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Original article

Down-regulation of ANXA7 decreases metastatic potential of human hepatocellular carcinoma cells in vitro

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ABSTRACT

We report for the first time the influence of ANXA7 gene on human hepatocellular carcinoma cells (HCC). We down-regulated ANXA7 in human HCC cell line (HepG2) using siRNA method. By Western Blot analysis, we confirmed about 70% down-regulation of the gene in the shRNA-ANXA7 transfected cells (shRNA-ANXA7-HepG2) compared to the non-specific sequence shRNA transfected cells (control-shRNA-HepG2) and the un-manipulated-HepG2 cells. We used CCK-8 cell proliferation kit and observed about 65% reduction in the shRNA-ANXA7-HepG2 cells where the two controls exhibited comparable cell proliferation rates. Also, by using PI staining followed by flow cytometry, we noticed a cell cycle arrest at G_0/G_1 with more than one fold reduction of shRNA-ANXA7-HepG2 cell population in the S-phase of the cell cycle. Also of particular note was a significant aneuploidy in the controls compared to zero aneuploidy in the ANXA7 down-regulated cells. Migration of the cells was detected using Boyden's transwell chamber and scratch wound healing assay which showed 50% and 30% respective reductions in shRNA-ANXA7-HepG2 cells migration. Furthermore, the control-shRNA-HepG2 cells and the un-manipulated-HepG2 cells invaded through the ECM-coated transwell plates two times more than the shRNA-ANXA7-HepG2 cells. We have found ANXA7 to be functioning like a tumour promoter in HepG2 human hepatocellular carcinoma cells and could have a potential as a therapeutic window into the management of liver cancer.

1. Introduction

Hepatocellular carcinoma (HCC) is an aggressive disease with usually less than one year survival after diagnosis. It is more prevalent in the Asian and African populations occurring in men twice as often as in women with its trends on the ascendancy over the past 10 years [1]. Globally cancer is the leading cause of death with liver cancer being only next to lung and stomach cancers in worldwide mortality [2].

Annexins are a family of calcium-dependent phospholipid-binding proteins, widely distributed in different tissues including the liver [3,4]. Since their discovery, the annexins have been linked to the cancer "organized crime committing syndicate", and are postulated to have a complex set of functions that may have a therapeutic potential for malignant disease treatment [5].

Annexin A7 (synexin), the first annexin to be described as an isolated, purified protein is expressed in 47 kDa and 52 kDa isoforms [6] and interferes with galactin-3 action of promoting cell cycle progressing and survival [8]. This is seen to be one of the mechanisms of Annexin A7 involvement with cell proliferation. In fact, the chromosomal location of ANXA7 (10q21) has long been hypothesized to carry a gene likely to be a tumor suppressor [7].

Lack of expression of Annexin A7 was found to be associated with high proportions of metastatic prostate cancer in humans [8] and is also a marker for the less invasive phenotype of malignant melanoma [9].

Because Anxa7 has been hypothesized to be a putative tumor suppressor gene, Srivastava et al. developed an Anxa7 gene knocked out mice to ascertain the validity or otherwise of the hypothesis. They discovered that the double hit model created, Anxa7(−/−), was lethal at early embryonic stage whilst the allelic insufficient type, Anxa7(+/−), demonstrated genomic instability, frequent loss of other tumour-suppressor genes, and was prone to cancer [10]. However, in sharp contrast to the earlier report by Herr et al. [11] also generated similar Anxa7 knock-out chimeric mice and their Anxa7 knock-out homozygous mice, Anx7(−/−), were healthy and fertile with no cancer prone tendencies.

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By immunohistochemical analysis, Sun et al. found Annexin A7 to be expressed in an increasing order from normal gastric tissue (25.0%), gastric cancer tissue without lymphatic metastasis (53.3%), to gastric cancer tissue with lymphatic metastasis (80.0%), indicating that the expression of Annexin A7 is positively correlated with the degree of malignancy as well as the lymphatic metastatic status of gastric cancer [12].

For the past few years, our lab has been conducting research on Anxa7 using two mouse syngeneic hepatocarcinoma cell lines (HcaF with 75% LNM and HcaP with 25% LNM) established by our lab and have demonstrated to be ideal in studying liver cancer in the mouse model [12–17].

These works have shown Anxa7 among other 33 genes to be highly expressed in the HcaF cell line with high lymphatic metastatic rate of 75% relative to the HcaP with 25% LNM, highlighting the involvement of Anxa7 with liver cancer and or lymphatic metastatic spread [13–15].

It is quite obvious from the forgoing that the "manhunt" for the putative role of Anxa7 in carcinogenesis is still slippery. More so, not much is known about the effect of ANXA7 on human hepatocellular carcinoma. We hypothesized that silencing Annexin A7 in a hepatocarcinoma cell line could lead to a reduction in the carcinogenic potential of the cells and could be of therapeutic benefit in liver cancer prevention and or treatment. In this paper, we silenced ANXA7 in HepG2 human hepatocarcinoma cell line and investigated the altered malignant properties under the lenses of cell proliferation, migration, invasion and cell cycle status. This is the first investigation addressing the role of ANXA7 in human hepatocellular carcinoma.

2. Materials and methods

2.1. Cell lines and cell culture

HepG2 liver cell line, obtained from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology (Shanghai, China), was cultured routinely in DMEM with high glucose (Gibco, USA) containing penicillin 100 units/ml, streptomycin 100 μg/ml and 10% FBS (PAA). The cells were maintained in an incubator at 37 °C with 5% CO_2 atmospheric condition.

2.2. shRNA design and plasmid construction

Five different pairs of shRNAs, four targeting the ANXA7 mRNA sequence and one with non-targeting sequence to be used as a control, were designed and inserted into pGPU6/GFP/Neo siRNA expression vectors containing neomycin resistance gene to enable antibiotic selection. All the expression vectors were constructed by Shanghai GenePharma co., ltd (Shanghai, China) and transfected into DH5 alpha *E. coli*. We transfected the expression plasmids into HepG2 cells and selected the siRNA with the best silencing effect by Western Blot analysis. The best ANXA7 silencing plasmid was named shRNA-ANXA7 and the negative control was named control-shRNA-HepG2. Their hairpin sequences were: 5′-GGATATGTA-GAAAGTGGTTTGTTCAAGAGACAAACCACTTTCTACATATCCTT-3′ for shRNA-ANXA7 and 5′-GTTCTCCGAACGTGTCACGTAAGAGAGAT-TACGTGACACGTTCGGAGATT-3′ for control-shRNA-HepG2.

2.3. HepG2 stable transfection

A day prior to transfection, 5×10^5 cells per well was plated into a 6-well plate in an antibiotic free DMEM with 10% FBS and placed in an incubator with a temperature of 37 °C with 5% CO_2. Transfection was performed the following day with 75 pmol of shRNA using Lipofectamine 2000 Reagent (Invitrogen, USA) according to the manufacturer's protocol. Transfection efficiency was assessed 24 hrs post-transfection by viewing the green fluorescing cells under an inverted fluorescence microscope. Prior to the transfection, we empirically determined G418 selection concentration to be 350 ug/ml of effective drug concentration, and 72 hrs post-transfection the cells were cultured in a medium containing this pre-determined G418 concentration until the entire cells in the non transfected wells died off. We continued to culture the cells under the selection pressure of the G418 until they became completely resistant to the drug. The stably transfected cells were then culture-expanded and the level of ANXA7 protein down-regulation was detected by Western Blot. The shRNA-ANXA7 transfected cells, the control-shRNA transfected, and the un-manipulated HepG2 cells were named: shRNA-ANXA7-HepG2, control-shRNA-HepG2 and un-manipulated-HepG2 respectively.

2.4. Detection of Annexin A7 expression by Western Blot

Cells in the log phase of growth were trypsinized, harvested and washed twice with ice-cold PBS. Total cell protein was extracted and then quantified by Nanodrop spectrophotometer (Thermofisher Scientific USA). Equal amounts of proteins prepared into equal volumes were loaded onto a gel (SDS-PAGE) and separated by electrophoresis. Guided by a pre-stained protein molecular weight ladder, portions of the gel corresponding to the molecular weights of Annexin A7 and GAPDH protein were sectioned out and transblotted onto a PVDF membrane (Invitrogen, USA). The membrane was blocked in 5% non-fat dried milk for one hour and then probed with monoclonal mouse anti Annexin A7 (Sigma-Aldrich USA, 1:1500) and anti GAPDH (ZSGB-Bio, China, 1:7500) primary antibodies for 1hr. After washing the membrane six times, Goat anti mouse secondary antibody, which is horseradish peroxidase-linked (1:4000 dilution), was then applied for 1hr followed by ECL (Beyotimes, China) and the bright bands were captured in the darkroom using X-Ray film.

2.5. Cell proliferation analysis

The effects of Annexin A7 down-regulation on cell proliferation was measured using Dojindo's CCK-8 cell proliferation kit (Dojindo Molecular Technologies, Japan). Briefly, triplicates of 3×10^3 cells/well of each of shRNA-ANXA7-HepG2, control-shRNA-HepG2 and un-manipulated-HepG2 cells were plated in 96 well plates. We added 10 ul of CCK-8/well at 24 hrs, 48 hrs, 72 hrs and 96 hrs and checked the absorbance at 450 nm using Multiskan Go spectrophotometer (Thermofisher Scientific, USA).

2.6. Flow cytometry analysis

Cells were synchronized at G_0/G_1 phase by growth in 100% confluence with reduced serum for three days [18,19]. The cells were then passaged and cultured for 24 hrs after which they were harvested in the log phase of growth, washed twice with ice-cold PBS and fixed in 75% cold ethanol overnight at 4 °C. The following day, the cells were washed twice with ice-cold PBS after discarding the ethanol, following which 50 μg/mL of RNase (Sigma, USA) was added for 30 min and then stained in 20 μg/ml of Propidium Iodide (Sigma, USA) overnight in darkness. The cells were analyzed by flow cytometry (Beckman Coulter, USA) and the data were analyzed by MultiCycle software (Phoenix Flow Systems, San Diego, USA) to get the cell cycle distributions.

2.7. Scratch Wound Healing assay

We plated 1×10^5 cells per well in 24 well plate and allowed the cells to form 100% confluence overnight. Thereafter, the cells