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BRIEF ARTICLE

siRNA-targeted inhibition of growth hormone receptor in human colon cancer SW480 cells

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Abstract

AIM: To determine the effects of RNAi-mediated inhibition of the growth hormone receptor (*GHR*) gene on tumors and colon cancer cells *in vivo*.

METHODS: Construction of a eukaryotic vector for human GHR expression, the pcDNA™6.2-GW/EmGFP-small interfering RNAs (siRNAs)-GHR plasmid, was used to inhibit GHR expression. Thirty-six BALB/c nude mice were randomly divided into groups and treated with normal saline (NS), recombinant plasmid (G_2), growth hormone (GH), 5-fluorouracil (FU), G_2+FU or G_2+FU+GH. Each nude mouse was subcutaneously inoculated with 1×10^7 human colon cancer SW480 cells; the nude mice were weighed before inoculation and on the 2nd, 5th, 8th, 11th, 14th and 17th day after inoculation. All nude mice were sacrificed after 17 d. Each subcutaneous tumor was removed and studied. Tumor volume was measured on the 5th, 8th, 11th, 14th and 17th day after inoculation. The expression of GHR protein in the tumor tissue was detected by Western blotting analysis, and the differences in GHR mRNA expression in the tumor tissue were detected by real-time quantitative reverse transcription-polymerase chain reaction.

RESULTS: Compared to the control group, the weights of the inoculated nude mice on the 17th day after inoculation were: G_2: 21.60 ± 0.71 g, GH: 21.64 ± 0.45 g, FU: 18.94 ± 0.47 g, FU+G_2: 19.40 ± 0.60 g, G_2+FU+GH: 21.04 ± 0.78 g *vs* NS: 20.68 ± 0.66 g, $P < 0.05$; the tumor volumes after the subcutaneous inoculation were: G_2: 9.71 ± 3.82 mm^3, FU: 11.54 ± 2.42 mm^3, FU+G_2: 11.42 ± 1.11 mm^3, G_2+FU+GH: 10.47 ± 1.02 mm^3 *vs* NS: 116.81 ± 10.61 mm^3, $P < 0.05$. Compared to the GH group, the tumor volumes were significantly decreased in the experimental groups. The GHR protein expression (G_2: 0.39 ± 0.02, FU: 0.40 ± 0.02, FU+G_2: 0.38 ± 0.01, G_2+FU+GH: 0.39 ± 0.01 *vs* NS: 0.94 ± 0.02, $P < 0.05$) and the GHR mRNA expression (G_2: 14.12 ± 0.10, FU: 15.15 ± 0.44, FU+G_2: 16.46 ± 0.27, G_2+FU+GH: 15.37 ± 0.57 *vs* NS: 12.63 ± 0.14, $P < 0.05$) were significantly decreased and increased, respectively, in the experimental groups.

CONCLUSION: Inhibition of GHR in human colon cancer SW480 cells resulted in anti-tumor effects in nude mice.

Key words: Growth hormone receptor; Small interfering RNAs; Colon cancer; Gene therapy; Signaling pathway

Core tip: Human growth hormone receptor (GHR) is highly expressed in colon cancer tissues. GH/GHR plays an important role in colon cancer emergence and development. After specific binding of GH to GHR in tumor tissues, the JAK-STAT signaling pathway is activated, resulting in improved cell growth and proliferation. small interfering RNAs (siRNAs)-targeted inhibition of the human *GHR* gene was used to investigate

its impact on the emergence and development of colon cancer and to determine how human colon cancer cells respond to GHR suppression. The siRNA-containing plasmid could suppress GHR expression in colon cancer cells and exhibited anti-tumor effects in nude mice.

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INTRODUCTION

As shown previously by our team, human growth hormone receptor (GHR) is highly expressed in colon cancer tissues. Additionally, less differentiated tumor tissues have higher levels of GHR expression. During tumor development, the expression of GHR demonstrates an upward tendency[1,2]. Some researchers[3-6] believe that the expression of GHR in tumor tissue is linked with the vegetative state of the tumor and that GH and GHR play important roles in the emergence and development of colon cancer. After specific binding of GH to GHR in tumor tissues, the JAK-STAT signal transduction pathway is activated, resulting in improved cell growth and proliferation[7-10]. Signal transduction therapy is a commonly used chemotherapy strategy, and currently, treatment often involves the use of small interfering RNAs (siRNAs) that target different signal transduction pathways[11-13].

In this study, siRNA targeting the human *GHR* gene was used to investigate the impact that GHR has on the emergence and development of colon cancer and to determine how human colon cancer cells respond to the suppression of GHR expression.

MATERIALS AND METHODS

Experimental animals and cell lines

Thirty-six 8-wk old, female BALB/c nude mice, weighing between 20 and 22 g, were purchased from Vital River Laboratories (VRL) with license No. SCXK (Jing) 2006-0009. The mice were kept in the SPF environment of the animal experiment center in Kunming Medical University. The human colon cancer cell line SW480 was obtained from the Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Science.

Laboratory reagents

The HQ high purity plasmid extraction kit was purchased from Invitrogen (Invitrogen, Carlsbad city, California, United States). The BCA protein concentration kit (Tiangen Biology and Chemistry) and the molecular mass albumin standard were purchased from Tiangen Biology and Chemistry (Fermentas Company). The mouse monoclonal anti-human GHR antibody was obtained from R and D Company (MAB1210), and the goat secondary anti-mouse IgG-HRP antibody was purchased from Abmart Company. RNase H was obtained from Invitrogen, and the Golden Taq PCR kit was purchased from Tiangen Biology and Chemistry. SYBR Green-Real Master Mix was purchased from Tiangen Biology and Chemistry, and all primers used in the study were obtained from Invitrogen.

Preparation of cell suspension

Colon cancer SW480 cells were cultivated in RPMI 1640 nutrient solution supplemented with 10% fetal calf serum (FCS), 10.0×10^3 U/L penicillin, and 100 mg/L streptomycin in a 37 ℃ incubator with 50 mL/L CO_2. This is an adherent cell line. Cells in the exponential growth phase were harvested using 0.25% trypsin, and the cells were resuspended using a machine. The cells were then centrifuged at 2000 rpm for 5 min. Then, the supernatant was removed, and the cells were resuspended in physiological saline at a concentration of 1×10^7 cells/mL. Trypan blue staining was used to ensure that cell viability was above 95%; after resuspension the cells were stored in an ice bath.

Construction of siRNA and eukaryotic expression vectors

siRNA oligonucleotides were designed against the mRNA sequence of human *GHR* found in GenBank, which had a total length of 4414 bp (Accession No.: X06562, GI: 31737). We used the RNAi Designer website (http://bioinfo.clontech.com/rnaidesigner) to design the siRNA that targeted the hGHR mRNA (527-547: GTCAGTTTAACTGGGATTCAT). Using the BLAST search program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to search the EST database, we found that the siRNA was not homologous to another gene and would be an effective siRNA sequence. The complementary single strand primer was incubated at 94 ℃ in annealing buffer solution for 3 min, and the oligonucleotides were annealed at 37 ℃ for 1 h. The annealed oligonucleotides were then phosphorylated at 37 ℃ for 30 min with T4 DNA-PNK. The oligonucleotides were then ligated into the linearized (BamHI/HindIII) pcDNA™ 6.2-GW/EmGFP-GHR-siRNA plasmid using T4 DNA ligase. The final product was transformed in competent *E. coli* DH5α cells, and the transformants were spread on transformation plates containing 50 μg/mL spectinomycin dihydrochloride (Sigma, Catalog No. S4014). The plates weres kept in a 37 ℃ incubator overnight, and three coenobium clones were picked from each plate and subcultured. The plasmids were extracted using plasmid extraction kits, and the plasmids were confirmed by restriction enzyme digestion with *EcoR* I , *Sac* I and *Sal* I . The transformation liquid was also sequenced to ensure that recombination had not occurred in the insert fragments during the cloning process. Finally, the pcDNA™6.2-GW/EmGFP-GHR-siRNA plasmids were constructed successfully and are referred to as G_2 throughout the paper. Additionally, the plasmids were extracted and diluted in

DMEM as a precaution.

Groups and method of drug distribution

We subcutaneously injected 1 × 10^7 human colon cancer SW480 cells into BALB/c nude mice. On the first day after the injection, the mice were divided into six groups and administered the indicated drug and dose. (1) Normal saline (NS): 10 μL of NS was injected into the abdominal cavity of each mouse; (2) Plasmid (G_2): 10 μg of the G_2 eukaryotic expression plasmid was injected subcutaneously into each mouse; (3) Growth hormone (GH): 2 IU/kg rhGH, a physiological dose of GH, was hypodermically injected into each mouse; (4) 5-fluorouracil (FU): 20 mg/kg 5-FU was injected into the abdominal cavity of each mouse; (5) 5-FU+plasmid (FU+G_2): 20 mg/kg 5-FU and 10 μg of the G_2 plasmid were injected into the abdominal cavity of each mouse; (6) 5-FU+GH+plasmid (FU+GH+G_2): 20 mg/kg 5-FU, 10 μg of the G_2 plasmid, and 2 IU/kg rhGH were injected into the abdominal cavity of each mouse; and (7) Each of the above groups was treated every 5 d for three rounds of treatment.

Observation index

Observation of weight and tumor volume of nude mice: The weight of the nude mice was recorded before injection of the SW480 cells and on the 2nd, 5th, 8th, 11th, 14th and 17th day after injection. The length and the minimum diameter of the tumors were recorded, and the tumor volume was calculated on the 5th, 8th, 11th, 14th and 17th day after hypodermic injection of human colon cancer SW480 cells The following equation was used to calculated the tumor volume: $V = (A \times B^2)/2$, where A represents the major diameter and B represents the minimum diameter.

Expression of GHR in tumor tissues as detected by Western blotting analysis

A sample of each tumor was removed from the nude mice and cut into pieces. Cleanser lysate solution containing PMSF (400 μL) was added to the tumor sample in a homogenizer. After the cells were lysed for 30 min, the homogenate was centrifuged at 12000 rpm for 5 min at 4 ℃. The supernatant was removed, placed in 0.5 mL centrifuge tubes, and stored at -20 ℃. Then, 20 μL of the lysate sample was separated and analyzed using SDS-PAGE; the proteins were electrotransferred onto nitrocellulose membranes and detected using a chemiluminescent detection system. Beta-actin was used as a loading control. The images were analyzed using the BandScan5.0 program. The ratio of GHR expression to beta-actin expression was analyzed using the integral optical density value (RV) of the band in the same sample. The results are shown as the expression of GHR relative to beta-actin, and the results were measured as, mean ± SD.

Expression of GHR mRNA in tumor tissue was detected by quantitative reverse transcription-polymerase chain reaction

Twenty microliters of the lysate sample was centrifuged at 5000 rpm for 10 min. Prechilled PBS was used to wash the cells, and total RNA was extracted using the Trizol reagent in a one-step method. For first strand synthesis, 1 μg of total RNA was combined with 1 μL of 0.5 μg/μL oligo primer and 12 μL of deionized water. The mixture was then incubated at 70 ℃ for 5 min. Then, the samples were quenched in a bath of ice water and centrifuged at 5000 rpm for 4 s. Next, 4 μL of 5 × reaction buffer, 1 μL of 20 U/μL ribonuclease, and 2 μL of 10 μmol/μL dNTPs were added, and the mixture was incubated at 37 ℃ for 5 min. Then, 1 μL of 200 U/μL reverse transcriptase was added, and the reaction was incubated at 42 ℃ for 60 min, followed by a 10 min incubation at 70 ℃. After the reaction, the cDNA was incubated at 0 ℃ and was stored at -20 ℃. Real time PCR with the SYBR-Green fluorochrome was used to detect the expression of GHR mRNA. For this reaction, 10 μL of cDNA, 2 μL of both forward and reverse primers, 10 μL of buffer solution, 4 μL of ddH_2O, and 1 μL of the ROX fluorochrome were incubated at 95 ℃ for 10 min, followed by 35 cycles of 94 ℃ for 10 s, 56 ℃ for 30 s, and 72 ℃ for 30 s; a final extension time of 5 min at 72 ℃ ended the reaction. GAPDH was used as a negative control. The following primers were used in this experiment: GHR forward: GCAGCTATCCTTAGCAGAGCAC; GHR reverse: AAGTCTCTCGCTCAGGTGAACG; GAPDH forward: GGTCTCCTCTGACTTCAACA; and GAPDH reverse: GAGGGTCTCTCTCTTCCT. The levels of GHR mRNA in the transfection group and the control group were determined by quantitative PCR, and the △CT value of the GHR mRNA was determined between the two groups. It was demonstrated that a higher △CT value represented a larger inhibition of GHR mRNA.

Statistical analysis

Data were expressed as the mean ± SD. Univariate or multivariate data were analyzed using variance analysis and pairwise comparison *q* test by SPSS 18.0 statistical software package. Statistical significance was considered at $P \leqslant 0.05$.

RESULTS

Weight changes in the tumor-bearing mice

At the end of the experiment, all 36 tumor-bearing mice survived from inoculation 2 to 17 d, except for the NS group. After injection, there was a statistically significant difference ($P < 0.05$) in the weight of the mice compared with their weight before injection. The weight of the GH group increased after inoculation with SW480 cells, while the other groups decreased in weight ($P < 0.05$). The weight of the G_2, FU, and G_2+FU groups noticeably decreased ($P < 0.05$) compared with the NS group. The weight of the FU and G_2+FU groups decreased compared with the G_2 group; however, this change was not significant. After the addition of GH, the weight of the G_2+GH+FU group increased compared with the FU group in the same period ($P < 0.05$; Table 1).

Table 1 Weight changes of tumor-bearing mice (n = 6; mean ± SD)

Time	Weight (g, mean ± SD)					
	NS	G_2	GH	FU	FU+G_2	G_2+FU+GH
Pre-operation	20.69 ± 0.67	21.92 ± 0.70	20.67 ± 0.57	21.93 ± 0.58	21.86 ± 0.73	21.25 ± 0.79
Inoculation 2 d	20.64 ± 0.60	21.86 ± 0.79	20.82 ± 0.56	20.89 ± 0.66[1]	20.09 ± 0.55[1,3]	20.41 ± 0.85[1,3]
Inoculation 5 d	20.65 ± 0.49	21.56 ± 0.81	20.96 ± 0.54[1]	20.94 ± 0.67[1]	19.92 ± 0.58[1,3,5]	20.42 ± 0.76[1]
Inoculation 8 d	20.65 ± 0.65	21.53 ± 0.56[1]	21.18 ± 0.44[1]	20.41 ± 0.73[1,3]	19.92 ± 0.52[1,2,3,5]	20.53 ± 0.70[1,3]
Inoculation 11 d	20.67 ± 0.63	21.53 ± 0.73[1]	21.27 ± 0.53[1,4]	19.86 ± 0.57[1,3]	20.06 ± 0.52[1,2,3,5]	20.63 ± 0.81[1,5]
Inoculation 14 d	20.61 ± 0.62	21.60 ± 0.68[1,2]	21.51 ± 0.44[1,4]	9.46 ± 0.52[1,2,3]	19.86 ± 0.92[1,2]	20.86 ± 0.72[1,4]
Inoculation 17 d	20.68 ± 0.66	21.60 ± 0.71[1]	21.64 ± 0.45[1,2,4]	18.94 ± 0.47[1,2,3]	19.40 ± 0.60[1,2,3]	21.04 ± 0.78[1,4]

[1]Compared with pre-operation, $P < 0.05$; [2]Compared with NS group, $P < 0.05$; [3]Compared with G_2 group, $P < 0.05$; [4]Compared with FU group, $P < 0.05$; [5]Compared with GH group, $P < 0.05$. NS: Normal saline; G_2: Recombinant plasmid; GH: Growth hormone; FU: 5-fluorouracil.

Table 2 Tumor volume changes in tumor-bearing mice (n = 6, mean ± SD)

Time	Subcutaneous tumor volume (mm^3, mean ± SD)					
	NS	G_2	GH	FU	FU+G_2	G_2+FU+GH
Inoculation 5 d	7.72 ± 1.61	7.93 ± 1.74	8.11 ± 1.65	7.42 ± 1.51	6.51 ± 1.20	7.33 ± 1.32
Inoculation 8 d	20.19 ± 4.91[2,3]	13.44 ± 4.12[1,3]	33.28 ± 3.24[1,2,3]	17.51 ± 5.75[3]	15.12 ± 5.01[3]	15.44 ± 4.23
Inoculation 11 d	106.02 ± 6.61[2,3]	21.12 ± 4.04[1,3]	151.90 ± 8.31[1,2,3]	21.00 ± 5.07[1,3]	19.22 ± 4.33[1,3]	22.97 ± 4.95[1,2,3]
Inoculation 14 d	133.41 ± 6.43[2,3]	20.00 ± 4.75[1,3]	178.93 ± 3.11[1,2,3]	16.23 ± 6.51[1,3]	11.55 ± 4.11[1,2,3]	12.12 ± 3.11[1,2,3]
Inoculation 17 d	116.81 ± 0.61[2,3]	9.71 ± 3.82[1,3]	149.01 ± 3.02[1,2,3]	11.54 ± 2.42[1,3]	11.42 ± 1.11[1,3]	10.47 ± 1.02[1,3]

[1]Compared with NS group, $P < 0.05$; [2]Compared with G_2 group. $P < 0.05$; [3]Compared with GH group, $P < 0.05$. NS: Normal saline; G_2: Recombinant plasmid; GH: Growth hormone; FU: 5-fluorouracil.

Changes in tumor volume in the tumor-bearing mice of all groups

By the end of the experiment, the tumor volume of the mice in all groups increased compared to the fifth day after inoculation. The tumor volume of the GH group had the most dramatic increase, followed by the NS group. The tumor volumes of the G_2, FU, G_2+FU, and G_2+GH+FU groups only slightly increased. Compared with the NS group in the same period, the tumor volume of the experimental group obviously decreased, whereas that of the GH group significantly increased ($P < 0.05$). Compared with the G_2 group, the G_2+FU group had a more pronounced tumor inhibition ($P < 0.05$). There was no obvious difference in the tumor volume of the G_2+GH+FU group compared with the G_2, FU, and G_2+FU groups in the same period (Table 2).

GHR protein expression in the subcutaneous tumors of tumor-bearing mice in all groups

The expression levels of GHR protein in the tumors of the GH (0.94 ± 0.02) and NS (0.94 ± 0.02) mice were significantly higher than the GHR levels in the G_2 (0.39 ± 0.021), FU (0.40 ± 0.02), G_2+FU (0.38 ± 0.01) and G_2+FU+GH (0.39 ± 0.01) mice. However, there was no significant difference between the G_2 group and the FU, G_2+FU, and G_2+FU+GH groups ($P > 0.05$).

GHR mRNA expression in the subcutaneous tumors of tumor-bearing mice

Compared with the NS group and the control group that did not have a plasmid, the △CT value of the G_2, FU, G_2+FU, and G_2+FU+GH groups significantly increased ($P < 0.05$). In the experimental groups, the inhibition ratios of the FU+G_2 and FU+G_2+GH groups against GHR mRNA were higher than that of the G_2 group ($P < 0.05$; Table 3).

DISCUSSION

Colon cancer is one of the most common malignant tumors[14-16]. Currently, the treatment for colon cancer is surgery combined with radiotherapy and chemotherapy. However, most patients cannot undergo operation or do not respond to chemotherapeutics, leading to the failure of the therapy. Determining the appropriate tumor target spot related to gene and specific therapy has become a hotspot of research in tumor therapy[17]. Due to its action as an anabolic agent and mitogen, GH has a wide range of functions in substance metabolism and body fluid equilibrium, which can accelerate the use of nitrogen and improve the synthesis of liver and muscle proteins. The nutritional effect of GH has already been shown in cachexia[18]. Because GH can increase the brittleness of chromosomes, which, in turn, can cause malignant transformation of cells, and can increase tumor growth, GH has been excluded as a therapy option for the treatment of tumors[19,20]. It has been demonstrated by many epidemiological researchers that patients who receive long term treatment with growth hormone have an increased risk of colon cancer[3,21], and GHR expression in the colon might relate to the occurrence, development and metastasis of these tumors[1,22]. The presence of GHR in the local tissue is a prerequisite for GH to play its role, which means that when determining

Table 3 △CT value of growth hormone receptor mRNA expression detected by real-time reverse transcription-polymerase chain reaction in all groups

	NS	LP	Negative	GH	G_2	FU	FU + G_2	G_2 + FU + GH
△CT	12.63 ± 0.14	12.63 ± 0.43	12.67 ± 0.21	12.71 ± 0.39	14.12 ± 0.10[1]	15.15 ± 0.44[1]	16.46 ± 0.27[1,2]	15.37 ± 0.57[1,2]

[1]Compared with NS group, $P < 0.05$; [2]Compared with G_2 group, $P < 0.05$. NS: Normal saline; G_2: Recombinant plasmid; GH: Growth hormone; FU: 5-fluorouracil.

whether to use GH as a therapy, it is important to know the expression and distribution of GHR in a specific tumor cell. GHR is highly expressed in colon cancer tissues[1,2], and high GHR expression has been correlated with poor patient prognosis[23].

RNAi refers to complementary double-stranded RNAs (dsRNAs) that bind to specific endogenous mRNAs, resulting in the degradation of those mRNAs and the silencing of gene expression. Aiming at the relevant signal of the auxanodifferentiation of tumor cells for transduction and targeting the interference of the expression of crucial proteins in transduction pathways of cell signaling can inhibit the growth of a tumor specifically and highly efficiently[24,25].

In this research, we constructed plasmids containing siRNAs that targeted the expression of GHR in colon cancer cells, thereby decreasing the expression of GHR in the colon cancer tissues and blocking the GHR-induced signal transduction that promotes tumor cell growth. The results demonstrated that, compared to the control group, the tumor volume and the mRNA and protein expression of GHR in the tumor tissue significantly decreased. Additionally, the combination of GHR silencing and 5-FU treatment had an anti-tumor effect. After the siRNA blocked the expression of GHR in the tumor tissue, the addition of GH could bind to the GHR in the normal tissue. As a result, the weight and nutrition of the nude mice may improve, and GH treatment increases the nude mouse tolerance towards chemotherapy and increases the chemosensitivity of the tumor cells[9]. Compared with the other experimental groups, the FU+G_2+GH group had no significant difference in its reduced tumor volume or decreased expression of GHR protein and mRNA.

Our research showed that the siRNA-containing plasmid influenced the expression of GHR in the colon cancer cells and played an anti-tumor role in the nude mice.

COMMENTS

Background

Human growth hormone receptor (GHR) is highly expressed in colon cancer tissues. In addition, less differentiated tumor tissues have higher levels of GHR expression. During tumor development, the expression of GHR increases. Some researchers believe that the expression of GHR in tumor tissue is linked with the vegetative state of the tumor, and GH/GHR plays an important role in the emergence and development of colon cancer. After specific binding between GH and GHR in tumor tissue, the JAK-STAT signal transduction pathway is activated, leading to improved cell growth and proliferation.

Research frontiers

Signal transduction therapy is a commonly used chemotherapy strategy, and currently, treatment often involves the use of small interfering RNAs (siRNAs) that target different signal transduction pathways. RNAi refers to complementary double-stranded RNAs that bind endogenous mRNAs, resulting in the specific degradation of that mRNA, which leads to decreased expression of that gene. Aiming at the relevant signal of the auxanodifferentiation of tumor cells and targeting the interference of the expression of crucial proteins in transduction pathways of cell signaling can inhibit the growth of tumor specifically and highly efficiently.

Innovations and breakthroughs

In this study, siRNA-targeted inhibition of the *GHR* gene was used to investigate GHR's impact on the emergence and development of colon cancer and to determine how human colon cancer cells respond to the suppression of *GHR* gene expression.

Applications

The results showed that the siRNA-containing plasmid influenced the expression of GHR in the colon cancer cells and played an anti-tumor role in the nude mice.

Peer review

The manuscript is well designed and had appropriate methodology.

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