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Original article

Annexin A7 and its binding protein galectin-3 influence mouse hepatocellular carcinoma cell line in vitro

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ABSTRACT

Lymph node metastasis is recognized as an important mode of liver cancer metastasis. Our previous study has built two hepatocarcinoma cell lines, Hca-F with high (75%) and Hca-P with low (25%) incidences of lymph node metastasis, and has indicated that annexin A7 is an important factor in the lymphatic metastasis of tumors. There is evidence that galectin-3 is the binding protein of annexin A7 and works in protein complexes. Our current study shows that both annexin A7 and galectin-3 express higher in Hca-F than Hca-P. Annexin A7 was successfully down-regulated in Hca-P by RNA interference, and this resulted in concomitant reduction of galactin 3 expression in annexin A7 down regulated compared to the control and N-control cells. Using CCK-8 assay, the expression level of annexin A7 and galectin-3 were found to have correlation with the proliferation ability; Transwell assay showed annexin A7 and galectin-3 are involved in cell migration and invasion regulation in mouse hepatocellular carcinoma cell lines, immunofluorescence assay indicate annexin A7 and galectin-3 were co-located annexin A7 and galectin-3 played roles in DNA damage and cell proliferation cycle checkpoint arrest pathway. Those phenomena indicated that annexin A7 influences lymphatic metastasis of tumors by interacting with galectin-3 through the regulation of tumor cell proliferation, attachment, migration and invasion.

1. Introduction

Liver cancer is one of the common malignant tumors. Lymph node metastasis (LNM) is an important factor resulting in poor prognosis and high mortality. Tumor metastasis includes four steps: proliferation, attachment, migration and invasion. Figuring out liver cancer lymph node metastasis-related proteins will be of potential benefit to decreasing the relapse and death rates of cancer patients and contribute new ideas to clinical treatment.

Annexin A7 (ANXA7) belongs to calcium binding protein families. It is described as a tumor suppressor gene. Annexin A7 was found to be a candidate gene related to lymphatic metastasis of hepatocarcinoma in mice [1,2]. Annexin A7 knockout mice have a high frequency of spontaneous tumors [3]. Previously our lab employed suppressive subtracted hybridization (SSH) technique to identify differentially expressed genes in Hca-F and Hca-P. The combination of HPLC/nESI-MS/MS, 2-D DIGE and Gene Chip micro array were used to screen, quantify and identify lymphatic metastasis-associated markers in mouse hepatocarcinoma cell lines. Transwell assay also show that down-regulated annexin A7 could decrease cell invasion and motility as well as proliferation. Annexin A7 as a protein spots was investigated to be associated with lymph node involvement in tumor metastasis [4–6]. Galectin-3 (GAL-3) is a 30 kDa protein that plays important roles in lots of activities of cancer malignant behaviors, such as adherence and growth. It is also involved in tumor occurrence and progression.

Annexin A7 was also found to interact with galectin-3 and affects it's release progress, but the mechanism of how annexin A7 and galectin-3 regulates tumor is still unclear [7]. Studies on annexin A7 and it's protein complexes with galectin-3 will help to reveal the role of annexin A7 in the mechanism of tumorigenesis and its lymphatic metastasis, which could be of therapeutic benefit.

2. Material and method

2.1. Animals and cell lines

Six hundred and fifteen mice were provided by the SPF animal center of Dalian Medical University (license number: SCXK [Liao] 2008-0002). Male mice were selected and fed till 6–8 weeks old and weigh 18–22 g. Mouse hepatocarcinoma cell lines Hca-F and Hca-P were established and stored by key laboratory of tumor metastasis in Liaoning Province. The shRNA-ANXA7 (Hca-$P_{ANXA7\text{-}down}$) cell line was established by transfection with shRNA. Three kinds of shRNA

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were designed and inserted into the pSilencer-shRNA vector. Vectors were selected based on the result of RT-PCR and Western Blot. The Hca-P cells were transfected with the most effective pSilencer-shRNA.

2.2. Cell culture

Hca-F and Hca-P tumor cells were injected at 2×10^6 cells in 0.1 ml cell suspension into inbred 615 mice and were grown 7 days. These cells were drawn and injected again in other 615 mice and allowed to grow 5 days. Two passages were done in order to harvest large number of cells. The cells in ascites were drawn and seeded into vials for culture in 90% RPMI 1640 medium supplemented with gentamicin/streptomycin 100 U/ml. 10% fetal bovine serum (Gibco) for 24 h in a 5% CO_2 atmosphere at 37 °C in a humidified atmosphere. Cell viability was determined by Trypan blue exclusion test.

2.3. Stable transfection of cells

pGPU6/GFP/Neo-shRNA-Annexin A7 (shRNA-ANXA7) and pGPU6/GFP/Neo (N-control) plasmids were transfected into Hca-P cells. The cells (4×10^5 cells/well) were added into a 24-well plate 1h prior to transfection, and transfected with 2 ug of two different plasmids using Sofast™ transfection reagent (Xiamen Sunma Biotechnology Co., Ltd., China) according to the instructions of the manufacturer. Transfection efficiencies were determined by fluorescence microscopy at 24 and 48 h. The stably transfected cells were obtained by growing them in 400 ug/ml G418 (gibco by life technologies, USA) for three weeks. Those plasmids were transfected in Hca-P cells stably. The expression of annexin A7 mRNA and protein were detected by real time quantitative polymerase chain reaction (qRT-PCR) and western blot analysis.

2.4. RT-PCR analysis

Cells were divided into four groups: group 1: Hca-F, group 2: Hca-P, group 3: $P_{ANXA7\text{-}control}$, group 4: $P_{ANXA7\text{-}down.}$ Total RNA was isolated from the four groups of cells using TRIzol (Invitrogen, USA), according to the instructions of the manufacturer. Before reverse transcription, 1.0 ul gDNA Eraser for 600 ng total RNA was added to eraser DNA in genome (condition: 2 min at 42 °C). Reverse transcription of purified RNA was performed using PrimeScript RT Enzyme Mix 1 and RT Primer Mix, reaction solution mixed on the ice. This step on the condition: 15 min at 37 °C then 5 s at 85 °C. The sequences of GAPDH gene primers were as follows: forward primer, 5′-TGTGTCCGTCGTGGATCTGA-3′; reverse primer, 5′-TTGCTGTTGAAGTCGCAGGAG-3′. The sequences of annexin A7 gene primers were as follows: forward primer, 5′-TTTCGGGA-TACGTTGAAAGTGG-3′; reverse primer, 5′-TCTgATACATCTGGGT-GAACATCTG-3′. The sequences of galectin-3 gene primers were as follows: forward primer,5′-CATTGTGTGTAACACGAAGCAGGAC-3′; reverse primer, 5′-CTGCAGTAGGTGAGCATCGTTGA-3′. Two stages standard SYBR Green qPCR progress were performed. Stage 1, one cycle (30 s at 95 °C). Stage 2, 40 cycles (95 °C for 5 s, then 60 °C for 31 s). I also added dissociation stage: one cycle (15 s at 95 °C and 60 s at 60 °C then 15 s at 95 °C). The quantification of gene transcripts was performed by qRT-PCR using SYBR green I dye and normalized with GAPDH as the internal control.

2.5. Western blotting

The cells were lysed according to the standard methods. In the process of protein extraction, phosphatase inhibitor was added. Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit with bovine serum albumin as a standard.

Equal amounts of protein from each group were separated for GAPDH/annexin A7/galectin-3 expression using 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA). They were incubated with a polyclonal antibody against annexin A7 (Sigma, USA; 1:1500) galctin-3 (abcam, Hong Kong; 1:1000) GAPDH (ZSGB-BIO, China; 1:1000) in 5% dried milk for 1 h, and then incubated with Alexa Fulor 680 donkey anti-mouse IgG (H + L) (molecular probes life technologies, USA, 1:15000) for 1 h at 37 °C.

2.6. Cell proliferation assays

Cell proliferation was assessed by cell counting kit-8 (CCK-8, Dojindo, Japan) according to the protocol of the manufacturer. Triplicates of 1×10^4 cells/well of each Hca-F/Hca-P/$P_{ANXA7\text{-}down}$/$P_{ANXA7\text{-}control}$ cells were plated in 96 well plates. 10 ul of CCK-8/well were added at 24 hrs, 48 hrs, 72 hrs, and 96 hrs. Multiskan Go Spectrophotometer (Thermofisher Scientific, USA) was used to check the absorbance of cells at 450 nm.

2.7. Cell migration assay

The Boyden-transwell assay (8 um pore size) was performed to analyse cell migration ability. Cells were suspended in RPMI1640 medium without serum and harvested at the concentration of 4×10^5/ml cells. The cells were put in the incubator of 5% CO_2 at 37 °C for 24 h. After incubation, 100 ul cells were laced in the upper chamber, each kind of cell was made in triplicate. Then, 500 ul RPMI-1640 medium which contains 20% FBS was placed in the lower chamber. Chambers were put in the incubator of 5% CO_2 at 37 °C for 24 h. The medium and the cells left in the upper chamber were removed by cotton swab, and the chambers were kept in room temperature for 30 min to dry. Chambers were immersed in 1% crystal violet solution for 20 min, and then washed with PBS several times. Cells migrated on the underside of membrane were counted by light microscope. The determinations were done in five visual fields.

2.8. Cell invasion assay

Cell invasion was measured with transwell units coated with ECM (sigma, USA) by incubating at 37 °C for 1 h to generate the artificial basement membrane. The membranes were rehydrated with 10 ul serum-free RPMI1640 with the further steps as performed for the cell migration assay.

2.9. Immunofluorescence assay

The Hca-P cells were fixed 10 min in acetone. The cells were washed with PBS and then blocked by incubating in 1% BSA for 1 h. The cells were then incubated with first antibody (monoclonal to galectin-3: abcam, Hong Kong, 5 ug/ml; monoclonal to annexin A7, sigma, USA, 1:200) for 1 hour at 37 °C. After washing cells with PBS three times for 5 min each, the second antibodies, (ZSGB-BIO; China): Rhodamine (TRITC)-Conjugated AffiniPure Goat Anti-rabbit IgG (H + L) and Fluorescein-Conjugated AffiniPure Goat Anti-mouse IgG (H + L) were used at 1:50 dilution for 40 min at 37 °C. DAPI (sigma; USA) at a concentration of 5 ug/ml was used to stain the cells' nuclei for 5 min.

2.10. Data analysis

SPSS software version 13.0 for Windows (SPSS Inc, IL, USA). Statistical analysis of the data, expressed as mean ± S, was performed using standard one-way analysis of variance (ANOVA) or one-way ANOVA for repeated measures. Statistical significance was set at $P < 0.05$.