γ agonist rosiglitazone, irreversible PPAR- γ antagonist (GW9662), was obtained from Cayman Chemical (Ann Arbor, MI, USA). The drugs were dissolved in DMSO immediately before use.

The human gastric cancer cell line AGS was obtained from ATCC (Manassas, VA, USA) and cultured in RPMI-1640 (Invitrogen) with 10% fetal bovine serum (FBS; JRH Biosciences, KS, USA) at 37°C in a humidified atmosphere of 95% room air and 5% CO₂. After 24 h, cultures of ~60% cell confluence were treated with various concentrations of rosiglitazone (0, 1, 5, 10, 20 and 50 μM) and incubated for 48 h. GW9662 (5 μM) was added to the cell cultures 6 h prior to rosiglitazone treatment.

Statistical analysis. Data were expressed as means \pm SD. Results from experiments with only two groups were analyzed by the Mann-Whitney U test and multiple groups were analyzed by one-way ANOVA to identify differences among means. A two-sided P-value of <0.05 was considered statistically significant.

Results

Overexpression of HCaRG in rosiglitazone-treated group. Using microarray analysis, 79 genes were found to be significantly up-regulated by >5-fold in rosiglitazone-treated groups as compared to the MNNG control (Table I). Among them, there was a >25-fold increase in the abundance of HCaRG transcripts in the gastric tumors of the rosiglitazone-treated group. The microarray data were further validated by using RT-qPCR in another set of rosiglitazone-treated tumors. In keeping with the microarray data, HCaRG was significantly increased in rat gastric tumors of the rosiglitazone group when compared with the MNNG group (Fig. 1).

Group	No.	HCaRG/ β -actin expression
Control	10	0.427 \pm 0.277 ^a
MNNG	20	0.091 \pm 0.122
MNNG + rosiglitazone (0.8 mg/kg)	20	0.294 \pm 0.363 ^a

Data expressed as $\bar{x} \pm SD$ (mean \pm SD). ^avs. MNNG group; $P < 0.05$.

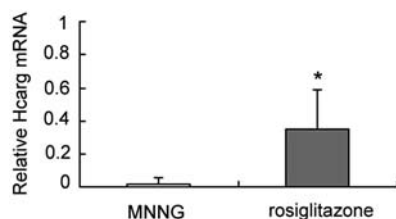


Figure 1. HCaRG expression in gastric tumor. Relatively high levels of HCaRG mRNA (normalized to β -actin) markedly increased in rat gastric carcinoma of the rosiglitazone-treated group (n=5) when compared with the MNNG group (n=6); * $P < 0.05$.

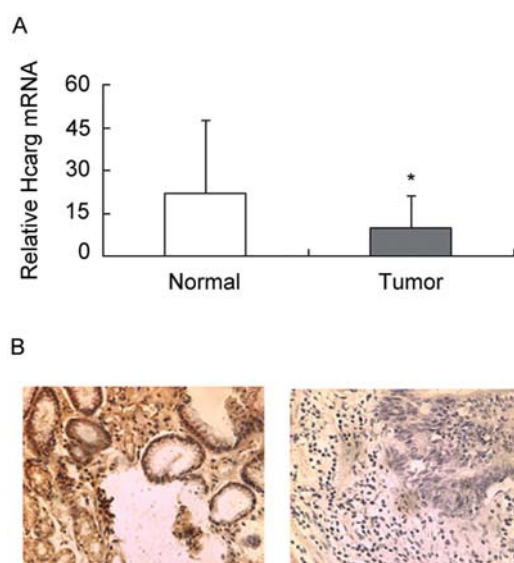


Figure 2. HCaRG expression in human gastric carcinoma and normal gastric mucosa. (A) Relatively lower levels of HCaRG mRNA (normalized to β -actin) in gastric carcinoma (n=16) and in adjacent normal gastric mucosa (n=15); * $P < 0.05$. (B) Immunohistochemistry of HCaRG expression. HCaRG immunoreactivity was mainly detected in the nucleus and cytoplasm of the normal gastric mucosa gland (left) and there was minimal expression in cancer cells (right). Magnification, x400.

We also determined the expression of HCaRG in the non-cancerous stomach of MNNG- and rosiglitazone-treated rats (Table I). The HCaRG expression was markedly reduced in the non-cancerous stomach of the MNNG group when compared to control rats not treated with MNNG ($P < 0.05$).

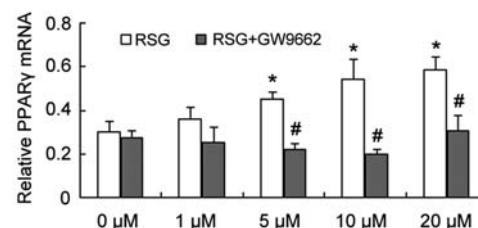


Figure 3. Expression of PPAR γ mRNA in AGS cells. PPAR γ mRNA levels were determined by using real-time RT-PCR. After exposure to rosiglitazone (RSG) for 48 h at different concentrations (0, 1, 5, 10 and 20 μ M), PPAR γ mRNA levels markedly increased. Pre-treatment with GW9662 (5 μ M) significantly reduced RSG-induced PPAR γ expression. The ratio of PPAR γ mRNA to β -actin mRNA at each time point is expressed as the mean \pm SD (n=3). (*) vs. control; $P < 0.05$ and # vs. rosiglitazone; $P < 0.05$.

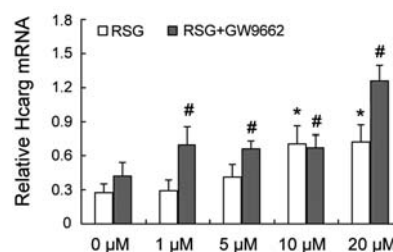


Figure 4. Expression of HCaRG mRNA in AGS cells. HCaRG mRNA levels were determined by real-time RT-PCR. After exposure to rosiglitazone (RSG) for 48 h at various concentrations (0, 1, 5, 10 and 20 μ M), there was an increase in HCaRG mRNA levels. Pre-treatment with GW9662 (5 μ M), however, did not attenuate the rosiglitazone-induced HCaRG up-regulation. The ratio of HCaRG mRNA to β -actin mRNA at each time point is expressed as the mean \pm SD (n=3); (*) or # vs. control; $P < 0.05$.

Notably, treatment with rosiglitazone induced the expression of HCaRG in the non-cancerous stomach of rat ($P < 0.05$).

Expression of HCaRG in human gastric adenocarcinoma. We determined the levels of HCaRG mRNA in paired human gastric adenocarcinoma and their adjacent normal tissues. The HCaRG level was significantly lower in tumor tissues compared with the adjacent normal gastric mucosa by RT-PCR (Fig. 2A). Similarly, HCaRG immunoreactivity was mainly detected in the nucleus and cytoplasm of normal gastric mucosa, whereas loss of HCaRG expression was observed in gastric cancer tissues by immunohistochemistry (Fig. 2B).

Rosiglitazone-induced PPAR γ and HCaRG activation in AGS human gastric cancer cells. To evaluate the effect of rosiglitazone on HCaRG expression in the human gastric cancer cell line, we examined the mRNA expression of PPAR γ and HCaRG by RT-qPCR in the human gastric cancer cell line, AGS. Exposure to rosiglitazone for 48 h produced a dose-dependent increase of PPAR γ (Fig. 3) and HCaRG (Fig. 4) mRNA expression.

Effect of antagonist on PPAR-activation and HCaRG expression level. Irreversible PPAR γ antagonist GW9662 was used to inhibit the ligand-induced PPAR γ activation. Pre-treatment with GW9662 markedly reduced rosiglitazone-

induced PPAR γ mRNA expression (Fig. 3) but did not blunt the HCaRG expression (Fig. 4).

Discussion

Gastric cancer is the second leading cause of cancer-related death in the world (18) with >1 million cases each year. The prognosis of gastric cancer is generally poor and the 5-year survival is <20% in patients with advanced cancer stage. A promising approach to reducing the risk of gastric cancer development is through chemoprevention (19).

Due to its anti-proliferative, pro-apoptotic and differentiation-promoting activities, PPAR γ has been extensively evaluated as a potential target for anti-cancer therapy in preclinical models (2). Our previous study confirmed that rosiglitazone can prevent gastric carcinogenesis in cell lines (8) and rats (15). In this study, we elucidated the anti-cancer mechanisms of rosiglitazone by using gene expression profiles of the MNNG-induced gastric cancer in rats to identify potential novel target genes. We specifically focused on genes up-regulated by rosiglitazone since PPAR γ usually acts as a transcriptional regulator of downstream target genes. Among the 79 genes that were up-regulated in rosiglitazone-treated gastric tumors, we identified the *HCaRG* gene which showed prominent responses to rosiglitazone-treated tumors as compared to tumors of the MMNG group. An increased expression of *HCaRG* mRNA was confirmed by quantitative real-time polymerase chain reaction in the same group of samples. We further showed that there was a significant reduction in *HCaRG* mRNA in human gastric cancers when compared with their adjacent normal gastric mucosa. These findings raise the possibility that *HCaRG* expression was lost in the gastric carcinogenic process.

HCaRG mRNA levels decreased in many cancerous cell lines as well as in primary glioblastoma, renal carcinoma, and hepatocellular carcinoma (16). This is the first study to demonstrate loss of *HCaRG* in human gastric cancer and the up-regulation of *HCaRG* gene expression by rosiglitazone. Although similar studies in other cell types have yet to be reported, these findings suggest that one possible mechanism through which rosiglitazone suppresses gastric cancer growth is via the up-regulation of HCaRG. *HCaRG* encodes for the copper metabolism gene MURR1 domain 5 (COMMD5) protein. MURR1 is a multifunctional protein that inhibits nuclear factor κ B (NF- κ B), a transcriptional factor with pleiotropic functions affecting innate and adaptive immunity, apoptosis, cell cycle regulation, and oncogenesis (20). NF- κ B inhibition is an attractive mode of preventing inflammation-induced cancer, including gastric (21). However, investigations have shown that *HCaRG* mRNA levels declined rapidly in the kidney after ischemia and reperfusion while there was a reciprocal increase in c-myc mRNA. *HCaRG* mRNA levels negatively correlate with the proliferative status of the kidney cells (16). Moreover, HEK293 cells were stably transfected with plasmid containing *HCaRG*. *HCaRG*-overexpression induced growth suppression which was associated with a reduced specific growth rate and mitotic index, cell cycle G₂M arrest, increased levels of p21^{Cip1/WAF1} and decreased levels of p27^{Kip1} (22). Collectively, rosiglitazone-derived

HCaRG is responsible for the suppression of gastric carcinogenesis involving cell proliferation and cell cycle signaling. While *HCaRG* is homologous to MURR1, our findings also raise the possibility that the up-regulation of *HCaRG* inhibits NF- κ B activity. The possible interaction between HCaRG and NF- κ B in gastric cancer needs to be addressed in future studies.

We observed that rosiglitazone induced a dose-dependent increase in mRNA expression of PPAR γ and *HCaRG* in AGS cells. Irreversible suppression of PPAR γ by the PPAR γ antagonist GW9662, however, did not reduce rosiglitazone-induced HCaRG expression. These observations show that rosiglitazone induced the increased expression of HCaRG in AGS cells through PPAR γ -independent signals. Similarly, rosiglitazone inhibited NSCLC (non-small cell lung carcinoma) cell growth, by activating TSC2 (tumor sclerosis complex-2) signaling (23); and decreased microsomal prostaglandin E synthase (mPGES)-1 expression, an inducible enzyme, in rat chondrocytes (24). The above-mentioned effects were exerted in a PPAR γ -independent signaling pathway. In addition, studies utilizing the irreversible PPAR γ -selective antagonist GW9662, have revealed PPAR γ -dependent and -independent mechanisms of growth inhibition in various human cancer types (25-28).

In conclusion, we have identified a novel pathway through which rosiglitazone inhibits gastric cancer development. Our results show that HCaRG was significantly down-regulated in human and rat gastric cancer tissues. Treatment with rosiglitazone induced the expression of HCaRG in cancer cell lines and the stomach of rat. Further study is needed to characterize the therapeutic potential of modulating HCaRG in gastric cancer prevention and therapy.

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