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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).

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Quantification of protein expression after western blot was achieved by using ImageJ software. (International Institute of Health, USA).

2.7. Immunocytochemistry

Hca-F and Hca-P cell lines (1×10^6 cells of each) separately respectively spread on poly-L-lysine coated slides, air dried and antigen retrieved by 0.01 M citrate buffer (pH 6.0) under low microwave temperature for 20 min. Hydrogen peroxide (3%) was added after slides were cooled, washed three times with cold PBS and incubated with reagent (A) of an IHC kit (ZSGB-BIO, China) for 10 min at room temperature. The cells were then incubated overnight with 1 μg/ml Anti-Ezrin monoclonal rabbit anti-mouse primary antibody (Abcam, USA) at 4° C. Afterwards they were washed three times with cold PBS; cells were incubated with reagent (B) of an IHC kit at 37 °C for 10 min, washed three times with PBS and incubated with reagent (C) of an IHC kit at 37 °C for 10 min. Finally, diamino-benzidine (DAB), (ZSGB-BIO, China) was used to develop visuals of the antigen-antibody reaction.

2.8. Immunofluorescence technique

Cover glasses were treated in 1 M HCL overnight, washed two times with distilled water, followed by absolute ethanol, and poly-L-lysine solution (1 mg/ml) for five minutes as adherent media. Cover glasses were then air dried, placed in a six-well plate and sterilized under ultraviolet light for two hours. (NC Hca-F, NC Hca-P, cDNA Hca-F and shRNA Hca-P) cells 5×10^6 of each were added to separate cover glasses and incubated in 5% CO_2 at 37° C overnight. Cells were fixed in 10% formalin and permabolized using 5% triton-X100. The cells were then blocked with 1% BSA for 1 h and incubated with 1 μg/ml of Anti-Ezrin monoclonal rabbit anti-mouse primary antibody (Abcam, USA) overnight at 4° C. Finally, cells were incubated with goat anti rabbit IgG (H + L) and fluorescence-conjugated Rhodamine (TRITC)-conjugated Affinipure as secondary antibody (1:100 dilution) (ZSGB-BIO, China) for one hour at room temperature and detected under a fluorescence microscope (Olympus, Japan).

Note: We did not use nuclear stain because the subcellular fractionation data suggested that Ezrin was partially located in the nucleus, and however the target was the total expression of Ezrin in different cell lines.

2.9. Migration and invasion

To determine the migration and invasion ability of the cell lines under study, 2.5×10^5 cells of each (NC-Hca-F, NC-Hca-P, Hca-F A7 up-regulated and Hca-P A7 down-regulated) cell line in 200 μl FBS-free RPMI-1640 medium was added to Boyden (0.8 μm) transwell membrane. The procedure for invasion assay was the same as the migration assay except that the inner chambers of the transwell were protected with 100 μl of extracellular matrix (ECM) (Sigma, USA). But, was added and overnight incubated in 4 °C before added the cells in invasion assay. 750 μl of 10% FBS medium was added to the bottom chamber of the transwell and incubated at 37 °C in 5% CO_2 humidified atmosphere for 14 h. Migrated and invaded cells were examined under a 40 x light microscope (Leica, Germany).

2.10. Statistical analysis

SPSS version 17 (IBM, USA) and Graph pad Prism version 5 (Graph Pad Software. Inc., USA) statistical software was employed for analysis of the data. ANOVA, Chi-square, Pearson correlation and *t*-test were appropriate conducted. The data are presented as Mean ± SEM and statistical significance was set at $p < 0.05$.

3. Results

3.1. Ezrin and A7 expressions are higher in Hca-F cells than in Hca-P cells

To determine the role of Ezrin in LNM-HCC, the expression of Ezrin and A7 were determined in Hca-F and Hca-P cell lines. The expression of Ezrin was significantly higher in Hca-F as compared with Hca-P cells ($p < 0.0001$) at the gene and protein expression levels as confirmed by qRT-PCR and western blot (Fig. 2A, B, Table 1), immunocytochemistry and immunofluorescence (Fig. 4A, B). The expression of A7 was however expressed in both cell lines, except that A7 was increased in Hca-F cells, but decreased in Hca-P cells ($p = 0.0007$) (Fig. 2A, B, Table 1). There was in direct correlation with the expression of Ezrin. However the variation on Ezrin and A7 in Hca-F was insignificant ($p = 0.1690$), but it is significant in Hca-P ($p = 0.0285$) (Fig. 2A, Table 1). Hence, Ezrin and A7 are highly expressed in high metastatic HCC as compared with low metastatic HCC; depicting a possible role of these proteins in LNN-HCC.

3.2. Ezrin and A7 expression and localization in Hca-F and Hca-P

The cellular location of Ezrin and A7 was determined in the two metastatically different HCC cell lines by subcellular fractionation followed by western blot (Fig. 3) and immunocytochemistry analysis (Fig. 4A). The expression and location of Ezrin in Hca-F cells were higher in the cytoskeleton and nucleus followed by cytoplasm. In Hca-P cells, nucleus Ezrin expression was highest, followed by cytoskeleton and cell membrane in (Fig. 3, Tables 2 and 3). With A7, subcellular fractions reveal that it is concentrated in the nucleus followed by cell membrane and cytoskeleton in Hca-F cells, but found increased in the cell membrane followed by nucleus and cytoplasm in Hca-P cells (Fig. 3, Tables 2 and 3). Thus, in high metastasis, Ezrin was highly expressed and predominantly abundant in cell cytoskeleton in high metastatic HCC whereas it is suppressed and largely found in the nucleus in low metastatic HCC. A7 on the other hand was highly expressed in Hca-F than in Hca-P cells and was highly expressed in the cell nucleus and moderately expressed in the cell membrane of Hca-P compared with Hca-F.

3.3. The expression of Ezrin is negatively correlated with A7 modulation in LNM HCC

To confirm the association of A7 and Ezrin, A7 expression was modulated and the resultant effect on Ezrin expression was evaluated. The effect of A7 on Ezrin in Hca-F cells and Hca-P cells was determined by selective stable up-regulation of A7 in Hca-F cells (Hca-F A7 up-regulated) ($p = 0.0057$) (Fig. 2) and A7 down-regulated Hca-P cells (Hca-P A7 down-regulated) ($p = 0.0131$) (Fig. 1, Table 1) and modulated expression modules were confirmed by western blot ($p = 0.0002$) (Fig. 2A, B). We observed

Table 1
Expression level of lymphatic metastasis associated Ezrin and Annexin 7 proteins in different HCC cell lines (%).

Cell lines	Erin(EZR)	Annexin A7(A7)
Hca-F UP	23.42	31.28
Hca-F	29.93	28.50
Hca-P	19.46	21.52
Hca-PD	28.18	18.70

F UP: Hca-F Annexin A7 up regulated, F: Hca-F, P: Hca-P and P D: Hca-P Annexin A7 down regulated.

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Table 2
Expression level of lymphatic metastasis associated proteins (Ezrin and Annexin 7) in each subcellular fraction (%).

Proteins	EZR	EZR	EZR	EZR	A7	A7	A7	A7
Cell lines	F UP	F	P	P D	FUP	F	P	P D
Cytoplasm	53.90	40.31	32.00	91.01	47.21	41.71	60.09	39.42
Membranes	35.48	28.61	59.56	65.63	38.19	58.19	99.01	33.53
Nucleus I	51.02	42.35	43.38	76.11	50.63	62.32	49.10	39.64
Nucleus II	37.19	53.10	89.19	38.01	39.10	62.17	66.00	32.27
Cytokeratin	44.27	73.01	72.37	42.82	74.37	54.81	30.91	25.14

Table 3
Location of proteins in different subcellular fractions.

Proteins	EZR	EZR	EZR	EZR	A7	A7	A7	A7
Cell lines	F UP	F	P	P D	F UP	F	P	P D
Cytoplasm	+++	++	++	++++	+++	++	+++	++
Membranes	++	+	+++	+++	++	+++	++++	++
Nucleus I	+++	++	++	+++	+++	+++	++	++
Nucleus II	++	+++	++++	++	+++	+++	+++	++
Cytoskeleton	++	+++	+++	++	+++	+++	++	+

(++++) very high expression (80% and above), (+++) high expression (50%–80%), (++) Very high expression (30%–50%), (+) Slight expression (bellow 30%) and (−) No expression.5.6. Up and down regulate of Annexin A7 level influenced the Ezrin mRNA expression.

that, A7 up-regulation in Hca-F cells and down-regulation in Hca-P cells is negatively correlated with Ezrin expression ($R^2 = -0.0015$) −0.9580 to 0.9640 (Fig. 4B, Table 2). Ezrin is observed suppressed in Hca-F A7 up-regulated cells ($p < 0.0001$), but elevated in Hca-P A7 down-regulated cells ($p = 0.0211$), indicating a negative correlation of Ezrin and A7 (Figs. 2 and 4 B). On the other hand, we observed a significant difference on Ezrin and A7 expression in A7-up and down-regulated cells as follow ($p = 0.0021$, 0.0007).

3.4. A7 alteration affected Ezrin expression and subcellular location in LNM HCC cell lines

Subcellular fractions and cellular location of Ezrin and A7 in A7 up-regulated in Hca-F cells and Hca-P A7 down-regulated cells were analyzed compared to NC Hca-F/Hca-P cells for cellular location and expression levels of Ezrin and A7. In Hca-F A7 up-regulated cells, Ezrin was concentrated in the cytoplasm followed by the nucleus while A7 was mostly found in the cytoskeleton and nucleus (Fig. 4A, Tables 2 and 3). In Hca-P A7 down-regulated cells, Ezrin was highly expressed in the cytoplasm followed by the nucleus, but A7 was mostly found in the nucleus followed by the cytoplasm and cell membrane. The abundance of A7 in Hca-F A7 up-regulated cells in the cytoskeleton as compared to its aplenty in the nucleus in Hca-F cells resulted in suppressed expression of Ezrin. On the other hand, the abundance of A7 in Hca-P A7 down-regulated cells in the nucleus, as opposed to its dominance in the cell membrane in Hca-P cells is associated with increased expression of Ezrin in Hca-P A7 down-regulated cells.

3.5. Modulation of A7 influence migration and invasion ability of HCC cell lines

HCC cells migration and invasion behavior was assessed by Boyden transwell method. The data showed an uncharacteristic migration and invasion pattern as associated with parental Hca-F and Hca-P cells. The migration and invasion abilities of Hca-P-A7 down-regulated cells were suppressed. Whereas, the migration and invasion abilities of Hca-F-A7 up-regulated cells were enhanced (Fig. 5).

4. Discussion

Lymphatic metastasis is known to be the first step of tumor metastasis and it is observed in 33.8% of all metastatic tumors, and specifically 7.45% of metastatic hepatocellular carcinoma [3]. Ezrin works as a protein-tyrosine kinase substrate and functions in cell surface structure, adhesion, migration, proliferation and organization. The cellular location of Ezrin and its associated expression in different cancers makes it a potential protein to influence HCC [11–13]. However, to determine Ezrin role in LNM HCC we expertized that; Ezrin expression could be used as diagnostic or/and prognostic biomarker in LNM-HCC and could also have interaction with Annexin 7, which may provide a therapeutic target for LNM-HCC.

Annexin 7 is a member of calcium/phosphor-lipid binding protein family, and conserved structural protein. It is reported to participate in physiological endocytosis, exocytosis and control of

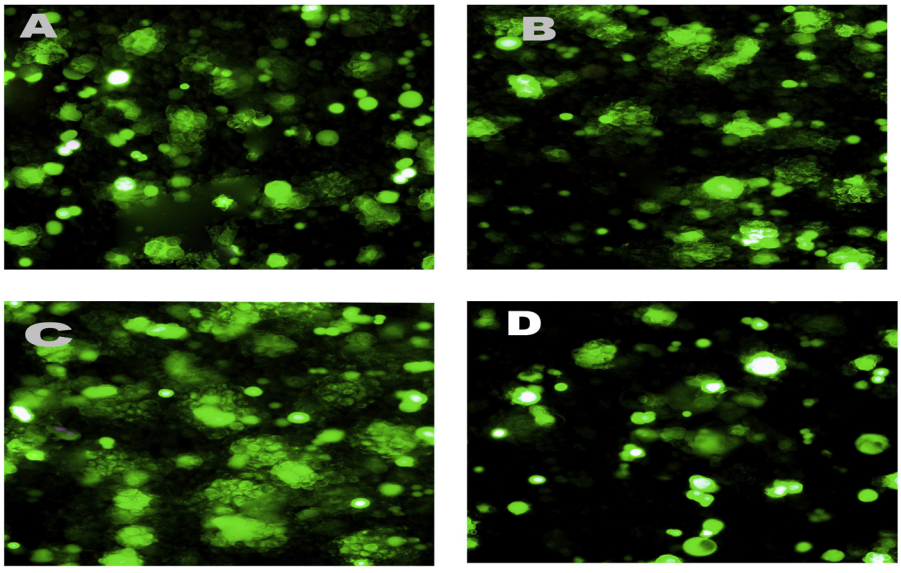

Fig. 1. A7 gene transfection in (Hca-F and Hca-P) HCC cell lines. A: Hca-F A7 up-regulation, B: Hca-F-GAPDH, C: Hca-P A7 down regulation and Hca-P-GAPDH.

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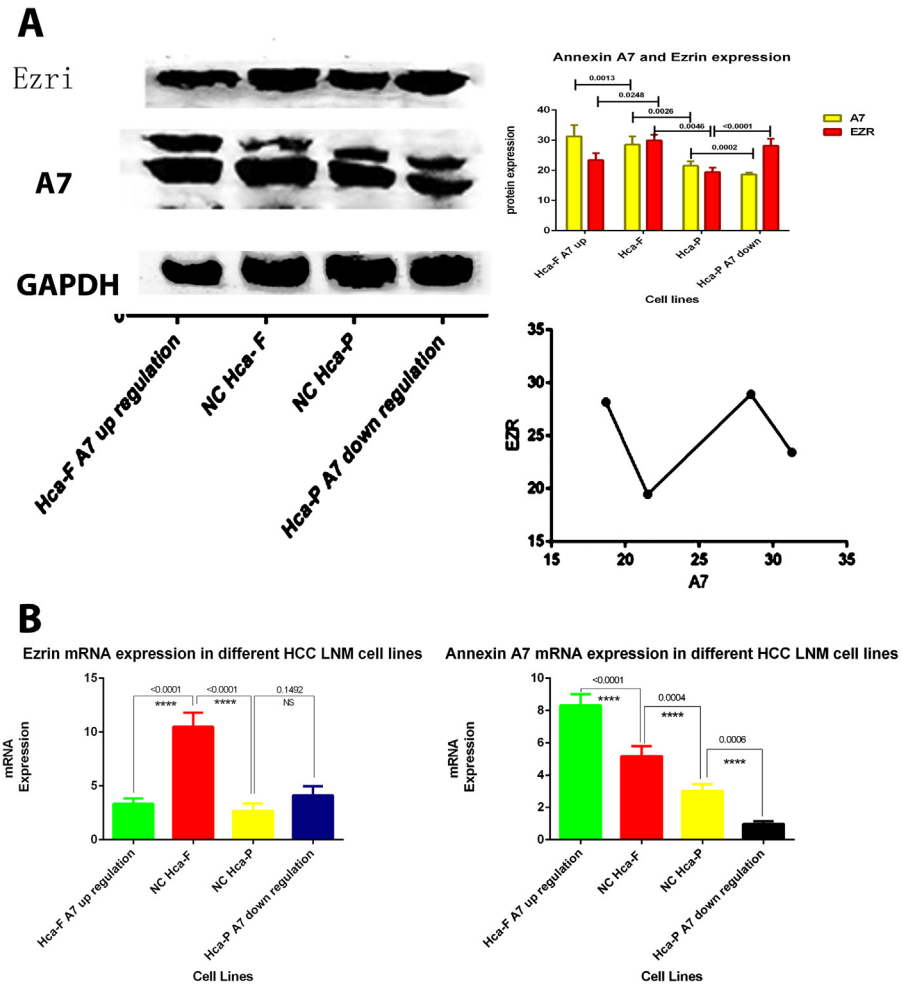

Fig. 2. A: Western blot evaluation of Ezrin and A7 proteins in LNM-HCC cell lines (Hca-F A7 up-regulation, NC-Hca-F, NC-Hca-P and Hca-P A7 down-regulation) cell lines. B: qRT-PCR, Expressions of Ezrin and A7 mRNAs of different LNM-HCC cell lines (Hca-F A7 up-regulated, NC-Hca-F, NC-Hca-P and Hca-P A7 down regulated).

cellular activities in malignant tumors [5,7,23]. Previous works in our lab have demonstrated significantly increased expression of A7 in Hca-F cells (high metastatic), but decreased A7 expression in Hca-P cells (low metastatic) [2–5]. Suggestive that decreased A7 expression may be associated with less metastasis. Subcellular fractionation study also revealed A7 was expressed in all cell fractions [2–5]. However, A7 was located more in the cytoplasm, nucleus and cytoskeleton in Hca-F cells, whereas in Hca-P cells, A7 was more in the membranes and nucleus fractions. In this study the finding is in agreement with the findings of Qazi et al. [5]. It is known that A7 has an ability to bind with F-actin. This could explain the localization of A7 in the cytoskeleton and may contribute to cellular membrane organization and facilitates A7 Ca^{+2} and phospholipid binding to subsequently modulate cellular trafficking procedure [4,5]. However, the localization of A7 on cytoplasm and nucleus might be related to the cytoplasmic Ca^{+2} homeostasis which changes during cellular molecular and cellular proliferation activities. Moreover, the cytoskeletal proteins could support the intracellular Ca^{+2}, modulate membrane stability and metastasis [3–5]. As well as in our experiments findings, A7 was higher in Hca-F than in Hca-P in both gene and protein levels. The findings of migration and invasion abilities were increased in case of A7 up-regulation.

The expression of Ezrin was determined in LNM-HCC cell lines (Hca-F and Hca-P) to ascertain its involvement in LNM-HCC. Our findings revealed high expression of Ezrin in Hca-F cells, but decreased expression in Hca-P cells. High expression of Ezrin had been reported in many cancers [1,17]. Also, high Ezrin expression was shown to be associated with lymphatic metastasis and was observed to correlate with intensity as well as the level of lymphatic metastasis in HCC, melanoma, pancreatic carcinoma and colorectal carcinoma [1,14,19,24,25]. In a similar study by Ningning [1], Ezrin was noted to be up-regulated in Hca-F cells which collaborates our finding of high Ezrin expression in high metastatic HCC [1]. The high level of Ezrin in high lymphatic metastasis (Hca-F >70% metastasis) and low expression in low lymphatic metastasis (Hca-P <30% metastasis) depicts its correlation with HCC progression. Thus, Ezrin could be a candidate marker for both diagnostic and/or prognostic in HCC. Ezrin and A7 have been investigated independently in many cancers including hepatocellular carcinoma, breast cancer, colon cancer, endometrial and ovarian cancers, brain tumors and soft tissues carcinomas. [26,27]. The findings of our study demonstrate that A7 and Ezrin are inversely related. This inverse association could be due to several factors including the activation and/or association between P85 and P53 proteins with A7 and Ezrin. P53 was found to be down-regulated through the Akt pathway, when Ezrin was co-expressed with P85 [28]. It is possible that A7 has a role in Ezrin phosphorylation. However, further studies are needed to clarify the mechanisms behind the negative relationship between Ezrin and A7.

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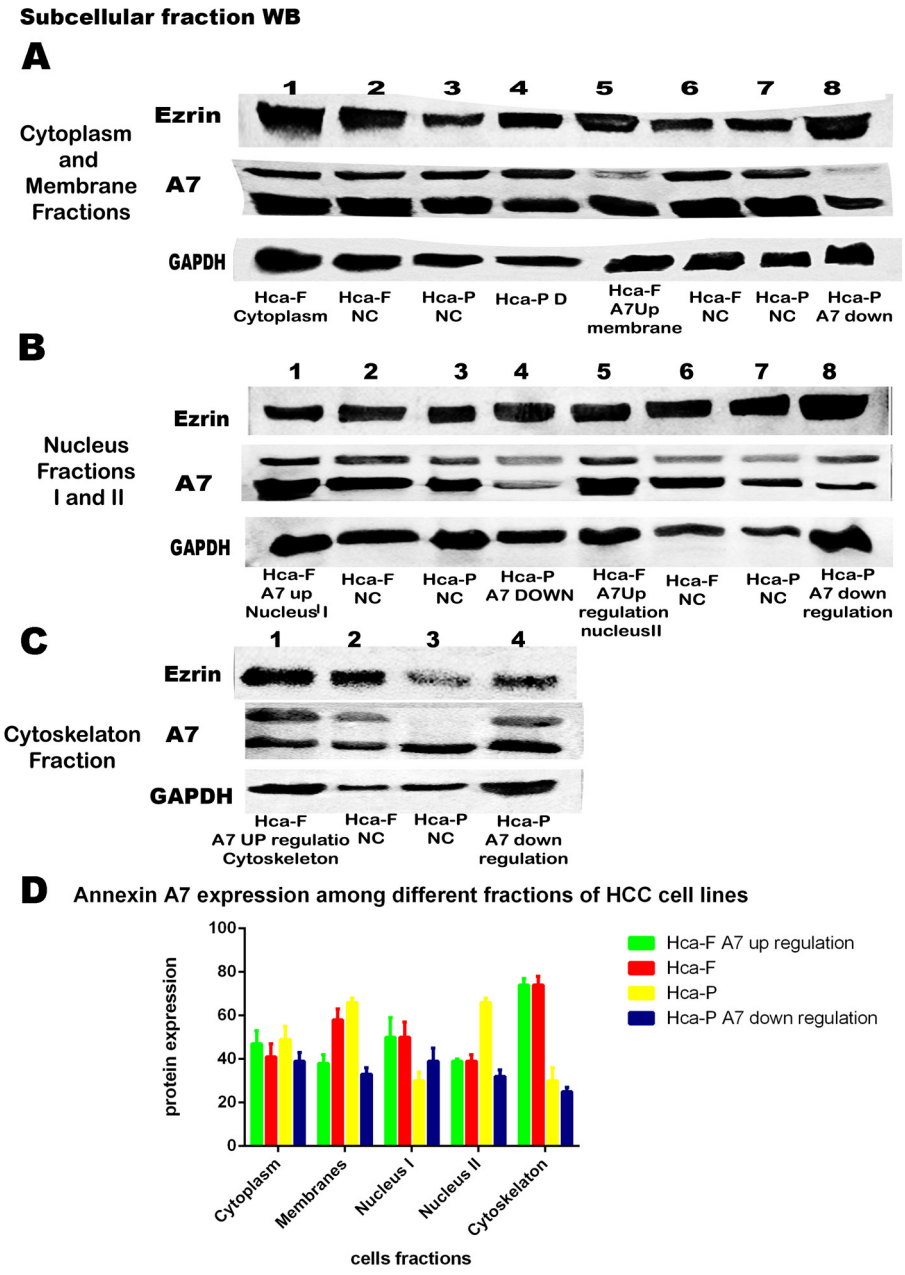

Fig. 3. Ezrin and A7 expressions in subcellular fractions of different LNM-HCC cell lines (Hca-F A7 up-regulation, NC-Hca-F, NC-Hca-P and Hca-P A7 down-regulation). However, the comparing of Ezrin and A7 expressions among each subcellular fractions as follow, A: (1–4): cytoplasm fraction, A: (5–8): membranes fraction, B; (1–4): nucleus fraction I, B: (5–8): nucleus fraction II and C: (1–4): cytoskeleton fraction.

Moreover, mutant Ezrin (Y353F) in the assembly of PI3K results in reduced production of functional enzyme and this impairs apoptosis [13], this could justify our observations of Ezrin expression in LNM-HCC-A7 modulated cells. Since Ezrin is a known proto-oncogene and its' expression was associated with bad prognosis [17]. It can as well be used as a potential biomarker of HCC. In addition, A7 was found to suppress the expression of Ezrin suggesting that it has tumor suppressor activity.

The cellular location of a protein would reveal it has a probable role in the cell homeostasis and function [5]. The location of Ezrin was analyzed in subcellular fractions of HCC cells. In Hca-F cells, Ezrin comparably was highly concentrated in the cytoskeleton comparably. However, in Hca-P cells, it was mostly located was mostly in the membranes and nucleus. Recently, it has been suggested that the main function of Ezrin could be cytoskeleton-dependent in high metastasis, whereas high nuclear localization coupled with low cytoskeletal Ezrin expression in low metastasis. The features of cytoskeleton-dependent and filament size, flexibility, concentration and cross-linkage are necessary for cellular appearance and cellular interactions [5,17,29]. Cytoskeletal Ezrin might play a role in the regulation of dynamic reformation of actin cytoskeleton cross-linkage and subsequent cellular activities as cells migration and invasion. The linkage between high total Ezrin, high cytoskeletal Ezrin and high HCC-LNM as compared with low total Ezrin, low cytoskeletal Ezrin and low HCC-LNM suggest as major role for Ezrin in lymphatic metastasis mechanisms. Many studies explained that more Ezrin correlated to malignant transformations increased proliferation, decreased apoptosis, enhanced motility and metastasis ability in some lesions [25,30]. This

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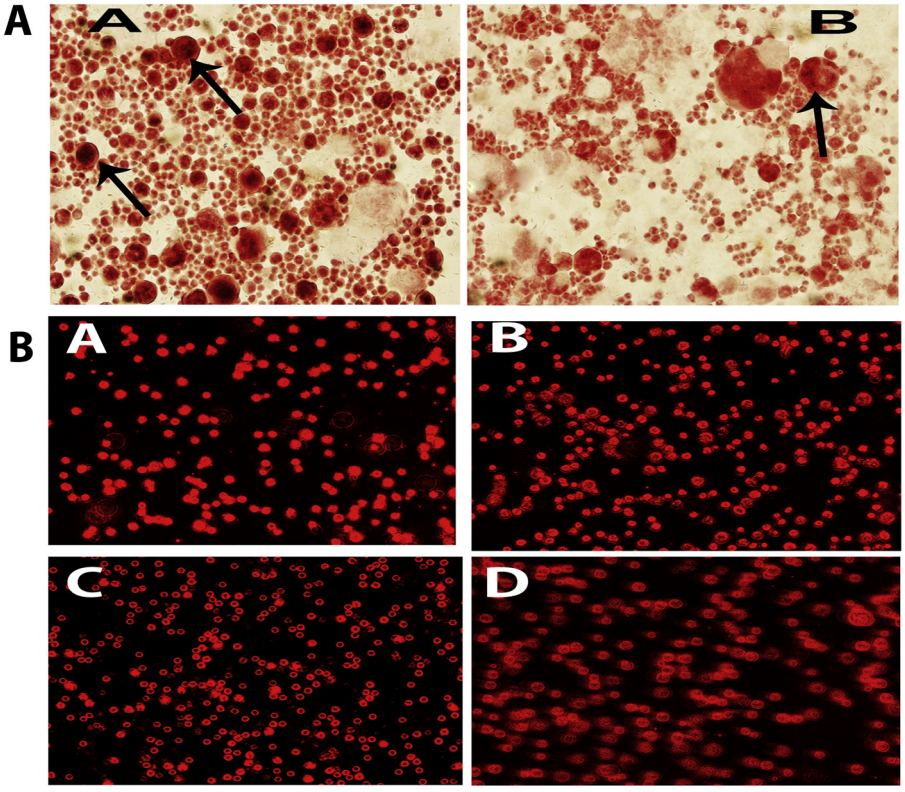

Fig. 4. A Immunocytochemistry expression of Ezrin in high and low LNM-HCC cell lines (A: Hca-F and B: Hca-P). B: Immunoflourescence evaluation of Ezrin expression in different LNM-HCC cell lines: (A: Hca-F A7 up regulated, B: NC-Hca-F, C: NC-Hca-P and D: Hca-P A7 down regulated) cells.

collaborates our findings that Ezrin was highly associated with high lymphatic metastatic cells, and vice versa.

Ezrin and A7 expressions and subcellular locations could play a critical role in growth and output of uterine endometrial adenocarcinoma as well as high metastatic osteosarcoma, epidermis carcinoma cell line and colon carcinoma [29–31]. Collectively, previous studies have determined the effect(s) of total Ezrin and A7 in benign and malignant lesions [1,3,14,19,24–26]. However, according to our data, the subcellular localizations of molecules are very important and have hallmark meanings in determining their cellular/biological activities and behaviors. The demonstration that both Ezrin and A7 are differentially distributed with the cell under different disease states is intriguing and requires further studies to understand the dynamics.

Based on the initial expression profiles of Ezrin and A7, we envisaged a probable regulation of Ezrin with A7. To confirm this, A7 was stably up-regulated in Hca-F cells (where its expression is usually high) and down-regulated in Hca-P cells in (where A7 is usually low). A stunning observation was seen, A7 was largely located in the cytoskeleton when up-regulated. The location of A7 in the cytoskeleton may position it to participate in a critical role such as cell trafficking mechanisms which due to its suppressive influence on Ezrin, the putative oncogene, may resulted in decreased lymphatic metastasis through suppression of Ezrin and Ezrin induces tumor promoting mechanisms. The suppression of Ezrin by A7 could be one of the mechanisms for the effect of A7 on the interactive and interactive mechanisms of LNM-HCC. Moreover, the lowest cytoskeletal A7 level was associated with A7 down-regulated Hca-P cells combined with the lowest cells ability to migrate and invade. In addition, previous studies revealed significant effects for A7 on LNM HCC cells migration and invasion [4,23].

Furthermore, we compared and discussed the translocation of Ezrin as an affected with A7 regulation in (Hca-F and Hca-P) cells. Moreover, the different subcellular location of Ezrin in different metastatic levels of colon adenocarcinoma cell lines; which differ in cellular subunits as plasma membrane and cytoplasm, which clearly varies according to metastatic level and behavior; migration and invasion and cell dynamic mechanism. Also, Ezrin phosphorylation (activation) is needed Ezrin to move from the cytoplasm to plasma membrane of microvillus which correlated to cell migration and invasion [5,20,31].

Lymphatic metastasis is a more complicated process which includes many factors that enhance cancer cells to migrate through lymphatic system [2,3]. According to our findings in migration, invasion assays and subcellular fractionation in LNM-HCC cell lines had been clearly influenced by A7 gene regulation. Migration and invasion were enhanced when A7 up-regulated and suppressed in the case of A7 down-regulated followed by extra changes in Ezrin expression and locations in different LNM HCC cell lines [3,26]. Depending on the fact; extra factors needed to activate Ezrin [31]. Ezrin might play direct or indirect critical role(s) in A7 activation process, cellular biological activities and behaviors of LNM HCC.

Collectively, our study demonstrates that Ezrin expression in the subcellular compartments of LNM HCC is directly associated with the grade of lymphatic metastasis. However, A7 is inversely associated with cytoskeletal Ezrin expression, cell migration and invasion, and this could be a contributing factor to lymphatic metastasis of HCC.

5. Conclusion and recommendations

Phenotypically, the data demonstrated the role of Ezrin as a proto-oncogene, and its expression might be associated with high lymphatic metastasis. A7 however, suppress the expression of

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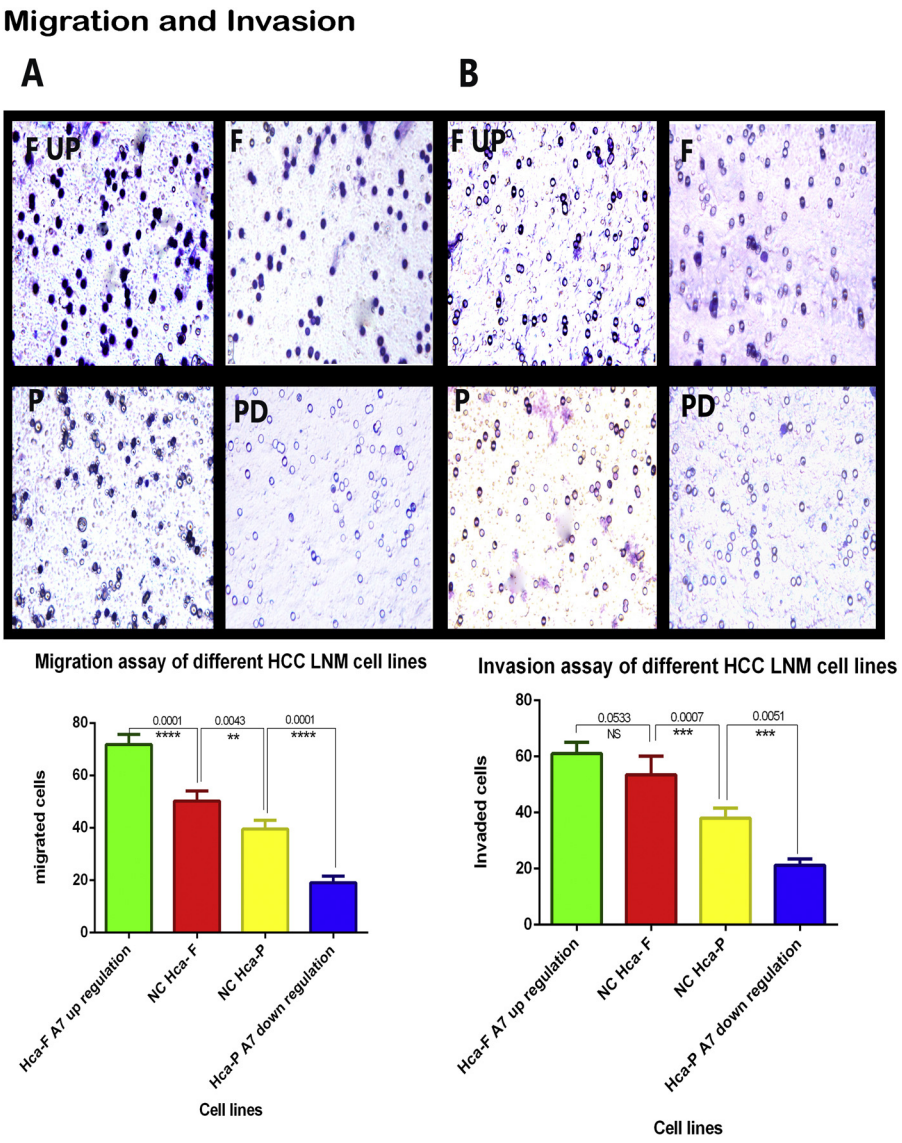

Fig. 5. Migration and invasion ability of different LNM-HCC cell lines. A: Migrated HCC-LNM (Hca-F A7 up-regulation, NC-Hca-F, NC-Hca-P and Hca-P Annexin A7 down regulated) cells. B: Invraded HCC-LNM (Hca-F A7 up-regulation, NC-Hca-F, NC-Hca-P and Hca-P A7 down-regulated).

Ezrin to suggest that it might have tumor suppressor activity and could be targeted for direct or indirect therapeutic activities.

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