Original Article

Effect of prostaglandin reductase 1 (PTGR1) on gastric carcinoma using lentivirus-mediated system

Shuo Yang, Fen Luo, Jun Wang, Xiang Mao, Zongyou Chen, Zhiming Wang, Fenghua Guo

Department of General Surgery, The Affiliated Huashan Hospital of Fudan University, Shanghai 200040, China

Received August 31, 2015; Accepted October 21, 2015; Epub November 1, 2015; Published November 15, 2015

Abstract: Gastric carcinoma is a digestive related malignant tumor with poor diagnosis and prognosis for advanced patients. PTGR1 (prostaglandin reductase 1), as a potential cancer biomarker, has not been reported in gastric carcinoma occurrence. To investigate the role of PTGR1 on gastric carcinoma cells, human PTGR1 was efficiently silenced by lentivirus-mediated system in MGC-803 cells confirmed by quantitative real-time PCR (qRT-PCR) and western blot. Then cell proliferation, colony formation and cell cycle were determined after knockdown of PTGR1 by MTT assay, colony assay and flow cytometry, respectively and data suggested that PTGR1 down regulated MGC-803 cells significantly suppressed the proliferation and colony formation ability and induced cell cycle arrest in the G0/G1 phase compared to controls (P < 0.001). Further investigation demonstrated knockdown of PTGR1 influenced cell proliferation and cell cycle via activating p21 and p53 signaling pathway described by Western blot assay. Our findings indicate that PTGR1 may be an oncogene in human gastric carcinoma and identified as a diagnosis and prognosis target for gastric carcinoma.

Keywords: Gastric carcinoma, prostaglandin reductase 1, RNA interference, cell cycle

Introduction

Gastric carcinoma, derived from gastric epithelial cells, is a digestive related cancer with low survival. It has been considered as the second common cancer in China [1] and main causes of cancer-related death worldwide [2]. At present, the treatment and prognosis is quietly poor for advanced gastric carcinoma [3], which can be ascribed to a variety of factors, including genetic regulation and interaction [4]. Even though investigators have made great effort in clarifying the molecular diagnostic and prognostic markers for gastric carcinoma, the results are far from enough.

PTGR1 (prostaglandin reductase 1) is a nitroalkene reductase [5] and plays a key responsible for biological inactivation of prostaglandins [6, 7]. It has been identified as a band of 33 kDa exclusively in tumor and evaluated as a potential biomarker in liver cancer [8]. In addition, prostaglandins are involved in metabolites of arachidonic acid through COX (cyclooxygenase) pathway and demonstrated to contribute to the development of lung cancer [9, 10]. Available evidences also indicate that Cox-2 derived prostaglandins could stimulate the proliferation colorectal carcinoma [11] and pancreatic cancer [12]. In the tumor samples, PTGR1 is thought as the top-ranked protein and possesses dual activity [13] and its overexpression has been shown to increase cell viability [14]. Despite recent obtained advances in understanding the biology of PTGR1 on tumor progression, there is poor report about the effect of down-regulation of PTGR1 on gastric carcinoma diagnosis and prognosis.

Lentivirus-mediated RNA interference technique is more and more applied to specifically and efficiently down regulate the expression level of a target gene [15-17]. In the present study, to investigate whether PTGR1 functions as potential biomarker in gastric carcinoma, one stable knockdown of PTGR1 cell line model was constructed by means of lentivirus-mediated RNA interference technique. Based on constructed PTGR1 silencing cell model, we further determined the effect of PTGR1 silencing on gastric carcinoma cell proliferation and growth,
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cell cycle regulation as well as downstream target proteins' expression level.

Materials and methods

Lentiviral vector construction

Two short hairpin RNA (shRNA) sequences (S1, 5′-CTTGGATTTGATGTCGTCTTTCTCGAGAAAGAC-GACATCAAATCAAAGTTTTT-3′ and S2, 5′-CTAT CCTACTAATAGTGACTTCTCGAGAAAGTCACTATT AGTAGGATAGTTTTT-3′) were specifically designed for PTGR1 (NM_001146108.1). The sequence of control shRNA was 5′-GCGGAGGGTTTGAA-AGAATATCTCGAGATATTCTTTCAAACCCTCCGCTTTTT-3′. Three nucleotide sequences were inserted into the between NheI and PacI restriction sites of pFH-L lentiviral vector with green fluorescent protein (GFP), named shPTGR1 (S1), shPTGR1 (S2) and shCon.

Cell culture and transfection

Human gastric carcinoma cell line MGC-803 and embryonic kidney cell 293T were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 and Dulbecco's modified Eagle's medium (DMEM), respectively, with 10% fetal bovine serum (FBS). Both of them were maintained in a humidified atmosphere containing 5% CO₂ at a temperature of 37°C. Then the reconstructed plasmids were transfected into 293T cells with the pHelper of pVS-VG-I and pCMVΔR8.92 (Shanghai Hollybio, China). Subsequently, packed lentiviruses were harvested and then stably transfected to MGC-803 cells (6 × 10⁵ cells/well) at a multiplicity of infection (MOI) of 60. Fluorescence microscopy was used to observe the GFP expression and PTGR1 knockdown efficiency was further confirmed by quantitative real-time polymerase chain reaction (qRT-PCR) and western blot analysis.

RNA extraction and qRT-PCR analysis

Total RNAs were extracted from MGC-803 cells infected with shPTGR1 (S1), shPTGR1 (S2) and shCon, respectively, and reverse-transcribed into complementary DNA (cDNA) from 1 μg RNA. Subsequently, primers for PTGR1, including forward: TCCTCTTGACCCCTTCCGG and reverse: AAAGGGTGTAACGCAACTA were designed to perform qRT-PCR analysis on BioRad Connet Real-Time PCR platform. For each PCR reaction, total 20 µl mixture was prepared that included 10 µl 2 × SYBR premix ex Taq, 0.5 µl primers (2.5 µM), 5 µl cDNA and 4.5 µl ddH₂O, then using the following amplified procedure: 1 min initial denaturation at 95°C and 40 cycles consisted of 5 s denaturation at 95°C denaturation at 95°C and 40 cycles consisted of 15 s annealing at 60°C and 30 s extension at 72°C. The 2⁻ΔΔCt formula [18] was used to determine the relative expression of PTGR1.

Protein separation and western blot assay

The MGC-803 cells were collected and lysed in 2X SDS Sample Buffer, containing 10 mM EDTA, 100 mM Tris-HCl (pH 6.8), 4% SDS and 10% glycerol after 5 days’ transfection. The protein extract was detected by bicinchoninic acid (BCA) protein assay. Total 30 μg protein was subjected to polyacrylamide-sodium dodecyl sulfate (SDS-PAGE) electrophoresis, and then transferred onto polyvinylidene fluoride (PVDF) membranes. After blocking with 5% nonfat milk with phosphate-buffered saline with 0.05% Tween (PBST) for 1 h at room temperature, the membranes were blotted with primary antibodies, including rabbit anti-PTGR1 (1:1000, #ap5941c, Abgent), rabbit anti-p21 (1:500, #2947, Cell signaling), mouse anti-p53 (1:1000, sc-126, Santa Cruz) and rabbit anti-GAPDH (1:50000, 10494-1-AP, Proteintech), followed by incubation with HRP-conjugated goat anti-rabbit (1:5000, Santa Cruz, SC-2054). Immunoblot bands were detected by ECL kit (Pierce) according to the manufacture’s instruction.

Cell growth trend analysis by MTT

MGC-803 cells (2000 cells/well) were cultured into 96-well plates after 90 h transfection. Each well was added 20 µl MTT solution (5 mg/ml) and incubated for 4 h at 37°C, and added 100 µl acidic isopropanol (10% SDS, 5% isopropanol and 0.01 mol/L HCl). After removal of the medium, the absorbance of each plate was measured at a wavelength of 592 nm by spectrophotometer.

Colony formation assay used to determine cell proliferation potential

MGC-803 cells were seeded into six-well plates and cultured for 7 days after 120 h transfection with a density of 400 cells per well. According
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According to previously described methods [19], the cells were washed with PBS and fixed in 4\% paraformaldehyde, and then stained with crystal purple for 20 min, followed by counting the number of colonies under Fluorescence microscope. Per colony was defined as more than 50 cells.

\textbf{Cell cycle distribution analysis}

MGC-803 cells were seeded into 6 cm dish after 96 h transfection with a density of \(1.0 \times 10^6\) cells per dish, and then cultured for 4 days to reach 80\% confluence. These cells were collected, stained with propidium iodide (PI), and subjected to flow cytometric analysis.

\textbf{Statistical analysis}

All the data were expressed as mean \pm standard deviation (SD) using SPSS software. Student’s \(t\)-test was used to compare the different values between two groups and a value of \(P\) less than 0.05 was considered as significant difference.

\textbf{Results}

\textit{shPTGR1} decreased expression of \textit{PTGR1} in MGC-803 cells

After transfected with \textit{shPTGR1}, more than 80\% cells were observed GFP positive expression, suggesting a higher efficiency recombinant lentivirus (Figure 1A). As shown in Figure 1B, qRT-PCR analysis for \textit{PTGR1} showed that there was an 80\% and 89\% decrease in MGC-803 cells transfected with \textit{shPTGR1} (S1) and \textit{shPTGR1} (S2) compared with \textit{shCon} group, respectively, indicating a significant knockdown efficiency (\(P < 0.001\)). Further western blot confirmed \textit{PTGR1} protein expression was remarkably down regulated in MGC-803 cells transfected with \textit{shPTGR1} (S1) and \textit{shPTGR1} (S2) compared with \textit{shCon} group.

\textit{shPTGR1} suppressed MGC-803 cells’ growth trend

To determine the effect of \textit{PTGR1} knockdown on cell growth, MTT assay was conducted. As
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depicted Figure 2A, the optical density (OD) values of sh\textit{PTGR1} (S1) and sh\textit{PTGR1} (S2) infected MGC-803 cells were significantly reduced to $0.339 \pm 0.007$ and $0.806 \pm 0.013$, respectively compared with $0.241 \pm 0.022$ for shCon infected MGC-803 cells at the fifth day transfection, indicating there was an obvious suppressed growth trend in MGC-803 cells transfected with sh\textit{PTGR1} (S1) and sh\textit{PTGR1} (S2) compared with shCon group ($P < 0.001$).

\textit{shPTGR1 inhibited MGC-803 cells' proliferation potential}

To further confirm the \textit{PTGR1} knockdown affected cells' proliferation, colony formation assay was used to the study. As depicted as in Figure 2B, a smaller and fewer colonies were observed in sh\textit{PTGR1} (S1) infected MGC-803 cells than those in shCon by fluorescence microscope and crystal violet staining. Statistical analysis (Figure 2C) also demonstrated there was a significant proliferation inhibition in MGC-803 cells infected with sh\textit{PTGR1} (S1), contrasted with those in shCon group ($P < 0.001$).

\textit{shPTGR1 blocked cell cycle progression}

To uncover the cause of the inhibition of \textit{PTGR1} knockdown on MGC-803 cells proliferation, flow cytometry was conducted on distinguishing cells in different cell phases. As shown in Figure 3A, there existed an obvious effect on cell cycle progression after \textit{PTGR1} knockdown. Data showed the percentage of cells in G0/G1 phase increased from $54.98 \pm 0.66$ in MGC-803 cells infected with shCon to $69.22 \pm 0.63$ in MGC-803 cells infected with sh\textit{PTGR1} (S1), while that in S phase decreased from $28.18 \pm$
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0.71 in MGC-803 cells infected with shCon to 14.8 ± 0.93 MGC-803 cells infected with shPTGR1 (S1). Statistical analysis suggested that knockdown of PTGR1 significantly blocked cell cycle at G0/G1 phase (P < 0.001) (Figure 3B), which may be related with cell proliferation inhibition.

Cell cycle regulatory protein analysis using western blot assay

To further explain cell cycle progression resulted from PTGR1 knockdown, cell cycle regulatory proteins, including p21 and p53, were chosen to determine their expression level. Based on the result of western blot, the protein expression of p21 and p53 were obviously upregulated in MGC-803 cells infected with shPTGR1 (S1) compared that in MGC-803 cells infected with shCon (Figure 4).

Discussion

PTGR1 has been reported to be involved in many cancers’ progression, including bladder cancer [20], colorectal carcinoma [21]. However, its function in gastric carcinoma was rarely investigated. In the present study, we used RNAi approach to specifically silence the expression of PTGR1 in human gastric carcinoma MGC-803 cells. Our results indicated that down regulation of PTGR1 inhibited cell proliferation in time-dependent manner by MTT assay and suppressed colony formation ability, and the underlying molecular mechanisms might be correlated with cell cycle arrest. Further we found PTGR1 silencing influenced cell-cycle progression, induced G0/G1 phase arrest. Take this fact into account, we therefore turned to study the possible regulatory mechanism of PTGR1 and found cell-cycle arrest accompanied with p53 signaling activation.

Figure 3. A. Cell cycle distribution determined by flow cytometer. B. Statistical analysis of cell cycle in the PTGR1 knockdown group of MGC-803. ***, P < 0.001.

Figure 4. Expression analysis of downstream protein, including p21 and p53 using western blot assay.
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Cell growth is mainly regulated by cell cycle control and many cycle regulatory proteins are involved in this process, including p21 and p53 [22]. The p21, as cyclin dependent kinase (CDK) inhibitor, has been demonstrated to play an inhibitory role in expression of CDK4/6, CDK1 and cyclin-CDK2 leading to cell cycle arrested at G1 and S phase [23, 24]. The p53 is considered as a tumor suppressor gene with 63 kDa molecular weight and could tightly control the expression of p21 [25]. The accumulation of p53 could lead to cell cycle arrest and apoptosis in colorectal cells [26]. Some literatures pointed out up-regulation of p21 and p53 could arrest cell cycle progression in gastric cancer cells [27, 28]. In p53 independent manner, p21 is significantly up regulated by suppressing cyclin-dependent kinase leading cell growth inhibition in gastric cancer cells [29, 30]. More importantly, the expression of both p53 and p21 are up regulated in human gastric carcinoma MGC-803 cells dealt with honokiol [31], which is consistent with our results. Related study show that the gene expression level of PTGR1 is increased during cancer development as measured by qRT-PCR and Western blot assay, suggesting it was positively correlated with cancer progression [8]. All these evidences are in line with our results that knockdown of PTGR1 was negatively correlated with gastric cancer development via up regulating tumor suppressor gene, like p21 and p53.

In summary, PTGR1 silencing could significantly suppressed cell proliferation potential along with cell cycle arrest via partially activating p53 signaling and up regulating p21 expression. Based on these preliminary results, it seemed reasonable to highlight PTGR1 could play an important role in the diagnosis and prognosis of gastric carcinoma.

Disclosure of conflict of interest

None.

Address correspondence to: Drs. Zhiming Wang and Fenghua Guo, Department of General Surgery, The Affiliated Huashan Hospital of Fudan University, 12 Urumqi Middle Road, Shanghai 200040, China. Tel: +86-13917975388; +86-13764090893; E-mail: wzhm824@126.com (ZMW); gfh0816@163.com (FHG)

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