·BASIC RESEARCH·

Inhibitory Effect of IGF1R siRNA on the Growth of Human Liver Cancer SMMC7721 Cell Xenograft in Nude Mice

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[ABSTRACT] **BACKGROUND & OBJECTIVE:** Using small interfering RNA (siRNA) to inhibit mammal gene expression becomes an effective technique in studying gene function. This study was to investigate the effect of insulin-like growth factor 1 receptor (IGF1R) siRNA on the growth of human liver cancer SMMC7721 cell xenograft in nude mice. **METHODS:** siRNA targeting IGF1R was designed, and plasmid SMMC7721-IGF1R-siRNA was constructed and transfected into SMMC7721 cells (SMMC7721-IGF1R-siRNA cells); the cells transfected with SMMC7721-IGF1R-mutation (SMMC7721-IGF1R-mutation cells) were used as negative control, and untransfected cells as empty control. Stable cell clones were screened by G418, and transplanted into nude mice to establish cancer xenograft. Tumor growth was monitored. Tumor morphology was observed with HE staining. The expression of IGF1R protein in tumor tissues was detected by Western blot. Microvessel density (MVD) in tumor tissues was detected by SP immunohistochemistry. Cell apoptosis was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay. **RESULTS:** The tumor volume was significantly smaller in SMMC7721-IGF1R-siRNA group than in SMMC7721-IGF1R-mutation group and SMMC7721 group ($P<0.05$). Necrosis and cell apoptosis were found in SMMC7721-IGF1R-siRNA group. The expression of IGF1R protein was significantly lower in SMMC7721-IGF1R-siRNA group than in SMMC7721-IGF1R-mutation group and SMMC7721 group ($P<0.05$). MVD was significantly lower in SMMC7721-IGF1R-siRNA group than in SMMC7721-IGF1R-mutation group and SMMC7721 group (11.3±4.4 vs. 36.7±7.6 and 28.4±6.5, $P<0.05$). The apoptosis rate of tumor cells was significantly higher in SMMC7721-IGF1R-siRNA group than in SMMC7721-IGF1R-mutation group and SMMC7721 group [(50.2±6.4)% vs. (5.4±1.0)% or (6.0±2.1)%, $P<0.05$]. **CONCLUSION:** IGF1R siRNA can inhibit the growth of SMMC7721 cell xenograft in nude mice.

KEYWORDS: RNA interference; Human insulin-like growth factor receptor 1; Microvessel density; Gene expression; SMMC7721 cells; Xenograft tumor; Nude mouse

1. Introduction

In cell cycle, once the cell enters into the G1 phase and lacks other growth factors, insulin like growth factor 1 (IGF1) can promote the cell cycle [1]. Double stranded RNA (dsRNA), which is named as RNA interference (RNAi) can specifically induce the decomposition of homologous messenger RNA (mRNA), thus to specifically silence the expression of relevant genes. Small interfering RNA (siRNA) provides a new measure to study the functions of unknown genes, and it also has

potential application values in human genetic therapy.

IGF1R protein is selectively expressed in primary liver cancer, making it a candidate target of genetic therapy against liver cancer [2, 3]. Human liver cancer cell line SMMC7721, with positive IGF1R expression, was established by the Secondary Military Hospital, Shanghai. This study was to investigate the effect of IGF1R siRNA on the growth of human liver cancer SMMC7721 cell xenograft in nude mice.

2. Materials and Methods

2.1 Experimental animals

Eighteen BALB/c nude mice were provided by Shanghai Experimental Animal Center, Chinese Academy of Sciences [animal certificate SCXK (Shanghai) 2006-0010]. The male and female mice weighing 18-20g, aged between 4 and 6 weeks, were fed under specific-pathogen free (SPF) conditions.

2.2 Major reagents

Rabbit anti-human IGF1R polyclonal antibody was bought from Santa Cruz Company, rabbit anti-human Ⅷ factor relevant antigen antibody was bought from DAKO company, histostain™-SP (streptavidin horseradishperoxidase) immunohistochemistry kit was bought from Beijing Zhongshan Biotechnology Co., Ltd., and TUNEL kit was bought from Fuzhou Maixin Biotechnology Co., Ltd.

2.3 Design and synthesis of IGF1R

IGF1R siRNA segment was designed and synthesized based on siRNA software on http://www.dharmacon sidesign center.com/: one was 5-ATACGGATCACAAGTTGAG-3, the other one was 5-CTCAACTTGTGATCCGTAT-3. One of the bases was mutated as the negative control: one was 5-ATACGGATCATAAGTTGAG-3, the other was 5-CTCAACTTATGATCCGTAT-3; the bases in shade were the mutated ones. The segments were synthesized by Shanghai Saibaisheng Biotechnology Company, and were named as SMMC7721-IGF1R-siRNA and SMMC7721-IGF1R-mutation, respectively.

SMMC7721-IGF1R-siRNA plasmid and human liver cancer cell line SMMC7721 were provided by National Genome Group South Research Center. The establishment of the plasmid and the stable strain was referred to the reference [4].

2.4 Establishment of the nude mouse xenograft model

Eighteen BALB/c nude mice were divided into three groups with five animals in each group, and one more mouse was put in each group in case of occidental death. Group A was SMMC7721 blank control group, group B was SMMC7721-IGF1R-mutation group, and group C was SMMC7721-IGF1R-siRNA group.

SMMC7721 cells were inoculated into DMEM medium containing 10% fetal bovine serum, and continuously cultured in a 37℃ incubator with 5% CO_2. Cells were digested with 1% pancreatic enzyme at the logarithmic phase (50% -70% area). Cells of 5×10^7 were collected, centrifuged at 1000r/min for 5 min. The supernatant was discarded. Cells were washed with DMEM containing no serum, centrifuged again. A small amount of DMEM was added, and mixed evenly with the cells. Prepared cell suspension of 0.1 ml (about 5×10^6 cells) was taken, injected into the forelegs of the mice subcutaneously to form a subcutaneous eminence with a diameter of approximately 3mm.

2.5 Drawing of the cancer growth curve

After implantation, the injection site was observed everyday to check whether there was infection or there was spontaneous regression. When the tumor appeared, the mass in each group (W) was observed and recorded. The nude mice were sacrificed 35 days after cell inoculation, the morphology of xenograft tumor was observed with naked eyes and pictures were taken. After the mice were sacrificed, autopsy was performed; the growth and the surrounding organs were observed. After removing, the tumor was cut into blocks of 1.0cmx1.0cm using a double face blade, and immersed into 4% paraformaldehyde to fix the tissues for later use. Cancer inhibition rate was calculated according to the following formula: cancer inhibition rate=$W_{\text{SMMC7721 or SMMC7721-IGF1R-mutation}} W_{\text{SMMC7721-IGF1R-siRNA}}/W_{\text{SMMC7721}} \times 100\%$.

2.6 HE staining of cancer tissues

After the specimens were dehydrated, decolorized, immersed and embedded, the tissues were cut into slices of 5m thick. The slides were dewaxed with paraformaldehyde, put into anhydrous ethanol for 10 min, immersed into hematoxylin for 5-10 min, and washed with tap water. The nucleus was flushed with light ammonia water for 3-5 min, and with 70%, 80% and 90% anhydrous

ethanol for 10 min, respectively. The slides were stained with eosin for 10 min, washed with 95% ethanol twice, 100% ethanol once, and dimethylbenzene thrice for 10 min. The slices were sealed with resin and routine HE staining was performed. Then the slides were observed under a microscope.

2.7 IGF1R expression in tumor tissues detected by Western blot

About 30mg of fresh tumor tissues were weighed and SDS-polyacrylamide gel electrophoresis was performed. After electrophoresis, the protein from gel was transferred to the PVDF membrane by an electrical transfer. The membrane was fixed with methanol, and incubated in washing buffer containing 5% skim milk at 4℃ overnight. The membrane was incubated in 10ml primary antibody (rabbit anti-human IGF1R polyclonal antibody) (1:1000 dilution) at room temperature for 2 h, and in 10 ml secondary antibody (mouse anti-rabbit polyclonal antibody) (1:5000 dilution) at room temperature for 2 h. The membrane was treated with ECL chemical luminescent kit and developed in dark room. Gray scanning was performed using UV spectrophotometer and a visible light analyzer (FR-200) (Furi Science & Technology, with software).

2.8 Microvessel density (MVD) in tumor tissues detected by immunohistochemistry S-P method

According to the method designed by Weidner *et al.* [5], vascular epithelial cells were marked by anti-Ⅷ factor relevant antigen monoclonal antibody using immunohistochemistry. The colored capillary and microvessels were calculated. The computer assisted image analyzed system was used, and the average vessel number in five randomly chosen fields was used to indicate the MVD of the tissue.

2.9 Apoptosis of xenograft by TUNEL

Frozen slices were fixed using freshly prepared 4% paraformaldehyde for 30 min at room temperature. After being washed by PBS, slides were incubated in 0.3% formaldehyde and hydrogen peroxide for 30 min at room temperature. After being washed again by PBS, slides were reacted with 0.1% TritonX-100 sodium citrate solution on ice for 2 min. Slides were incubated with 50 l TUNEL reaction mixture in a wet box for 60 min at 37℃. After being washed and dried, the slides were incubated with 50 l conversion agent POD in a wet box for 30 min at 37℃, then with 80 l DAB solution at room temperature, observed under a microscope. Color reaction was stopped using PBS buffer. The slides were routinely redyed, dehydrated, and stored after confining. Five visual fields were chosen, and the ratio of apoptosis cells was calculated. Apoptosis index (AI) = the number of positively stained cells / the number of total cells × 100%.

2.10 Statistical analysis

Determined values were expressed as mean ± SD. Fishers definite probability methods and variance analysis were applied to perform hypothesis test. $P<0.05$ was set as the significance level.

3. Results

3.1 Latency of the nude mouse xenograft model

The latency of SMMC7721-IGF1R-siRNA group was (25.0± 1.8) days, and the latencies of SMMC7721-IGF1R-mutation group and SMMC7721 group were (13.5± 3.1) days and (14.7± 2.5) days. The tumor formation time was obviously postponed in the test group ($P<0.05$).

3.2 Cancer growth in nude mice

In the control group, the flat node appeared at the injection site 13 days after transplantation. In SMMC7721-IGF1R-siRNA group, the subcutaneous tumors grew more slowly than those in the control group. When the study was ended, the tumor inhibition rate was 54.8% in SMMC7721-IGF1R-siRNA group, which was significantly higher than those in SMMC7721-IGF1R-mutation group and SMMC7721 group ($P<0.05$). No animals died naturally in the test group (Figure 1).

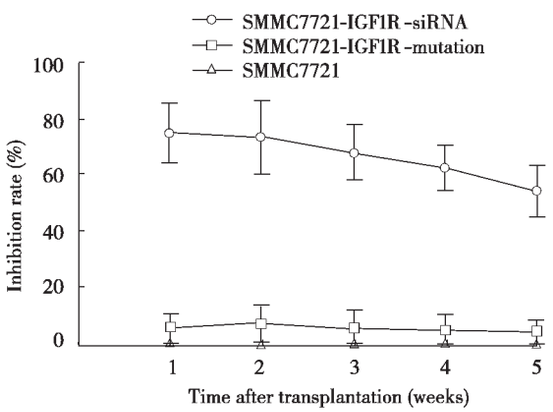

Figure 1 Inhibitory effect of IGF1R siRNA on the growth of human liver cancer SMMC7721 cell xenograft in nude mice

3.3 Gross observation of the xenograft tumors

The nude mice were sacrificed after the experiment, and the subcutaneous tumor tissues were removed and observed with naked eyes. It was found that the tumor mass was pink with uneven surface, in the form of a node or a lobe. The tumor was gray, fragile and easy to crack. In SMMC7721-IGF1R-siRNA group, the tumor was surrounded by the intact fibrous coat, which could be easily separated; while in the control group, the coat was not complete, and adhered to the surrounding tissues. No metastatic lesions were observed in the other tissues or organs (Figure 2).

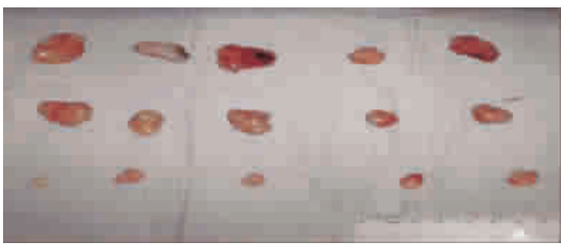

Figure 2 Gross observation of SMMC7721 cell xenograft in nude mice

Fiber capsules of the xenograft tumors in SMMC7721-IGF1R-siRNA group are complete, while fiber capsules of the xenograft tumors in SMMC7721-IGF1R-mutation group and SMMC7721 group are incomplete and adhere to surrounding tissues.

3.4 Pathological characters of tumors

On the HE stained slides, the volume of tumor cells were large. The cells had rich cytoplasm and the ratio of nucleus to cytoplasm was big. Cells were deeply stained, and megakaryocytes were observed. There were a lot of mitotic phases. The tumor nest was large, with a few interstitial tissues. A great number of sinusoids appeared. There were very thin connective tissue membranes separating the tumor from normal tissues, however, the coat was usually not complete. In contrast, in SMMC7721-IGF1R-siRNA group, there were less cancer cells, more distinctive cell borders, smaller cancer nests, richer interstitial tissues, rarer sinusoids. There were scattered pyknotic necrosis in some nucleus and cytoplasm, not accompanied with infection, and the fibrous coat of the node was obvious and intact (Figure 3).

3.5 IGF1R expression in tumor tissues

As shown in Figure 4, in SMMC7721-IGF1R-siRNA group, IGF1R was expressed more lowly than that in the control group. The amount of protein expression accounted for 18.0% in SMMC7721 group, while 17.0% in SMMC7721-IGF1R-mutation group ($P<0.05$).

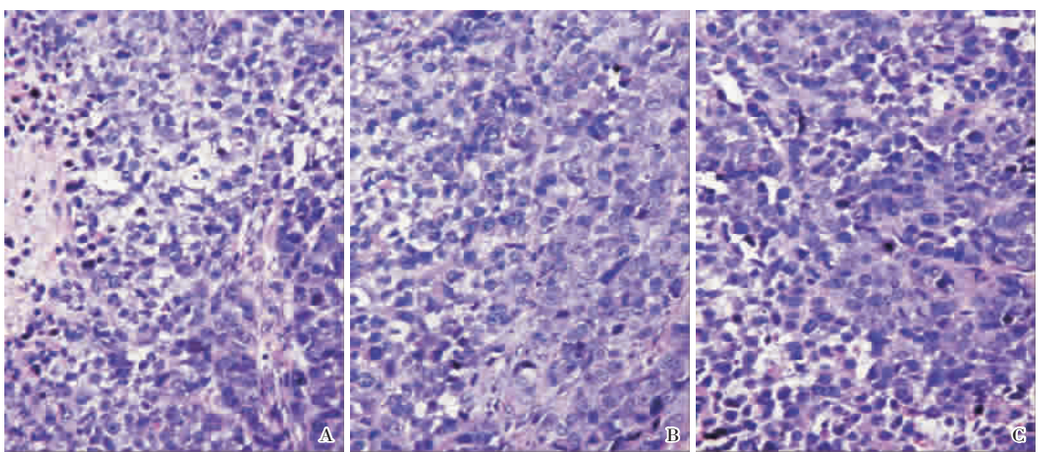

Figure 3 Morphology of the xenograft tumors in nude mice (HE ×200)

More necrosis tumor cells, less cancer nests and less blood sinuses are presented in SMMC7721-IGF1R-siRNA group (A); while tumor cells grow well, and more cancer nests and more blood sinuses are presented in SMMC7721-IGF1R-mutation group (B) and SMMC7721 group (C).

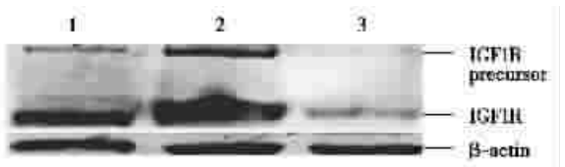

Figure 4 Expression of IGF1R protein in the xenograft tumors analyzed by Western blot

Lane 1: SMMC7721 cells; lane 2: SMMC7721-IGF1R-mutation cells; lane 3: SMMC7721-IGF1R-siRNA cells.

3.6 MVD in tumor tissues

The microvessel count was different in varied regions, with abnormal morphologies. Some of the vessels had no obvious lumen, and some were in the form of sprout. The MVD values in SMMC7721-IGF1R-mutation group (36.7± 7.6) and SMMC7721 group (28.4± 6.5) were obviously higher than that in

SMMC7721-IGF1R-siRNA group (11.3± 4.3) (P<0.05).

3.7 Apoptosis index of xenografted tumor tissues

In SMMC7721-IGF1R-siRNA group, some cells had positive reaction, and the AI was (50.2 ± 6.4)% . The AI values in SMMC7721-IGF1R-mutation group and SMMC7721 group were (5.4 ± 1.0)% and (6.0 ± 2.1)% , respectively, which were significantly lower compared to SMMC7721-IGF1R-siRNA group (P <0.05) (Figure 6).

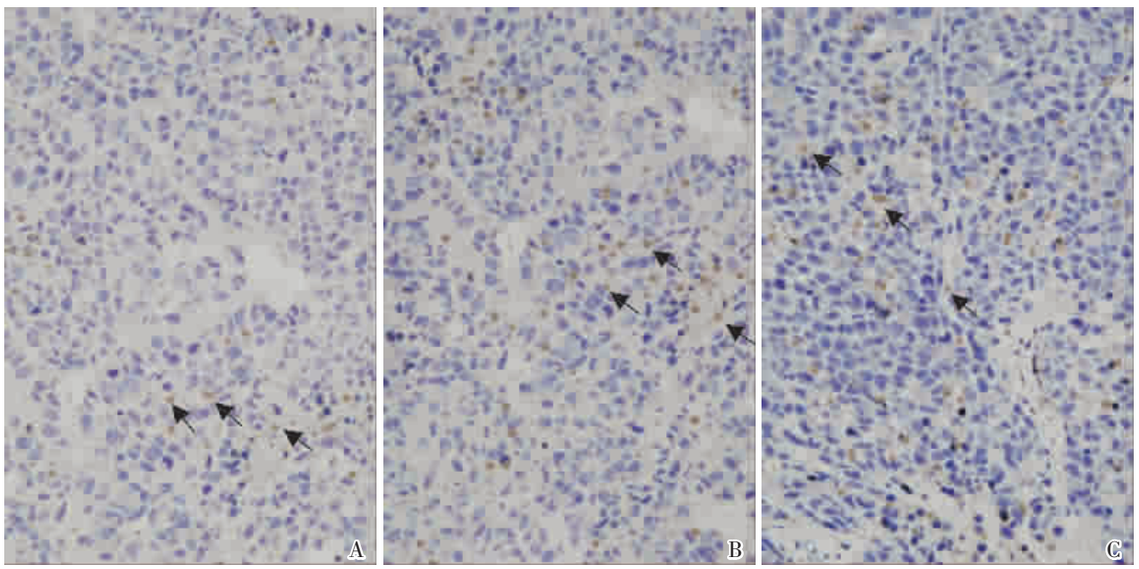

Figure 5 Microvessel density (MVD) in the xenograft tumors in nude mice (SP ×200)

MVD is lower in SMMC7721-IGF1R-siRNA group (A) than in SMMC7721-IGF1R-mutation group (B) and SMMC7721 group (C); arrows indicate pulmonary capillary endothelial cells.

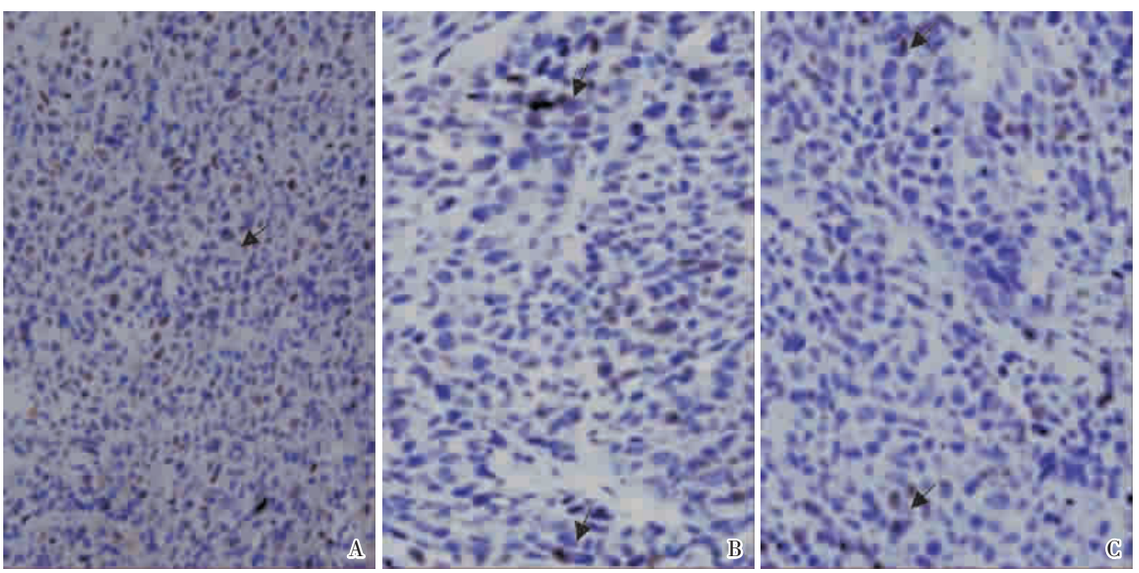

Figure 6 The tumor cell apoptosis in the xenograft tumors in nude mice (TUNEL ×100)

Scattered apoptosis cells with umber particles in nuclei are presented in SMMC7721-IGF1R-siRNA group (A); while much lesser apoptosis tumor cells are presented in SMMC7721-IGF1R-mutation group (B) and SMMC7721 group (C); arrows indicate apoptosis cells.

4. Discussion

In this study, when IGF1R expression was blocked by IGF1R-siRNA, the growth of SMMC7721 cell xenograft in nude mice was obviously inhibited. In IGF1R-siRNA group, the formation of sinusoids was obviously less than that in the control group, which indicates that, IGF1R-siRNA transfect is likely related to tumor angiogenesis. Under normal

conditions, angiogenesis is an ordered, strictly controlled and self-limited process. However, under pathological conditions, angiogenesis is significantly increased and not self-limited, and about 10% of the vascular epithelial cells actively proliferate. Pathological proliferation is a critical event in tumor growth and metastasis, which is closely related to the developing stage of cancer and vascular invasion. The MVD value of tumor tissues can effectively reflect angiogenesis, which can be used as an independent index to predict prognosis. In this study, immunohistochemistry was applied to determine the MVD value, and the results indicate that IGF1R-siRNA transfect exerted an inhibitory effect on angiogenesis. SMMC7721 cells transfected with IGF1R-siRNA could obviously inhibit the growth of subcutaneous tumors in nude mice, and the MVD value, which is closely related to cancer metastasis, was significantly decreased, demonstrating that blocking of IGF1R expression could significantly inhibit invasive capacity of liver cancer cells *in vivo*. IGF1R is selectively expressed in malignant cancer, therefore, it can be chosen as an effective target for cancer treatment [3, 6]. Resnicoff *et al.* [7] believed that, apoptosis was related with decreased amount of IGF1R. The development of cancer in nude mice was restrictively dependent on the amount of escaped and apoptotic cells and decreased IGF1R can induce cell apoptosis. It was indicated that, when fibroblasts were dispersed in mice subcutaneously, cells gradually underwent apoptosis; while the survival time of fibroblasts expressing IGF1R and primary cancer cell with increased expression of receptors was significantly prolonged. In addition, it is demonstrated in the study that, the amount of IGF1R is a critical factor on the survival of cells. Once it reached a certain level, a non-mitosis mode could be transformed into a mitosis mode [7].

We also confirmed that, when IGF1R-siRNA was transfected into SMMC7721 cells *in vitro*, cell proliferation could be inhibited [4]. Moreover, IGF1R-siRNA could down regulate IGF1R protein expression in cancer tissues, and inhibit cancer growth. The establishment of an ideal xenograft model is an important tool to study tumor biology and experimental treatment of cancer.

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