Full Length Research Paper

Gelsolin and Annexin 7 proteins correlates with lymph node metastasis (LNM) in carcinoma cell

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Accepted 25 November, 2013

Background: There has been a tendency to focus on subcellular proteomes concerning specific subcellular compartments and macromolecular structures of the cell. Subcellular proteomics, as an important step to functional proteomics, has been a focus in proteomic research. Aim: To find whether LNM associated proteins perform their functions in some association with each other as some binding or interacting partners. Comparison of ratio of difference of their expression levels on the basis of their sub-cellular expression sites. Methods: Sub-cellular structures were focused to investigate two calcium dependent phospholipid binding proteins. The effect of their expression levels on each other were analyzed by increasing and decreasing regulation of protein on each other and further evaluation by SDS-PAGE. Results: LNM associated proteins were expressed at different sub-cellular location with different expression levels with higher expression in high potential cell lines for LNM. Up-regulation has increased and down-regulation has decreased their expression levels. Conclusion: The separation of cells into its compartments can increase the probability of detecting even low expression levels of proteins at different sites which not only tells the whole protein content of that specific fraction rather helps in understanding their structures, functions and binding associations with each other.

Keywords: lymph nodes, metastasis, hepatocellular carcinoma, proteomics, subcellular fractions.

INTRODUCTION

Sub-cellular fractionation and purification of organelles had always been a challenge for cell biologists (Lukas et al., 2003). Proteomics is one of the leading technologies available to researchers in the post genomic era due to the central role of proteins and their interactions in cellular function (Cox and Mann, 2007). But limited resolution power of separation technologies presently applied in proteomics, additional steps are required for deeper analysis. Indeed protein's specific patterns and location of expression and monitoring expression levels at particular sites can reveal some hidden facts associated with cancer (Klose, 1975).

Lymphatic metastases are usually an early step, with lymph nodes often the first organ to develop metastasis (Audet et al., 2005). The proteome analysis represents an analytical strategy and tools for the comprehensive
identification of proteins (Huber, 2003). Main focus of our group is molecular mechanism of lymphatic metastasis of hepatocellular carcinoma (HCC). For more insight at this mechanism we tried to analyze proteins sub-cellularly using syngenetic cell lines Hca-F and Hca-P with high(above 70%) and low (below 30%) lymphatic metastatic potential, successfully developed in our lab from hepatocarcinomas in mice (Bryant et al., 2002; Santoni et al., 2000). Hca-F and Hca-P metastasize only to lymph nodes which have been proved to be the ideal models for studying lymphatic metastasis for hepatocarcinoma (Gygi et al., 1999). We found Gelsolin gene 1.9 times higher and the protein expression 1.7 times higher in Hca-F than in Hca-P (Kiselar et al., 2003; Kuijpers et al., 1992; Claudia et al., 2001). At both protein and gene level the results showed higher expression of Gelsolin in Hca-F cell line in liver ascites and can promote tumor cell migration and invasion in LNM of liver cancer (Wang shaqing unpublished data).

We have also observed Annexin 7 over expression at both gene and protein level in Hca-F, indicating its role in the tumor cell proliferation, apoptosis, migration and invasion. (Wang Zhiqiang; data unpublished). Annexin 7 expressions in cancer tissues was 53.3%, which was significantly higher than that 25.0% in normal samples while significantly lower than that 76.7% in lymphatic metastasis in primary Gastric cancer tissues (Helga et al., 2009). The repression of Annexin 7 inhibits the mobility and invasion abilities of Hca-F cell, increases the apoptosis rate of Hca-F cell (Sun et al., 1999).

In this study we have fractionated the cells to have better insight of LNM associated proteins Gelsolin and Annexin 7 sub-cellular expression patterns in the cells. We have also tried to figure out any binding association of both proteins by up and down-regulating their expression levels.

MATERIALS AND METHODS

Animals and cell lines

Inbred Chinese 615 mice (aged 6-8 weeks, weighing 18-22 g) were provided by the animal center of Dalian Medical University. All mice were maintained by the Institutional Animal Care Guidelines. The mouse hepatocarcinoma cell lines Hca-F with LNM rate above 75% and Hca-P with LNM rate below 30% were established and maintained by our department.

Cell growth for in vivo system

Four inbred 615 mice were randomly divided into two groups. 2x10⁶ cells / 0.1 ml of cell suspension were inoculated to 615 mice intra-peritoneally. After 7 days the ascites were collected under sterile conditions, re-inoculated into other 615 mice and allowed to grow for 5 days in abdominal cavity after the extraction of non-bloody ascites. Several passages were done in order to harvest large number of cells in around 2 weeks.

Cell growth for in vitro system

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 at pH7.2 for 24 h in a 5% CO₂ atmosphere at 37°C in a humidified atmosphere. Cellular passages were done until the cell number increases to desired amount of 3-5x10⁶ cells in each vial. In every passage the cells were washed with phosphate buffer solution (PBS, pH 7.4). Cell viability was determined by Trypan blue exclusion test. Lymph node metastatic rates were calculated in Hca-F and Hca-P cells by inoculating (2x10⁶ cells/mouse) among 20 mice in a group of 40 inbred 615 mice. On the 28th day post inoculation, their lymph nodes were collected and stained by HE and examined under microscope.

Stable transfection of cells

To establish a mouse hepatic cancer cell line Hca-F transfected with shRNA(F-down A7). Three sh RNA were designed and inserted into the pSilencer vector to silence Annexin 7 gene. The most effective P Silencer –shRNA vector was selected based on the result of RT-PCR and western blot. The Hca-F cells were transfected with the most effective P Silencer –shRNA and transfectants were selected by 400µg/µl G418 (Geneticin). To construct PcDNA 3.1 –Annexin 7 (P-up A7)and to transfect P-cells stably, Annexin 7 gene was amplified by PCR. Bam H1 and EcoR1 enzymes were used to digest the Annexin 7 gene and PcDNA 3.1 plasmid. This plasmid was transfected in P cells stably. The effectiveness was checked by genome DNA checkup and western blot analysis.

Protein sample preparation

Before fractionation cell suspension was made so that each aliquot will have 3-5x10⁶ cells/ frozen cell pellet. Calbiochem® proteo Extract® sub cellular Proteome Extraction Kit (S-PEK) (cat.no. 539790) was used for the differential extraction of proteins from mammalian cells in all four cell lines. During the complete extraction procedure, the conditions given in user protocol 539791 were strictly followed. The resultant supernatant after each step is the required protein sample.
Sub-cellular protein extraction from culture cell suspension

Before beginning of extraction procedure all buffers were mixed well by vortexing and kept on ice during the whole procedure except buffer IV and protease inhibitor cocktail at room temperature. Cell suspension was transferred to 4 ml centrifuged tube and pellet by centrifugation at 1500 rpm for 10 min at 4°C. Supernatant was aspirated and discarded. Pellet was washed twice with 2 ml ice cold wash buffer, re-suspended and incubated for 5 min at 4°C with gentle agitation, centrifuged in cold at 4°C at 1500 rpm for 10 min. 1 ml extraction buffer1 was mixed with 5µl protease inhibitor cocktail and this mixture was added to cell pellet, re-suspended and incubated for 10 min at 4°C with gentle agitation, centrifuged at 2900 rpm for 10 min in cold at 4°C. Supernatant was collected separately (fraction1) without disturbing the cell pellet. 1ml of Extraction buffer2 with 5µl protease inhibitor cocktail was mixed with cell pellet, re-suspended and incubated for 30 min at 4°C, centrifuged at 7840 rpm for 10min at 4°C. Supernatant (fraction2) was collected separately. In the pellet 500 µl of extraction buffer3 with 5µl protease inhibitor cocktail and 1.5µl Benzonase nuclease was mixed and added to cell pellet, incubated for 10 min at 4°C with gentle agitation. Mixture was centrifuged at 8720 rpm for 10min at 4°C, supernatant (fraction3) was collected separately. 500µl of extraction buffer 4 with 5µl protease inhibitor cocktail was added to pellet. Residual particles were re- suspended and mixed thoroughly (fraction4).

Determination of protein concentration

The concentration of protein in each fraction was calculated by Bradford assay using bovine serum albumin as standard. Subsequent measurements were taken at 595 nm with a spectrophotometer. The OD value was obtained by Bio-SenSC300 (Bio-Tech.)

SDS PAGE

The extracted proteins were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis in equal amounts, blotted on polyvinylidene difluoride membranes (Millipore), applied with antibodies for Gelsolin(Epitomics,1:1000) and Annexin 7 (SIGMA ALDRICH,1:1500) in 5%dried milk for 1hr then incubated with horseradish peroxidase-linked antibodies(1:2500) for 1 hr. Bands were detected by an ECL western blotting kit(GE Healthcare,USA) and analyzed by(Auto Chemi system Bio imaging UVP).For each fraction loading control proteins used were: for fraction 1 GAPDH (KANG CHEN),for fraction 2 cytochrome P450reductase (Abcam),for fraction 3 c-jun(Abcam) and for fraction 4 α-tubulin(Santa Cruz).

Data analysis

The data obtained was expressed as Mean± S.D. and analyzed statistically by SPSS. Version 13.0 for Windows (snow panther, SPSS Inc, IL, USA) using independent t-test, paired t-test and one-way ANOVA at (P<0.05).

RESULTS

In vitro and in vivo cell growth

Hca-F and Hca-P cells were grown well in vivo environment and cause the metastasis of tumor to lymph nodes with different rates to metastasize. The rates were calculated as75% for F-cells and 25% for P-cells. Our lab has proved by separate comparative proteomic analysis of Hca-F and Hca-Pcells that both the cell lines have different metastatic potential which make them ideal models for studying metastasis of lymph nodes (data not shown). Cells were regenerated without contamination under strict conditions of cell culture and aliquoted after regular and successful passages in vitro.

Determination of metastatic rate

The implanted tumors both in Hca-F and Hca-P tumor-bearing mice were palpable on the 7th day post-inoculation. The rate of tumor formation in both groups was 100%. On the 28th day post –inoculation, 18 out of 20 mice for F cells and 4 out of 20 for P cells developed lymph node metastasis indicating the metastasis rate as 80% in F-cells and 20% in P-cells.

Down regulated expression in Hca-F

The P-silencer Annexin 7 expressing vectors were transfected into Hca-F cells successfully. The expression levels between F-cells and F downA7 cells were noted by western blot and the ratios of difference in expression levels were found to be 2.47 for Annexin 7.The expression levels were found to be 73% for F-cells and 60% for FdownA7 cells. The transfected cells were selected further by G418 medium.

Plasmid insertion increases the expression level in Hca-P

Western blot analysis was carried out to evaluate Annexin 7 protein levels. Hca-Pcells transfected with PcDNA3.1 plasmid showed higher expression levels than
in Hca-P-cells. The ratios of difference among expression levels were found to be 1.40 between P and PupA7-cells. The expression levels were found to be 59% for P upA7 cells and 47% for P-cells.

**Sub cellular fractionated protein samples**

Hca-F, Hca-P, F-downA7 and P-upA7 cells were successfully established, regenerated and aliquoted after successful passages. Each aliquote have had 3-5 x10⁶ cells/ frozen cell pellet. In all fractions expression level were checked and in cell lines with different metastatic potential. The four fractions which were obtained and served as protein sample were cytoplasm, membrane and membrane bound organelles, nuclear material and cytoskeleton.

**Distribution of protein concentration**

The concentration of protein was determined for each sub cellular fraction by Bradford assay. The values determined for each fraction were as follows (figure 1)

### Table I. Comparative summary of Gelsolin and Annexin 7

<table>
<thead>
<tr>
<th>Distribution pattern</th>
<th>Gelsolin</th>
<th>Annexin 7</th>
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<tbody>
<tr>
<td></td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td><strong>Expression level (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F high low nil moderate</td>
<td>high low nil moderate (51KDa/47KDa)</td>
<td></td>
</tr>
<tr>
<td>P high nil nil moderate</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ratio of difference</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F/P 1.2 -- -- 1.5 2.3/1.5 1.8 -- 1.6</td>
<td></td>
<td></td>
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<tr>
<td>t-test values</td>
<td></td>
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<tr>
<td>F 0.84±0.03 -- -- 0.62±0.02 0.08±0.001/ 0.41±0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P 0.70±0.05 -- -- 0.40±0.06 0.03±0.002/ 0.25±0.05</td>
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</tbody>
</table>

a=cytoplasm, b=cellular membranes, c=nuclear material, d=cytoskeleton

Expression level % = % calculated for ten values of expression levels in each fraction

t-test values expressed as mean±SD and calculated at P <0.05°

**Distribution patterns of lymphatic metastasis associated proteins**

Distribution patterns of each protein under study according to sub cellular fractionation and in each cell line under study is summarized in table 1. All the results indicate that there might be a positive correlation with the lymphatic metastasis proteins under this study and lymphatic metastasis progression and development. All were expressed at high levels in lymphatic metastasis cell lines with some differences indicating their strong involvement in tumor metastasis. Annexin 7 two isoforms were detected only in cytoplasm but not in other fractions of its expression. Both proteins mentioned above have shown their involvement in lymphatic metastasis yet both have no prominent relation with each other for their functions but still minor binding relations can be hypothesized.
Figure 2a. In each parts (i,ii,iii,iv) line 1,2,4,5 shows internal controls for fractions 1,2,3,4 labeled as a,b,c,d. line 3 indicates test sample (Annexin 7) expression for each fraction. Part (i,ii,iii,iv) also shows expression in F,F-down, P,P-up cell lines, respectively.

Figure 2b. In each parts (i,ii,iii,iv) line 1,2,4,5 shows internal controls for fractions 1,2,3,4 labeled as a,b,c,d. line 3 indicates test sample (Gelsoline) expression for each fraction. Part (i,ii,iii,iv) also shows expression in F,F-down P,P-up cell lines, respectively.

Figure 3a, b. Represents Gelsolin (a) and Annexin 7 (b) with their expression levels in each cellular fraction.

Annexin 7 and Gelsolin expression levels

Annexin 7 was detected in cytosolic fraction1, membranous fraction2 and cytoskeletal fraction4. The expression levels were detected by western blot analysis. The expression level was highest in Hca-F cells compared to other cell lines (figure 2a). Hca-P showed minimum expression level among all four cell lines analyzed. Expression levels were 72.6%, 62.5%, 48.8%
Figure 4. Comparison between ratio of difference among all cell lines under study for Gelsolin and Annexin 7.

Figure 5. Comparison of each fraction among cell lines with different metastatic potential (F,F-down,P,P-up) respectively. Centre lines shows internal controls for each fraction. First line in each figure part shows fraction 1,2,3,4 for Gelsolin in all comparative cell lines. Third line in each figure part shows fraction 1,2,3,4 for Annexin 7 in all comparative cell lines.

and 59.8% (47KDa) and 9.9%, 4%, 2% and 2.86% (51KDa) for fraction 1 respectively. Fraction 2 has 36.3%, 28%, 22% and 25.5% whereas fraction 4 has 52.5%, 48%, 39.5% and 43.8% respectively. The relative protein expression level in Hca-F cells showed higher expression of Gelsolin in cytosol, membrane fraction and cytoskeleton (figure 2b) which were 84%, 19% and 69% compared with Hca-P cells for cytosol and cytoskeleton which were 76% and 36% respectively. Yet in P cells expression was not detected on membranes and membrane bound organelles. (figure 3a and b).

Statistical analysis of expression levels

The statistical comparison was made between the four cell lines analyzed for Annexin 7 and among the four fractions of cells. The values were 0.99 ± 0.015, 0.02 ± 0.005, 0.04 ± 0.009 and 0.26 ± 0.016 for 51 KDa isoform and 0.73 ± 0.064, 0.47 ± 0.035, 0.62 ± 0.080 and 0.58 ± 0.069 for 47 KDa isoform in fraction 1 of F-cells, P-cells, F down A7 and P upA7 respectively. 0.34 ± 0.096, 0.21 ± 0.018, 0.26 ± 0.017 and 0.23 ± 0.014 in fraction 2 and 0.53 ± 0.019, 0.37 ± 0.015, 0.44 ± 0.015 and 0.43 ± 0.017 for fraction 4 respectively. Statistically, expression levels of both cell lines were found as 0.84±0.05, 0.69±0.061 for F-cells and 0.76±0.05, 0.36±0.049 for P-cells for fraction 1 and 4 respectively. Gelsolin expression was not found in fraction 2 of P-cells but only in fraction 2 of F-cells. All the data was found significant at P < 0.05. Ratio between fraction 1 of F and P cell lines was calculated as 1.1 and between fraction 4 of F and P cell lines as 1.9 (figure 4).

Comparison of similar sub-cellular location

Gelsolin and Annexin 7 were found expressed almost at same locations although the expression levels were different. In order to see their role and dependency Annexin 7 in F-cell lines was silenced and Gelsolin expression level was checked from 20µg to 90µg. It was noted that increasing amount of protein will increase the expression level till 40-50µg but diminished above 70µg. Similarly analysis was done also by enhancing Annexin 7 expression in P cell lines and checked from 20µg to 90µg. Protein expression was prominent till 30µg but increasing beyond 45 µg it was diminished. Gelsolin expression in F shRNA (Annexin 7) was 77%, 30% and 58% for fraction 1,2 and 4 respectively. (figure 5) In P cDNA 3.1 (Annexin 7) for Gelsolin was 64% and 22% (figure 3a,3b).
Annexin 7 and Gelsolin correlates with LNM of HCC

Western blot analysis was performed to measure the protein expression level of Annexin 7 and Gelsolin in cell lines with different metastatic potential (table1). The expression level values were higher in high metastatic potential cell line and lower in low metastatic potential cell line that indicates its positive correlation with LNM of HCC. For further investigation and confirmation the expression was down-regulated in high potential cell line and up-regulated in the low potential cell line. Results indicated that down-regulation caused decreased expression whereas up-regulation caused increased expression. (figure 6a, b)

DISCUSSION

The identification of subsets of proteins at the sub-cellular level is an initial step towards the understanding of cellular function (Huber, 2003). Cancerous cell undergoes lot of morphological changes during the progression of disease. However, it is hypothesized that different proteins especially the tumor-associated proteins are compartmentalize at different locations and expressed at different levels depending on their functions during the progression of cancer metastasis. To check this hypothesis sub-cellular fractionation is the major step as it can provide possible clues about their exact location and level of expression and in turn can provide possible clues about the specific roles of these proteins (Bryant et al., 2002). There are subsets of proteins that are associated with sub cellular structures only in certain physiological states, but localized elsewhere in the cell in other states. For example, there is protein translocation between different compartments, cycling of proteins between the cell surface and intracellular pools (Santoni et al., 2000). In other terms ‘sub cellular proteomics’, can
be used as a multifunctional tool in cell biology (Gygi et al., 1999).

The resemblance observed in the expression levels and distribution pattern within the lymphatic metastatic cells during this study provide evidence that both Gelsolin and Annexin 7 are cytoplasmic, calcium-regulated and actin-modulating proteins. Both functions in calcium dependent manner (Kisler et al., 2003; Küppers et al., 1992). It seems that both translocate during the metastasis stage, they reside and express to different location in the cells indicating their multiple functions in the metastasis. Annexin 7 has been proposed to function in the fusion of vesicles, acting as a Ca\(^{2+}\) channel and as Ca\(^{2+}\)-activated GTPase, thus inducing Ca\(^{2+}\)/GTP-dependent secretory events (Claudia et al., 2001). To understand their role in tumor metastasis we have targeted and down-regulated its expression in the mice and the resulting expression level was decreased in a metastatic cell (Helga et al., 2009). Gelsolin's activity is stimulated by calcium ions. Although the protein retains its overall structural integrity in both activated and deactivated states (Sun et al., 1999).

Our experiments showed that Annexin 7 and Gelsolin both expressed in higher levels in cytoplasm as its major site of expression in the Hca-F-cells than with the progression of tumor it start expressing in cytoskeleton. Gelsolin is secretory in nature, it relocate itself to cell periphery to increase invasive phenotype which is most malignant feature of the disease (Kassis et al., 2001). This means with the progression of cancer Gelsolin expression level and distribution at different sub cellular location increases the conversion of a non-invasive tumor to an invasive tumor. Annexin 7 is found in the vicinity of secretory vesicles, on sub-cellular membranous structures, and on plasma membranes (Cachuy et al., 1996) suggesting a possible role in Ca\(^{2+}\)-mediated exocytosis. Gelsolin and Annexin 7 both are found expressed at noticeable level in cytoskeletal fraction during our experiments supporting their involvement in cellular transformation, which is a characteristic of metastasis (David et al., 2005).

Our results are supportive for the concept that Gelsolin and Annexin 7 are distributed to different sub cellular locations during hepatocellular carcinoma. Expression level was highest in cytoplasm but with the progression of disease they start acting on actin filament of cytoskeleton they effect the morphology of cancerous cell (Shepard et al., 2006) that is an important phenomenon of lymphatic metastasis. We have also observed that in high metastatic potential cell lines Gelsolin expressed at membranes only but not in low metastatic potential. This finding is indicating that Gelsolin start membrane ruffling and protein shuttling (Wells et al., 2000) only with high potential metastasis that may depend on acquiring a certain level of Gelsolin expression. 47 KDa form of Annexin 7 is almost expressed in all tissue type but 51 KDa, its expression in liver cell indicates this isoform as playing important roles in lymphatic metastasis of HCC.

During our comparative studies we down-regulated Annexin 7 expression in F-cells and observed Gelsolin expression in it. We have noticed it only at membranes. Gelsolin expression has increased than in Hca-F cells around 50ug of proteins but this level is diminished at higher amount of proteins. In other two fractions expression levels were noticeable but not exceeded than that in Hca-F. Similarly we up-regulated Annexin 7 expression in Hca-P cells and observed the expression levels which were noticeable around 30ug. After both up-regulation and down-regulation we have noticed that Gelsolin expression is noticeable in all fractions but only membranes have shown elevation, with all these observation we suggest that at membranes with specific amount of proteins (Ridley et al., 2003) they might serve as binding partners (Jones et al., 2005) during the progression of disease and down-regulation of Annexin 7 might enhance Gelsolin binding at membranes.

One limitation in the successful fractionation of cells is the production of an ideal homogenate, that is, the release of organelles and other cellular constituents as a free suspension of intact, individual components. Very often cytoplasmic aggregates, cytoskeletal elements and nuclei might release DNA during homogenization procedures (Sumner, 1981; Iwabu et al., 2004) get mixed so in order to avoid it, a fractionation kit(S-PEK) has been used that with the specific buffers and reagent mixture has preserved the sub-cellular structural integrity. It yields the total proteome fractionated into four sub proteomes of decreased complexity. Each fraction was checked and confirmed using Western blotting with normalization against specific housekeeping proteins. Clear bands of respective proteins for each fraction were observed indicating their fractional integrity.

Expression at different sub cellular location might link their multiple roles in progression of tumor metastasis (Zhou et al., 2006) Gelsolin and Annexin 7 both are found sharing almost the same sub cellular distribution pattern especially in high metastatic potential cell lines. These findings suggest that Gelsolin and Annexin 7 similar sub-cellular location may be a new target for limiting hepatocellular tumor progression.

**CONCLUSION**

Subcellular fractionation of cell has increased the accuracy of analyzing proteins interactions and their expression among organelles which are found associated with tumor progression. Detecting tumor lymphatic vessels proliferation is major step in understanding cancer.
Acknowledgement

This research work was supported by grants from National Natural Science Foundation of China (No.81071725) and educational department of Liaoning Province (No.2009S028). Special thanks to Ms. Huang Y and Mao Jun for their help.

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