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Original article

Ezrin expression is altered in mice lymphatic metastatic hepatocellular carcinoma and subcellular fractions upon Annexin 7 modulation in-vitro

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ABSTRACT

Ezrin and Annexin seven (A7) have been suggested to be involved in several roles in cancers metastasis. However, the role of Ezrin and the effect of A7 on Ezrin expression in lymphatic metastatic hepatocellular carcinoma (LNM-HCC) have not been extensively explored yet.

This study reports expression of Ezrin in high lymphatic metastasis (Hca-F >70%) and low metastatic metastasis (Hca-P <30%) HCC cell lines, and the effect of A7 on Ezrin expression.

Real-Time PCR, Western blot, Subcellular fractionation, Immunocytochemistry and Immunofluorescence were used to investigate Ezrin expression in addition to migration and invasion behaviors of A7 up-regulated Hca-F cells, A7 down-regulated Hca-P and in their respective negative control (NC) cells.

Ezrin expression was higher in high LNM-HCC than low LNM-HCC ($p = 0.0046$). Cell fractionation analysis reveals that Ezrin was highly present in the cytoplasm, nucleus and cytoskeleton of NC-Hca-F cells. However, Ezrin was highly observed in the cell membrane, nucleus and cytoskeleton of NC-Hca-P cells. A7 up-regulation in Hca-F suppressed Ezrin expression ($p = 0.0248$), but increase the migration and invasion, whereas Ezrin was mainly located in the cytoplasm and nucleus fractions. Down-regulation of A7 in Hca-P cells, enhanced Ezrin expression ($p < 0.0001$) in the cytoplasm and nucleus fractions, and suppressed migration and invasion.

In conclusion, Ezrin may play a role in LNM-HCC and might be inversely associated with A7 expression. The subcellular localization of Ezrin and A7 was varied according to the metastatic levels. Ezrin may thus be a potential diagnostic and/or prognostic biomarker for HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is an aggressive disease, ranking third in cancer-related deaths worldwide. Fifty percent of all HCC cases are found in China [1,2]. HCC is heterogeneous in nature, commonly resistant to chemotherapy and accounts for 90% of liver malignancies [3]. Lymph node metastasis (LNM) involves the movement of malignant cells from the primary tumor site to the lymph nodes and continuing to other organs through the lymphatic vessels in full accounts for approximately 7.45% of HCC metastatic cases. Hepatitis B virus (HBV) and Hepatitis C virus (HCV) infections are the main reported causes of the disease [1,3,4]. At initial stages, the disease does not show definitive signs and symptoms. Hence, it mostly diagnosed in the advanced stage, making it difficult to treat. The urgent need for specific and sensitive biomarkers for HCC diagnosis can therefore not be over emphasized.

For many years, we have been working on Annexin 7 (A7) and LNM-HCC using Hca-F high lymphatic metastatic (>70%) and Hca-P low lymphatic metastatic (<30%) hepatocellular carcinoma cell lines at both the genetic and proteomic level [3–5].

A7 is a member of the calcium-binding protein family [6]. It is located in the cytoplasm, and is found to be involved in cell proliferation, maturation, membrane fusion and selectivity [5,7]. A7 has two isoforms with molecular weights of 47 kDa and 51 kDa [8]. Down-regulation of A7 in Hca-P cells decreases invasion and

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migration potential in Hca-P cells [6]. On the other hand, A7 up-regulation in Hca-F cells suppresses the HCC progression [3].

Ezrin is a compartment of Ezrin/Radixin/Moesin (ERM) plasma membrane protein linking the cytoskeleton to the cell membrane [9]. Ezrin, cytovillin or villin-2 are encoded by the *EZR* gene [10]. Ezrin is located in the cytoplasmic peripheral membrane, Ezrin works as a protein-tyrosine kinase substrate and functions in cell adhesion, migration, proliferation, and organization [6,11,12]. Ezrin can bind sodium-hydrogen exchanger regulatory factor (NHERF) proteins, actin phosphatidylinositol biphosphate (PIP2) and/or membrane proteins like CD44 and ICAM-2 [11,13]. Such amplexes could play essential roles in cellular activities such as in signal transduction, growth control, cell-cell adhesion, cell surface tension, microvilli formation and migration [14]. Overexpression of Ezrin is reported in breast cancer [15], Lung cancer [16], Osteosarcoma [17], Leukemia [18], Cutaneous melanoma [19] and Colorectal carcinoma [20]. However, higher expression of Ezrin in osteosarcoma is not always associated with poor disease prognosis [17].

Ezrin and A7 are involved in various cellular and biological activities in several types of cancers [1,2,17,21]. The data on Ezrin expression in LNM-HCC is currently limited; additionally, the effect of A7 on Ezrin in HCC is not yet to be known. The present study sorted to determine the role of Ezrin in HCC-LNM and the effect of A7 on Ezrin in HCC-LNM.

2. Materials and methods

2.1. Cell lines and cell culture

Mouse synegic LNM-HCC high lymphatic metastasis (Hca-F, >70%) and low lymphatic metastasis (Hca-P, <30%) cell lines established in our laboratory, the Department of Pathology and the Key Laboratory for Tumor Metastasis and Intervention Studies of Liaoning Province, Dalian medical university, China, were used in this study based on the standard protocols [2–5].

Hca-F and Hca-P cell lines were cultured in 90% RPMI 1640 (Grand Island, USA) with 10% fetal bovine serum albumin (PAA, USA) for two days at 37 °C humidified incubator with 5% CO_2. Cells were intraperitoneally injected into 615 Chinese inbred mices from the Specific Pathogen Free Animal Center (SPF) in Dalian Medical University. After 10 days, the cells were harvested from fully palpable ascites developed in the mice and were used for various experiments [2–5].

2.2. Stable transfection of cell lines

The previously published works in our lab have demonstrated that A7 is highly expressed in Hca-F than in Hca-P [3–5]. We therefore up-regulated A7 in Hca-F and down-regulated A7 in Hca-P cell lines to investigate the A7 on Ezrin.

Complementary DNA 3.1 plasmid was amplified in PG-CMV/EGFP/Kan/Neo vector using a eukaryotic enzyme' to produce PG-CMV-EGFP-LNheI/Ecor A7 (Gene pharma, China) and PG-CMV-EGFP-Kan/Neo-GAPDH, as a positive control and the negative controls were plasmid free gene transfected cells. A7 was up-regulated in Hca-F cells by adding 2 μg of PG-CMV-EGFP-LNheI/Ecor A7 (cDNA-ANXA7) to 8×10^5 Hca-F cells per well in a six-well plate. To down-regulate A7 gene in Hca-P, 2 μg of PGPU6/GFP/Neo-shRNA-A7 (shRNA-ANXA7) was added to in 8×10^5 Hca-P cells per well in a six-well plate. Six microliter per well of sofast™ (Xiamen Sunma- Biotech, China) transfecting solution was added to each well. After 48 then after 72 h, transfection efficiency was assessed by fluorescence microscopy. Stable transfection was achieved by selective with 400 μg/μl of G418 (Geneticin) for three weeks. Transfection stability was confirmed by RT-PCR and western blot.

2.3. Subcellular fractionation

Subcellular fractionation kit for mammalian cells (Thermo scientific, USA) was used to separately fractionate NC-Hca-F, NC-Hca-P, A7 cDNA- Hca-F and A7-shRNA Hca-P cells into five subcellular fractions (cytoplasm; membranes and membrane's organelles; nucleus membrane and its soluble materials; nucleoli and chromatin bonds and cytoskeleton). An amount of 7×10^6 cells/ml of suspended cells were transferred into 1.5 ml tube and washed three times with cold PBS for three minutes at 500 rpm/g. Fractionation buffers were added to the tubes and centrifuged. The supernatant was collected and the remaining pellets were washed with PBS for each cell fraction. The volume of fractionation buffers and centrifuge speed were according to the manufacturer manual.

2.4. qRT-PCR

Total cellular mRNA was extracted using TRIzol reagent (Invitrogen, USA) according to manufacturer's instruction from the various cell lines. 500 ng of mRNA from each cell line was added to super mix 5× reagent (Transgen Biotech, China) with remover reagent to prepare cDNA in a thermocycler (Bio-Rad, Singapore) at 42° C for 15 min and then 85° C for 5.0 s. The quantification of Ezrin and A7 was performed by qRT-PCR using SYPR green II dye (Transgene Biotech, China) by three step PCR reaction condition; 94° C for 30 s, 94° C for 5.0 s, 55° C for 15 s and 72° C for 10 s for 45 cycles with a dissociation stage. The primers used were: GAPDH forward primer 5′-TGTGTCCGTCGTGGATCTGA-3′; GAPDH reverse primer 5′-TTGCTGTTGAAGTCGCAGGAG-3′; m-Ezr Forward primer 5′-GGTACTTCGGCCTCCAGTATGT-3′; m-Ezr-Reverse primer 5′-GTTCCTCGGCCACGTCTTC-3′; m-ANNXA-R Forward primer 5′-TCTGATACATCTGGGTGAACATCTG-3′ and m-ANNXA-R Reverse primer 5′-CATGAACAGCGCAAGGATTA-3′. GAPDH was used as endogenous gene control. The qRT-PCR results were analyzed with the MXP/CXP software (Agilent Technologies, Germany). The relative mRNA expression was calculated using the comparative $^{\Delta\Delta}$Ct [22].

2.5. Protein extraction

Proteins were extracted from NC-Hca-F, NC-Hca-P, shRNA-Hca-P and cDNA-Hca-F HCC cell lines using RIPA buffer, DTT, PMSF and ×100 antiprotease cocktail (Roche, CA, China) in a ratio of 95:1:1:3 respectively. The concentration of total and subcellular proteins concentrations was evaluated by spectrophotometer at 595 nm using bicinchoninic acid assay (BCA) kit (Thermo SCIENTIFIC, USA) with bovine serum albumin as the standard.

2.6. Western blot

Western blot technique was applied to confirm Ezrin and A7 expressions by loading equal amount of cell proteins (50 μg/well) in 10% sodium dodecyl sulphate-poly acrylamide gel electrophoresis (SDS-PAGE). The protein bands were transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, USA) and blocked with 10% skim milk (Claix, France) for two hours at room temperature. The membranes were then incubated with either 1 μg/ml Anti-Ezrin monoclonal rabbit anti-mouse primary antibody (Abcam, USA), A7 polyclonal rabbit anti-mouse primary antibody (Protein tech, USA) in 1:1000 dilution, and GAPDH polyclonal antibody (Protein tech, USA) as loading control, overnight at 4 °C. PVDF membranes were washed and proteins bands detected using the appropriate secondary antibody 1:10000 (LI-COR, USA) for an hour and visualized by *LI-COR* scanner (LI-COR, USA).