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Original Article

Effects of silenced KDR gene on the proliferation and apoptosis of gastric MGC803 cells

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Abstract: In this study, the expression of silencing KDR gene in gastric MGC-803 Cells was investigated, in order to discuss the effects of KDR gene on proliferation and apoptosis of MGC-803 cells. siRNA sequence of KDR gene was designed and synthesized; MGC-803 cells were transfected by Lipofectamine^{TM2000}. The expression of KDR after disturbance in mRNA and protein was detected by RT-PCR and Western blot; flow cytometry was used to detect the cell cycle; cell proliferation was detected by WST1 assay and cell apoptosis was detected by TUNEL assay. KDR expression in mRNA and protein of the observation group was significantly lower than that of the control group, and the difference was statistically significant ($P < 0.05$). The growth rate significantly slowed down in observation group; the cell cycle was arrested in the G0/G1 phase and the number of S-phase cells reduced; there was a statistically significant difference between the two groups ($P < 0.05$). The proliferation of MGC-803 cells transfected with KDR siRNA was significantly inhibited ($P < 0.05$), and cell apoptosis was significantly promoted, with a statistically significant difference ($P < 0.05$). Specific interference on KDR gene expression could inhibit the proliferation of MGC-803 cells and promote the apoptosis of tumor cells; therefore, siRNA sequences of KDR may be an effective target for the treatment of gastric cancer.

Keywords: Gastric cancer, KDR, siRNA, cell proliferation, apoptosis

Introduction

Gastric cancer is one of the most common gastrointestinal malignancies. There is no ideal treatment for gastric cancer in clinical. Especially, advanced gastric cancer is difficult to cure, so it is imperative to seek effective treatment and drug for gastric cancer [1-3]. Currently tumor gene is a hot pot, and studies have found that KDR could promote tumor cell apoptosis and decrease cell viability, which is expected to be a novel anticancer drug [4-6]. This study attempted to investigate the effects of KDR gene silencing by siRNA on the proliferation and apoptosis of MGC-803 cells, in order to provide a theoretical basis for targeting KDR gene therapy in gastric cancer.

Materials and methods

The main materials

MGC-803 cells were supplied by Nanjing KeyGEN Biotech Co., Ltd.; RPMI1640 culture medium and trypsin were purchased from Gibco Company, America; fetal calf serum was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd; TRIzol and Lipofectamine^{TM2000} liposomal transfection reagents were purchased from Invitrogen company, America; the RT-PCR two-step kit was purchased from TaKaRa company; AMV reverse transcriptase kit was provided by Chongqing future reagent Company; cDNA synthesis kit was purchased from Japan TOYOBO company; siRNA gene fragment was purchased from Guangzhou Ruibo Biotech Co., Ltd.; rabbit anti-human KDR polyclonal antibody was purchased from Shanghai Wei Feng Biotechnology Co., Ltd.; Lipofectamine III 2000 was purchased from Invitrogen Corporation, America; TUNEL apoptosis detection kit was purchased from Beijing Zhongshan Biotechnology Company; Ponceau red dye was purchased from Guangzhou Jing Xin biotech Co., Ltd. RIPA protein lysates and BCA protein quantitation kit was purchased from Jiangsu Biyotime Company; ECL kit was purchased from American Thermo Company.

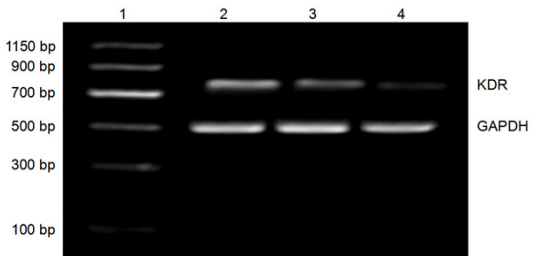

Figure 1. RT-PCR detection of MGC-803 cells KDR mRNA expression. 1: Marker; 2: Blank group; 3: The control group; 4: Observer group.

Design, synthesis and transfection of siRNA

According to the published literature [7], KDR siRNA sequence was composed by 5'-GCCACCAUGUUCUCUAAUATT-3' (sense strand) and 5'-UAUUAGAGAACAUGGUGGCAT-3' (antisense strand); the negative control siRNA SCR (scramble siRNA) sequence was composed of 5'-UUCUCCGAACGUGUCACGUTT-3' (sense strand) and 5'-ACGUGACACGUUCGGAGAATT-3' (antisense strand), both synthesized by Shanghai GenePharma Co, Ltd. MGC-803 cells were cultured in RPMI 1640 complete medium containing 10% fetal calf serum (37°C, 5% CO_2, saturated humidity). Passage was conducted every 3-4 d, and cells at logarithmic growth phase were used for the experiment. Gastric MGC-803 cells were seeded in 24-well plates, 1×10^5 cells/well; when the fusion rate was 70%, liposome transfection was performed. Transfections were divided into three groups: cells transfected with pGenesil-1-KDR-siRNA vector were the MGC-803/KDR siRNA group (observation group); cells transfected with control vector were MGC-803/control group (control group), and non-transfected gastric MGC-803 cells were the MGC-803 group (blank group). RNA interference effect was detected at 48 h after transfection.

Semi-quantitative RT-PCR

After abandoning the cell culture medium, cells washed with PBS for three times. Total RNA was extracted using TRIzol method, and cDNA was synthesized by reverse transcription using cDNA synthesis kit. KDR and internal control GAPDH were amplified respectively. Upstream primer of KDR was 5'-CTGGCATGGTCTFCTGTGAAGCA-3', and downstream primer was 5'-AATACCAGTGGATGTGATGCGG-3'; the amplified product was 795 bp. Upstream primer of internal reference GAPDH was: 5'-CGTGGAAGGACTCATGACCA-3', and downstream primer was: 5'-TCCAGGGGTCTTACTCCTTG-3'; the amplified product was 509 bp. PCR reaction conditions were: degeneration at 94°C for 2 min, annealing at 62°C for 1 min when degeneration at 94°C for 45 s, extension at 72°C for 1 min, totally 35 cycles, and then extension for 8 min at 72°C. 2% agarose horizontal slab electrophoresis was conducted at 100 V for 45 min. Automatic gel imaging and analysis system (France VI) was used to scan absorbance and KDR absorbance/that of GAPDH was taken as the relative intensity of mRNA expression.

The expression of KDR detected by westem blot

The cells were collected and proteins were extracted and tested by UV spectrophotometry. 3 × buffer was added after lysis, then the mixture was centrifuged (10.000 r/min, r = 400 mm, 5 min) before being placed in boiling water for 10 min. After 15% SDS-polyacrylamide gel electrophoresis, using the method of semi-drying transfer membrane, the proteins were transferred from the gel to a nitrocellulose membrane (NC) in the transfer tank, with a voltage of 20 V for 12 min. Then the proteins were pre-stained by Ponceau and molecular weight was labeled. The membrane was washed by TBST buffer, and the rabbit anti-human KDR polyclonal antibody (1:200) diluted in TBST buffer was added as the first antibody, incubated at 4°C overnight. Alkaline phosphatase labeled by IgG (1:2000) was added as the second antibody, incubated at room temperature for 2 h, washed with PBS, and then detected using ECL.

Cell cycle detection using flow cytometry

Cells were 0.25% trypsinized and centrifuged at 1000 r/min for 5 min (centrifuge radius of 17 cm) to remove the culture medium; then the remaining cells were washed with PBS once, and the supernatant was discarded and the cells were collected, which were washed two times with ice-cold PBS, and incubated in 80% ethanol overnight at 4°C; After being rinsed by PBS for three times, cells were fixed and washed with PBS, and then re-suspended in 0.1 mg/mL propidium iodide in the dark at room temperature for 30 min; cell cycle was detected by flow cytometry. The above experiments were repeated for three times.

Table 1. KDR mRNA expression of KDR protein and 48 h after transfection of siRNA gene silencing KDR ($\bar{X}$±S)

Group	KDR protein (KDR/GAPDH)	KDR mRNA (KDR/GAPDH)
Blank group	1.47±0.11$^{\Delta}$	0.48±0.05$^{\Delta}$
The control group	1.51±0.13*	0.47±0.03*
Observer Group	0.95±0.03*,$^{\Delta}$	0.33±0.02*
t Observer Group/Blank group	8.3653	6.5844
t Observer Group/The control group	7.4614	7.6863

Observation group and blank group, *P < 0.05; observation group and control group, $^{\Delta}$P < 0.05.

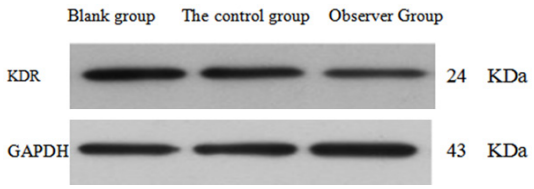

Figure 2. Western blot detection MGC-803 cells KDR protein expression.

Cell proliferation detected by water-soluble tetrazolium (WST-1)

Cells in KDR siRNA group, Control siRNA group and control group were respectively adjusted to the concentration of 1 × 10^4 cells/mL, and then 200 μL cell suspension was inoculated in 96-well plates, three well for each group. 20 μL WST-1 was added to each well at 24, 48 and 72 h after incubation. After incubation for another 4 h, optical density of each hole was detected at the wavelength of 450 nm using a microplate reader. Inhibition rate of proliferation was calculated. Proliferation inhibition rate = [1- D (450) of siRNA group/that of control group)] 100%.

TUNEL assay of apoptosis

After transfection for 48 h, cells in KDR siRNA group, control siRNA group and control group were collected for TUNEL staining (according to the product specification). The nuclei of apoptotic cells were brownish yellow. Apoptosis rate = the number of apoptotic cells/total number of cells × 100 %.

Statistical analysis

Measurement data were described as mean ± standard deviation. Differences between the groups were tested using SPSS 11.5 windows. Continuous variables between the two groups were compared using t test; comparative analysis of continuous variables among groups was conducted by analysis of variance and covariance correction; P < 0.05 was considered statistically significant. P < 0.05 was considered the difference is significant.

Results

KDR siRNA inhibition of KDR mRNA expression

KDR mRNA expression detected by RT-PCR in MGC-803/KDR-siRNA group (observation group) was significantly decreased compared to the control group and the blank group (both P < 0.05). It was shown in **Figure 1** and **Table 1**.

siRNA inhibition of the expression of KDR protein

Comparisons of KDR protein expression detected by Western blot showed that MGC-803/KDR-siRNA group (observation group) had significantly narrower bands, and there were statistically significant differences in the Gray value among groups (P < 0.05). It was shown in **Figure 2** and **Table 1**.

Cell cycle detection using flow cytometry

Flow cytometric analysis of cell cycle showed that compared with the control group and blank group; there was a slight increase in the G0/G1 phase and a slight decrease in S phase in the observation group. There were significant differences between observation group and control group, and the observation group and the blank group (P < 0.05). Cells in M phase had no significant changes and the difference was not statistically significant (P > 0.05), indicating a clear S-phase arrest (**Table 2**).

Inhibitory effect of KDR gene silencing on cell proliferation

KDR siRNA significantly inhibited MGC-803 cell proliferation, showing a significant time-dependence. Growth inhibition was found in observation group after transfection for 24 h, and the inhibition significantly enhanced after 48 h, with a statistically significant difference (P < 0.05). While plasmids in the control group and the blank group had no growth inhibition on MGC-803 cells (**Table 3**).

Table 2. siRNA inhibition of KDR expression of cell cycle MGC-803 ($\bar{X}$ ±S, n = 3)

Group	G0/G1 phase	S phase	G2/M phase
Blank group	55.4±2.8	36.4±2.4	8.4±0.2
The control group	55.3±2.1	36.7±2.3	9.6±1.1
Observer Group	69.5±2.8*	20.3±1.3*	10.1±1.4
t Observer Group/Blank group	6.1013	11.152	1.6865
t Observer Group/The control group	7.0271	10.754	1.4283

And the control group, the control group, *P < 0.05.

Table 3. KDR siRNA for MGC-803 cell proliferation inhibition rate (%)

Group	24 h	48 h	72 h
Blank group	0±0.3	0.3±0.26	0.5±0.42
The control group	0±0.4	0.3±0.25	0.4±0.53
Observer Group	11.6±2.9[a]	41.6±5.6[a,b]	51.6±6.6[a]
t Observer Group/Blank group	6.8913	12.534	12.989
t Observer Group/The control group	6.8632	12.534	13.004

[a]P < 0.05 compared with the control group and the control group; [b]P < 0.05 comparing with 24 h and 72 h.

Effect of KDR gene silencing on apoptosis

TUNEL assay showed that the apoptosis rates of the observation group, the control group and the blank group were (18.23±5.4)%, (7.29±2.12)%, and (7.46±2.36)% repectively; Apoptotic cells significantly increased in KDR siRNA group, and the difference was statistically significant (P < 0.05); while there was no significant difference in apoptosis rate between the control group and the blank group (P > 0.05, **Figure 3**).

Discussion

KDR is a Specific receptor which mediates VEGF and functions in tumor neovascularization [8]. KDR is not only highly expressed in human malignancies (including gastric cancer cells) but also expressed in endothelial cells, and the expression levels correlates with organizations and proliferation state of the cell [9-11]. Normally, vascular endothelial cells update slowly. The expression of KDR receptor is low, but the proliferation of vascular endothelial cell is more active, therefore they are mainly expressed in active proliferation of tumor cells and wounded healing tissue. They are almost not expressed in normal tissues [12, 13]. We design and synthesis KDR siRNA sequences by using the different expression level of vascular endothelial KDR in tumor and normal tissues. MGC-803 cells were transfected by Lipofectamine^{TM2000}. The results show that there is an obvious growth inhibition after 24 h transfection in the observation group. The inhibition effect enhanced after 48 h, but in the control and blank group, plasmids have no growth inhibition on MGC-803 cells.

KDR plays an important role in cell growth and differentiation. The autocrine form of KDR directly acts on tumor cells and promotes the cell proliferation and apoptosis [14, 15]. In this study, KDR siRNA transfected MGC-803 cells had typical features of apoptosis such as cell shrinkage, karyopycnosis and formation of apoptotic bodies. While in the non-transfected KDR siRN in the control group and study group, ppoptotic features, such as the formation of apoptotic body, did not occur. Therefore, we can inhibit the growth of tumor cells by utilizing the specificity of KDR siRNA gene, which was highly expressed in proliferating tumor cells and had little impact on normal tissue cells.

Studies have reported that KDR could regulate proliferation and apoptosis of human gastric cancer cells and vascular endothelial cells [16]. Zong-Hai Huang et al's study showed that KDR gene can induce apoptosis of gastric cancer cells by observing its impact on gastric cancer cells through cell membrane structure, DNA content distribution and other aspects of observation [17]. The results of this study further confirmed this view. In short, specific interference of KDR gene expression can inhibit the proliferation of gastric MGC-803 cells and promote apoptosis. Therefore, SiRNA sequence of KDR may provide new ideas for the treatment of gastric cancer.

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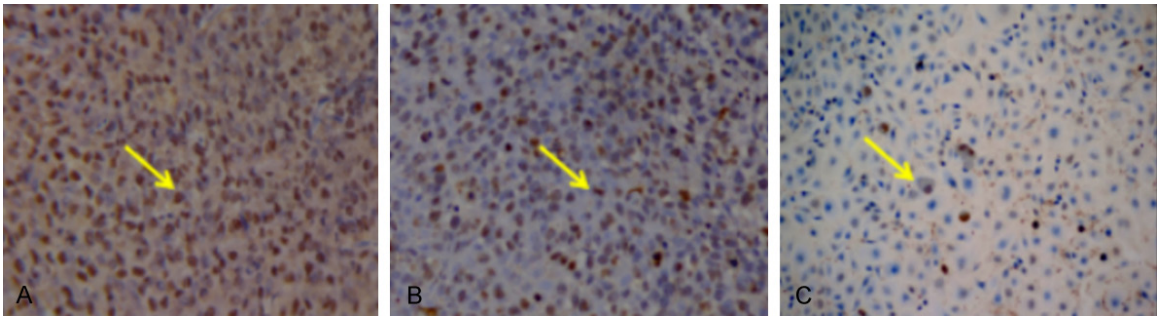

Figure 3. *TUNEL assay for apoptosis (× 200) A*: There was a large number of apoptotic cells in the observation group, and brown positive signals appeared in the nucleus. B, C: There were no apoptotic cells in the control group and blank group, shown in **Figure 3**.

Disclosure of conflict of interest

None.

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