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Original article

Subcellular proteomics: Determination of specific location and expression levels of lymphatic metastasis associated proteins in hepatocellular carcinoma by subcellular fractionation

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ABSTRACT

Background: Subcellular fractionation and proteomics form an ideal partnership when it comes to specific location and analysis of intracellular organelles and expression levels of multiprotein complexes. Lymphatic metastasis is the major complicated system involving multiple factors. However, to date lymphatic metastatic mechanism is poorly understood.
Aim: To specifically locate expression site by subcellular fractionation, based on expression levels, interpret the involvement of different lymphatic metastasis associated proteins in hepatocellular carcinoma cell lines with different lymphatic metastasis potential.
Method: Mouse hepatocellular cell lines Hca-F and Hca-P are used to evaluate the location and expression levels of some lymphatic metastasis-associated proteins in the cell by using subcellular fractionation kit and Western blot analysis. The proteins under studies were Gelsolin, JNK and Annexin 7.
Results: Gelsolin was sequestered in cytoplasm, membrane and cytoskeleton in F-cells but in P-cells, it was found in cytoplasm and cytoskeleton .JNK was located in nuclear fraction and cytoskeleton in F and P cells, Annexin7 was in cytoplasm with its two isoforms only at this location, cell membrane and cytoskeleton in F and P cells. With the high expression level of Gelsolin, JNK and Annexin 7 in Hca-F cell line than Hca-P cell line.
Conclusion: With subcellular fractionation specific location of Gelsolin, JNK and Annexin 7 at various cell sites during lymphatic metastasis were determined. High expression levels were found in high lymphatic metastasis potential cell lines which indicate their roles according to different expression sites in the disease.

1. Introduction

Present estimates of the number of genes in the human genome expressed in a particular cell type reach 10,000. However, the number of proteins in the entire human body is expected to be many times higher. Thousands of chemical modifications occur after proteins are created that alter their enzymatic activity, binding ability, how long they remain active, and so on [1]. These modifications and the still-underestimated rate of alternative splicing give rise to a human proteome size that is likely to be significantly larger than the number of estimated genes. Because of the limited resolution power of separation technologies presently applied in proteomics research, additional fractionation steps are required [2]. Therefore, proteomics research has become increasingly aware of techniques for analyzing subcellular proteomes of reduced complexity. Subcellular fractionation, allowing the separation of organelles based on their physical properties, was initially applied to separate organelles derived from rat liver [3]; one major 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 [4]. Very often, cytoplasmic aggregates or cytoskeletal elements with nucleus are observed, to ensure the reliable fractionation and to overcome these problems, we have used subcellular fractionation kit in this research.

HCC is a phenotypically and genetically heterogeneous polyclonal disease and resistant to most conventional chemotherapy. Ninety percent of malignant tumors are carcinomas, and lymph nodes are often the first organ to develop metastasis [5]. HCC is one of the globe's most common types of cancer and one of the most fatal [6]. At present, HCC is largely a Third World disease, especially Southeast Asia and Africa, China alone accounts for more than 50%of the world HCC cases [7]. Lymphatic metastasis is a complex process involving multiple genes and their products. However, molecular mechanism of metastasis remains poorly understood [8]. A mouse hepatocellular cell line Hca-F with high lymphogenous metastatic

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potential (above 70%), and its syngenetic cell line Hca-P with low lymphogenous metastatic potential (below 30%) have been successfully set up in our lab from hepatocarcinomas in mice [9,10]. Hca-F and Hca-P metastasize only to lymph nodes, and not to other organs, which have been proved to be the ideal models for studying lymphatic metastasis for hepatocarcinoma [11]. The proteome analysis at the level of subcellular structures (that can be enriched by subcellular fractionation) represents an analytical strategy that combines classic biochemical fractionation methods and tools for the comprehensive identification of proteins. Among the key potentials of this strategy is the capability to screen not only for previously unknown gene products but also to assign them, along with other known, but poorly characterized gene products, to particular subcellular structures. Furthermore, the analysis at the subcellular level is a prerequisite for the detection of important regulatory events such as protein translocation in comparative studies.

Our group has engaged in the research for molecular mechanism of lymphatic metastasis of HCC during the past decade. We have already screened out the lymphatic metastasis-associated candidates and found higher expression of some genes and proteins including Gelsolin, JNK, Annexin 7 in Hca-F than in Hca-P by cDNA microarray as well as by 2 –DIGE, mass spectrometry and liquid chromatography [12] and constructed a subtracted cDNA library in these cell lines using subtractive suppressive hybridization [13]. We have compared Hca-F cells and Hca-P cells between the expression of different genes by gene chip assays [14] and obtained the lymphatic metastasis associated proteins by using quantitative proteomics techniques [15].

With these technologies, we found Gelsolin gene in Hca-F cells 1.9 times higher than in Hca-P cells and the difference of Gelsolin protein in Hca-F cells and Hca-P cells is 1.7 times [13–15]. At both protein and gene level of Gelsolin in Hca-F and Hca-P cell lines, the results showed higher expression of Gelsolin in Hca-F cell line in liver ascites and can promote tumor cell migration and invasion in lymph node metastasis of liver cancer (Wang Shaoqing; unpublished data). In fact, in vivo and in vitro studies showed that not only invasion, migration and proliferation rather regulation of apoptosis also has an important role in tumor metastasis.

We also found that JNK1 expression was much higher in Hca-F than in Hca-P cell lines at both gene and protein levels. JNK1 protein has the same origin with mouse and humans in structure and function. Therefore, it is important to study the relationship between JNK1 and lymphatic metastasis of mouse hepatocellular carcinoma. In vitro, by Transwell technique, our results showed that the ability of migration and invasion of Hca-F cells decreased significantly after inhibition of JNK1 expression by RNA interference. This indicated that JNK1 could regulate behaviors of migration and invasion in lymphatic metastasis of HCC cells [16].

Our group findings for Annexin 7 showed its overexpression in Hca-F cells than that of Hca-P cells at both gene and protein level, indicating its role in the proliferation, inhibition of tumor cell apoptosis, enhanced tumor cell migration and invasion. (Wang Zhiqiang; unpublished data). Annexin 7 expression 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 [17]. The repression of Annexin A7 inhibits the mobility and invasion abilities of Hca-F cell, increases the apoptosis rate of Hca-F cell [18].

In this study, we have focused on Gelsolin, JNK and Annexin-7 subcellular location, compared their expression levels in the cells statistically and by Western blot analysis. Based on this data, we tried to figure out their involvement in lymphatic metastasis and its progression.

2. Materials and methods

2.1. Animals and cell lines

Inbred 615 mice were provided by the animal facility of Dalian Medical University. Mouse hepatocarcinoma cell lines Hca-F, F sh RNA (F cells transfected with shRNA targeting Annexin 7, down regulation), Hca-P and P c DNA3.1 (P cells transfected by P cDNA3.1-Annexin7, up regulation) were established and stored by our department. To establish a mouse hepatic cancer cell line Hca-F transfected with shRNA, three sh RNA were designed and inserted into the pSilencer vector to silence Annexin7 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 400 μg/μl G418 (Geneticin). To construct PcDNA 3.1 – Annexin 7and 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 PcDNA3.1 plasmid. This plasmid was transfected in P cells stably. The effectiveness was checked by genome DNA checkup and Western blot analysis.

2.2. Cell culture

Hca-F and Hca-P cells were injected at 2×10^6 tumor cells in 0.1 ml cell suspension into inbred Chinese 615 mice and were grown into mouse abdominal cavity for 7 days. These cells were drawn and injected again in other 615 mice and allowed to grow for 5 days. Two passages were done in order to harvest large number of cells in around 2 weeks. 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 than regular passages were done in order to grow large amount of cells in vitro. Cell viability was determined by Trypan blue exclusion test.

Hca-F and Hca-P cells were inoculated (2×10^6 cells/mouse) into the left foot pad of each mouse among 10 mice in a group of 20 inbred 615 mice. On the 28th day of postinoculation, the mice were euthanized and their lymph nodes were collected and stained by HE and examined under microscope. Therefore, the lymph node metastasis rate was calculated.

2.3. Subcellular fractionation

Before fractionation, cell suspension was made so that each aliquot will have $3–5 \times 10^6$ cells/frozen cell pellet. S-PEK (cat. no. 539790) was used for the differential extraction of proteins from mammalian cells according to their subcellular localization. The extraction procedure provided four fractions with decreased proteome complexity. Cell suspensions were washed with wash buffer (kit component no. KP 31250) and centrifuged twice according to manual's instruction.

2.4. Subcellular protein extraction from culture cell suspension

Before the beginning of extraction procedure, all buffers were mixed well by vortexing and kept on ice during the whole procedure. 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, resuspended 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 buffer 1 was mixed with 5 μl protease inhibitor cocktail and added to pellet, incubated for10 min at 4 °C, centrifuged at 2900 rpm for 10 min in cold at