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Cloning and functional research of renal cell carcinoma related novel gene—GYLZ-RCC18

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Abstract  After the renal cell carcinoma related novel gene fragment GYLZ-RCC18 was cloned by using suppression subtractive hybridization (SSH) method, we cloned the full length of GYLZ-RCC18 and performed chromosome location by the FISH method. RT-PCR was used to detect the expression of the first reading frame of GYLZ-RCC18 in different stages and grades of renal cell carcinoma tissue and other tissues. Also we transfected the antisense oligonucleotide of GYLZ-RCC18 to renal cell carcinoma cell line GRC-1, and analyzed the proliferation activity, growth speed, apoptosis and mortality changes in GRC-1. The results show that the full length of GYLZ-RCC18 (GenBank accession No.: BE825133) cDNA is about 3.5 kb long which is located at No. 14 chromosome. GYLZ-RCC18 has a higher expression in higher grades and stages of renal cell carcinoma than in the lower ones. The expression of GYLZ-RCC18 in renal cell carcinoma was much higher than that in normal kidney and other tissues. After transfection of GYLZ-RCC18 antisense oligonucleotide, the mortality of GRC-1 increased evidently, the proliferation activity and growth speed were inhibited remarkably at the same time. Also the antisense oligonucleotide can induce the apoptosis of GRC-1 all through the observation time. Our results indicated that GYLZ-RCC18 is an important novel gene related to renal cell carcinoma. Its overexpression would stimulate the growth and proliferation activity and plays an antiapoptotic effect in renal cell carcinoma. Transfection of antisense oligonucleotide could inhibit the generation and development of renal cell carcinoma. The study provides a new clue for the research of renal cell carcinoma, and also provides an instruction for special genetic diagnosis and the therapy of renal cell carcinoma.

Keywords: kidney neoplasm, carcinoma, gene, GYLZ-RCC18.

Renal cell carcinoma is the third commonest cancer in the urinary system. But up to now, the molecular mechanism for its generation and development has remained unknown. After constructing the human renal cell carcinoma cDNA subtractive library and obtaining renal cell carcinoma relative gene fragment GYLZ-RCC18 by using the suppression subtractive hybridization (SSH) method, we cloned the full length of GYLZ-RCC18 and studied its function, which laid the foundation for its further research.

1 Materials and methods

(i) Renal cell carcinoma and normal kidney tissues and cell lines. 30 cases of fresh renal cell carcinoma samples came from the operations between April 1999 and February 2000, normal kidney tissues (5 cm away from tumors) were excised as control samples, and all the samples were confirmed by pathology. The tumor samples were divided into Robson stages T1, T2, T3, each containing 10 cases respectively, and Gleason grades G1, G2, G3, each containing 12, 10, 8 cases respectively. The average age of the patients was about 47 years old. Renal cell carcinoma cell line GRC-1 was constructed by the Institute of Urology, Peking University. Normal kidney cell line HK-2 was presented by Fred Hutchinson Cancer Center, USA. The GRC-1 and HK-2 cell lines were routinely maintained in RPMI-1640 (Gibco) with 10% fetal bovine serum (FBS) at 37°C under 95% air and 5% carbon dioxide in T-75 flasks. When the cells reached 80% confluence (approximately 5×10^6 per flask) 24 h later, cultures were changed to a medium of RPMI-1640 with 1% FBS and the experimental conditions were imposed onto these cultures. Culture flasks were divided into different treatment groups with five flasks for each. Antisense and sense oligonucleotide of GYLZ-RCC18 were designed and synthesized by Shanghai Biotechnological Company. Design of the primers of GYLZ-RCC18 and β-actin, sequence of cDNA and analysis of full length of GYLZ-RCC18 were supplied by Sina Company.

(ii) Cloning of the full length of GYLZ-RCC18 by SMART RACE. According to the sequence of RCC18 cDNA, we designed the 5' and 3' gene-specific primers (GSP1): an antisense primer (GSP1) for the 5' RACE PCR and a sense primer (GSP2) for the 3' RACE PCR. We cloned the full length of RCC18 cDNA by applying SMART (switching mechanism at 5'end of RNA transcript) RACE technique in combination of SMART RACE cDNA Amplification kit (Clontech Laboratories Inc.) with the following modification: after isolating mRNA by using mRNA purification kit (Pharmacia), 5'-RACE-Ready cDNA and 3'-RACE-Ready cDNA were synthesized respectively by combining 1 μg poly(A)+ RNA with 1 μL 5'-RACE cDNA Synthesis Primer or 3'- RACE cDNA Synthesis Primer. The mixture was incubated at 70 °C for 2 min, then added with 2 μL 5 × first- strand buffer, 1 μL DTT (20 mmol/L), 1 μL dNTP (10 mmol/L), 1 μL (200 units) MMLV reverse transcriptase (Gibco) and then mixed and incubated at 42°C for 1.5 h in an incubator. We
diluted the first-strand reaction product with 250 μL Tricine-EDTA buffer. PCR reactions were prepared in a manner as below: 5'-RACE-Ready cDNA or 3'-RACE-Ready cDNA 2.5 μL, PCR-Grade water 34.5 μL, 10× Advantage 2 PCR buffer, dNTP MIX (10 mmol/L) 1 μL, 10 × Universal Primer Mix 5 μL (long strand: 5'-CTAATACGACTCATATAGGGC-3'; short strand 5'-CTA- ATACGAC-TCACTATAGGGC-3'), GSP1 or GSP2 (10 μmol/L) 1 μL, 50× Advantage 2 Polymerase Mix 1 μL. Thermal cycling commenced by using the following program for touchdown PCR cycles: 5 cycles at 94°C for 33 s, 72°C for 3 min 10 s; 5 cycles for 94°C for 33 s, 70°C for 33 s, 72°C for 3 min 10 s; 23 cycles at 94°C for 33 s, 68°C for 33 s, 72°C for 3 min 10 s. The 5' and 3' RACE cDNA products were analyzed by electrophoresing on a denaturing 1% formaldehyde agarose/EtBr gel.

(iii) Detecting the chromosome location of GYLZ-RCC18 by using FISH. The probe of GYLZ-RCC18 was 5' RACE fragment about 3.0 kb DNA, which was randomly primed and labeled with Digoxigenin-11-dUTP by using DIG-High Prime Labeling 1 and Detection Starter Kit (Riche), following the recommendations of the product. Chromosome sample (Boning, Beijing) was warmed at 50°C for 2 h and then denatured at 75°C in 70% methylamine for 3 min, and washed by 70%, 90%, 100% ethanol respectively for 5 min each. In situ hybridization: 2 μL denatured probe was mixed with chromosome sample and hybridized at 37°C for 18 h. Washing and signal detection: the sample was washed in 50% methylamine/2 × SSC, 1 × SSC and then blocked in blocking buffer (3% BSA/4 × SSC/0.1% Tween20), added with 100 μL sheep anti-digoxigenin-rhodamine (Gibco), incubated at 37°C for 120 min, washed for 3 times, and then added with chromomycin A3 and incubated for 3 min, then observed through a fluorescence microscope after stimulation in 260 nm laser.

(iv) Expression of GYLZ-RCC18 in renal cell carcinoma and normal kidney tissues and cell lines. Total RNA was isolated from 100 mg RCC and normal kidney tissue or 5×106 cell lines respectively by using the RNA Purification Kit (Gibco Company) guided by the recommendations of the manufacturer. The quantity and quality of RNA were determined by spectrophotometry (Pharmacia Biotech) and electrophoresing samples on 1.0% agarose/EtBr gel. The absorbance was A260/A280 > 1.90, and 28S/18S was over 1.8. RT-PCR ready cDNA was synthesized respectively by combining 3 μg total RNA with Oligo(dt) Primer 1 μL. The mixture was incubated at 70°C for 2 min, then 2 μL 5 × first-strand buffer, DTT (20 mmol/L) 1 μL, dNTP(10 mmol/L) 1 μL. Superscript TMI RNase H-MMLV reverse transcriptase (Gibco Company) 1 μL (200 units) were added to the two tubes of mixture, which was subsequently incubated at 42°C for 1.5 h. Then PCR reactions were prepared as shown below: PCR-ready cDNA 2 μL, PCR-Grade water 35 μL, 10× Advantage 2 PCR buffer, dNTP MIX (10 mmol/L) 1 μL, GYLZ-RCC18 (upstream: 5'TGGTACTACTTGTCGTC-CTG3', downstream: 5'CCACGTTGTCAAGTGTGA- GA3'), or β-actin (upstream: 5' GTGGGGGCGCCCGCG- GCACC3', downstream: 5'CTTCCTTAATGTCGCA-GACAGT 3'), primer 1 μL, Taq polymerase (Promega Company) 1 μL. Thermal cycling was then commenced by using the following program for touchdown PCR cycles: 38 cycles at 94°C for 60 s, 59°C for 60 s, 72°C for 120 s. The PCR products were analyzed by electrophoresing on a denaturing 1% formaldehyde agarose/EtBr gel in the Gel. Doc 1000 system (Bio-Rad Company). The relative absorbance (ratio of AGLYZ-RCC18/Agactin) was used as the expression quantity of the sample.

(v) Transfection of antisense oligonucleotide of GYLZ-RCC18 into renal cell carcinoma cell line GRC-1 and changes of growth speed, apoptosis, proliferation activity, mortality and morphology. A couple of compensative antisense and sense oligonucleotide of GYLZ-RCC18 were designed at the beginning of the first reading frame, which was about 20 bp long. The GRC-1 cells were divided into three groups, which acted as negative control, sense and antisense respectively, while the normal kidney cell line HK-2 was used as normal control group. GRC-1 and HK-2 cells (2 × 10⁴/mL) were seeded in a 96-well culture plate (100 μL/well) and treated as described above. After 18 h treatment period, the sense and antisense cells were transfected by the method described in ref. [3] and recommendations of the manufacturer with the following specification/modification. A reagent: sense oligonucleotide (5'-GGCGAACTGCGTTCCGATGCA-3') or antisense oligonucleotide (5'-TGCACTCGGAACGCGTTCCGATGCA-3') 2 μg + 100 μL fetal serum free DMEM (Gibco Company, USA); B reagent: 10 μL LipofectAMINE 2000 Reagent (Gibco Company, USA) + 100 μL fetal serum free DMEM, incubated at room temperature for 15 min. A and B reagents were mixed and incubated for another 50 min, following an addition of 0.8 mL fetal serum free DMEM. The above-stated mixed reagent was then incubated to 96-well culture plate 20 μL/well with another 80 μL fetal serum free DMEM added. After an incubation in 37°C/5% CO2 condition for 5 h, 100 μL 20% fetal serum/DMEM 100 μL was added and cultured for further 18 h, then the culture medium was replaced by 200 μL 10% fetal serum/DMEM. Analyses were conducted on the changes of cells number, morphology, mortality, MITT test, apoptosis, eosin exclusion assay at 24, 48, 72, 96, 120, 144, 168 and 192 h respectively.