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Expression of embryonic liver fodrin (ELF) and stem cell markers in CD13 liver cancer stem cells

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Abstract. – OBJECTIVE: Investigating the molecular signaling pathways using CD13 as a marker for Cancer Stem Cells (CSCs) in human liver cancer.

PATIENTS AND METHODS: In the present study, liver carcinoma biopsies were obtained from the liver cancer patients, as well as healthy controls. Immunohistochemistry and Immunoblotting experiments were performed accordingly to conclude the data.

RESULTS: Immunohistochemistry along with immunoblot data showed the expression of Oct-4, STAT3 and interestingly Embryonic Liver Fodrin (ELF) in the CD13 positive liver cancer stem cells. Though embryonic liver fodrin (ELF) is a pro-differentiation protein, it was expressed in the CD13 positive liver cancer stem cells along with stem cell markers such as Oct-4 and STAT3.

CONCLUSIONS: Our finding concludes that an association of ELF expression was noted in liver cancer patients. Hence, ELF may have value as prognostic indicators and may facilitate the development of novel therapeutics for liver cancer.

Key Words:
Cancer stem cells, Liver cancer, CD13, ELF, Oct-4, STAT3.

Introduction

Worldwide, liver cancer is the fifth and eight most common cancers in men and women population, respectively[1]. Reports suggest that men have 2 to 4 times more susceptibility to liver cancer than women[1]. Various signaling molecules perform the process of differentiation and development of embryonic stem cells (ES) and also somatic stem cells into various functional cell lineages[2]. Cancer stem cells (CSCs) are the basics for many tumors, including one that of liver cancer[3-8]. Especially, the CSCs have an essential role in the action of metastasis[9]. But, the origin and the mechanisms of CSCs are still unclear, molecular mechanism behind is not manifested. Notably, many cancers have cancer stem cells in the tumor site or at the site of cancerous cells[3-18]. CSCs proliferate slowly when compared with normal somatic tissue-specific stem cells. This unique property is the important key for a possible generation of novel therapeutic agents to control the spreading of cancer. Searching for the molecular switch or its mechanism, which is unique to liver CSCs, is essential for specific therapies against liver cancer. Embryonic liver fodrin (ELF) is a kind of β-spectrin and performs the TGF-β signaling molecular pathway[19]. ELF has a link with Smad3 and thereby furnishing it to the cytoplasmic domain of the TGF-β Type I receptor complex, heteromeric complex formation with Smad4, nuclear translocation forwarded by specific gene activation[20]. Besides, homozygous mutant of $elf^{-/-}$ leads to defective liver development in mice. In addition, the heterozygous mutant of $elf^{+/-}$ mice shows to develop dramatic spontaneous Hepatic Cell Carcinomas (HCC)[2].

In the present study, we evaluated the regulatory role as well as expression pattern of ELF, and we studied its molecular link in connection with liver cancer stem cells. Our findings will facilitate the discovery of prognostic indicators and the development of novel therapeutics for liver cancer.

Patients and Methods

Patients and Sample Collection

For the present study, 18 biopsy samples were obtained from the Affiliated Hospital with proper consent from the patients and the approval of the Ethical Committee. Based upon the preliminary examination, liver biopsy samples were collected

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from the patients through the standard surgical procedure. The collected samples were stored accordingly in -80ºC deep freezer after careful labeling of the samples. The biopsy samples were accessed histologically, confirming to have >90% tumor cells by histology specialists. The samples will be used for immunohistochemistry analysis. A normal liver biopsy was used as controls, which was collected from donors.

Immunohistochemistry

The biopsy samples were treated with formaldehyde and processed for paraffin-embedding using standard procedure. The thin tissue sections (4 μm) were deparaffinized and hydrated. Antigens were retrieved in sodium citrate buffer at 95ºC for 30 min (pH 6.0). Non-specific staining was blocked by treating the thin sections with normal serum. The processed thin sections were incubated separately with primary antibodies (purchased from Sigma-Aldrich namely Anti-ELF (Cat. No. HPA003479), Anti-Oct-4 (Cat. No. ABE422) and Anti-STAT3 (Cat. No. SAB4300708) (Sigma-Aldrich, Oakville, Ontario, Canada) overnight at 4ºC followed by incubation with secondary antibodies. The slides were developed and observed under a microscope.

Immunoblot Analysis

Tissue lysate from the liver samples (control and cancer tissues) were prepared and resolved in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Immunoblot was performed with wet blot method using the standard procedure. Blocking step was performed with 2% Bovine Serum Albumin (BSA – Sigma-Aldrich, Cat. No. 05470, St. Louis, MO, USA). The membrane was further incubated with primary antibody. Following antibodies were purchased from Sigma-Aldrich namely Anti-ELF (Cat. No. HPA003479), Anti-Oct-4 (Cat. No. ABE422), Anti CD13 (Cat. No. SAB4700001), Anti-STAT3 (Cat. No. SAB4300708) and Anti-β actin (Cat. No. A1978) (Sigma-Aldrich, St. Louis, MO, USA) and diluted appropriately as mentioned in the manufacturer instruction. The non-specific binding of primary antibody is washed out and further incubated with the suitable secondary antibody. Later, the slides were developed and the signals were obtained and visualized using DAB (3,3'-diaminobenzidine) (Sigma-Aldrich, Cat. No. D8001, St. Louis, MO, USA).

Statistical Analysis

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) for Windows 11.0 (SPSS, Inc., Chicago, IL, USA). All experiments were performed in triplicate and the results are expressed as means, average, and standard errors of the mean. The intensity of the immunoblot was determined by scanning the blot in gel documentation system (Bio-Rad, Hercules, CA, USA). Statistical significance was evaluated by using the *t*-test, which was performed by measuring the mean intensity of the immunoblot using Bio-Rad imaging Software. p-value < 0.05 was considered as statistically significant.

Results

Collection of Samples and Analysis

ELF expression and its molecular link in connection with liver cancer stem cells were accessed in the biopsy samples from liver cancer patients. A careful preliminary histopathological observation was performed by the pathologist and it was confirmed that the samples had >90% liver cancer cells and, therefore, were included in the study. In addition, normal control liver samples were obtained from the donor with proper consent approval.

CD13 Positive Liver Cancer Stem Cells

CD13 expression was detected in all the liver cancer patient samples, but not in the normal liver tissues. The data (not shown here) confirms that the liver cancer sample has the liver cancer stem cells. The data is the preliminary step for the current experiments. Also, the data shows the expression of stem cell marker Oct-4 in the liver cancer samples but not in the normal samples (Figure 1B).

Immunohistochemistry Analysis

ELF, Oct-4, and STAT3 expression in the liver cancer tissue samples were confirmed by immunohistochemistry (Figure 1). The data (Figure 1C) shows the expression of ELF in liver cancer samples. Similarly, the data (Figure 1D-E) shows the expression of STAT3 and Oct-4. Both are stem cell markers, which was present in the liver cancer samples, respectively. The data suggests that the liver cancer samples that express Oct-4 and STAT3 are liver cancer stem cells. Data (Figure 1F) shows the overlay of ELF, Oct-4, and STAT3, respectively.

Immunoblotting Analysis

The immunohistochemistry data were validated by immunoblot analysis. CD13 expression was observed in the liver cancer samples and none in

the control samples. Based on the data (Figure 2), it was confirmed that the liver cancer tissue samples contain liver cancer stem cells. In addition, Oct-4, STAT3, and ELF were expressed in the liver cancer tissue samples and none in the control samples. The intensity of immunoblot was calculated and the data was statistically significant. Immunohistochemistry analysis in accordance with the immunoblotting confirmed and validated that ELF was expressed in the liver cancer stem cells.

Discussion

Liver cancer is common cancer ranked as fifth in the worldwide[21]. Liver cancer is a lethal malignancy form of cancer worldwide; therefore understanding the molecular mechanism is the key to solve this pathology. The high death rate is due to poor prognosis in early stages of some cancers, especially HCC[22]. Hence, we aimed to study the molecular link and expression pattern of ELF in liver cancer stem cells. Of note, in the present study ELF expression was detected in liver cancer samples but not in the control samples by immunohistochemistry (Figure 1) as well as immunoblotting analysis (Figure 2). Though CD13 is the liver cancer stem cell marker, it was expressed in the liver cancer tissue samples. In addition, the stem cell markers Oct-4 and STAT3 were expressed in the liver cancer cells, which imply that the liver cancer tissue samples have liver cancer stem cells (CSCs). The overlay image illustrated that Oct-4, STAT3, and ELF signals were obtained in the same cells. The data confirms that ELF was expressed in the liver cancer stem cells.

Experimental studies show the prevention of liver cancer angiogenesis through miR-126 molecular pathway[23]. Interestingly, though ELF is a pro-differentiation factor, its expression was noted in the CD13 liver cancer stem cells. It is may be due to tumor metastasis. Recently, it was re-

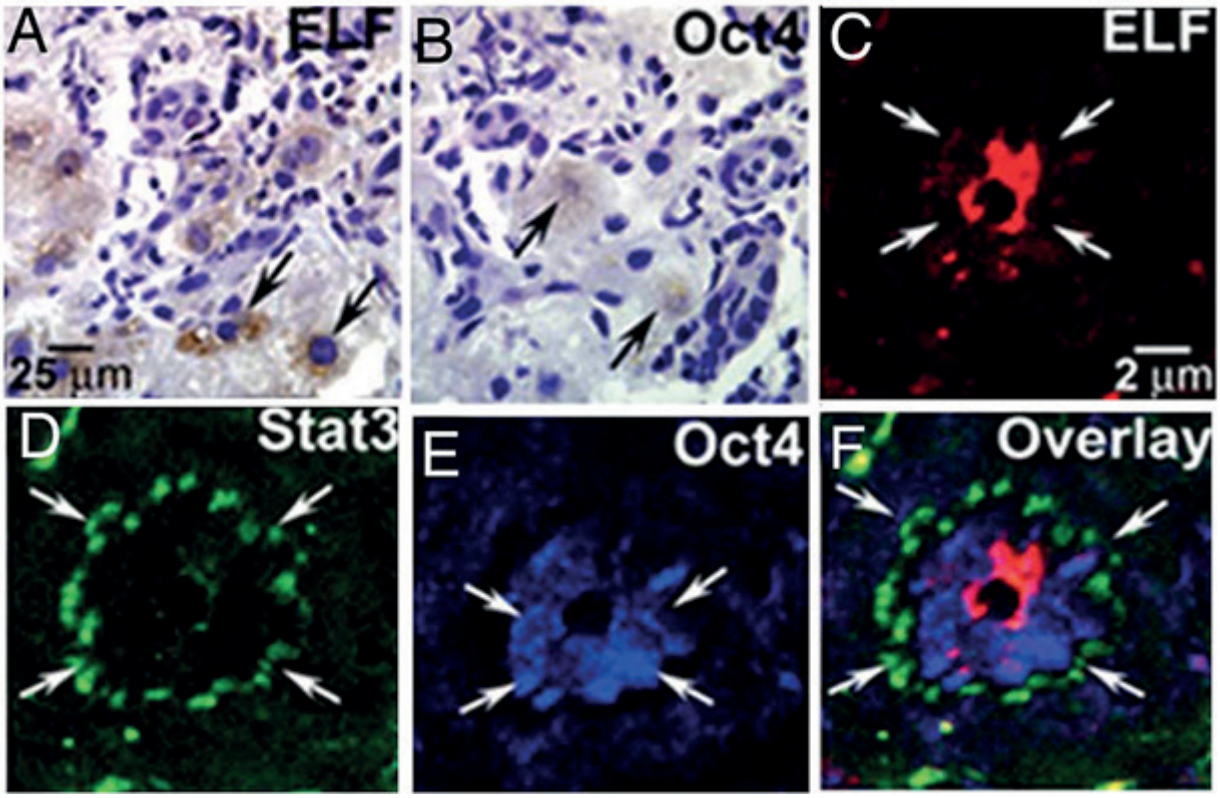

Figure 1. Immunohistochemistry analysis of liver cancer samples. ***A,*** Immunohistochemistry of liver cancer tissue samples stained with Anti-ELF antibody and the sections were counterstained with hematoxylin. ***B,*** Immunohistochemistry of liver cancer tissue samples stained with Anti-Oct-4 antibody and the sections were counterstained with hematoxylin. The positive signals were identified by brown color. ***C,*** Immunohistochemistry of liver cancer tissue samples stained with Anti-ELF antibody. ***D,*** Immunohistochemistry of liver cancer tissue samples stained with Anti-STAT3 antibody. ***E,*** Immunohistochemistry of liver cancer tissue samples stained with Anti-Oct-4 antibody. ***F,*** Overlay image of ELF, Oct-4, and STAT3. The slides were observed under microscope at 100X magnification. Scale bars – 2 µm.

ported that HIF-1α and KISS-1 expression was noted in liver cancer patients[24]. HIF-1α and KISS-1 are recently-discovered genes, closely related to tumor metastasis, whose expression was noted in liver cancer patients[24]. Further experiments are essential to confirm the expression of ELF in tumor metastasis. Hence, ELF may have value prognostic indicators in context to tumor metastasis and also may facilitate the development of novel therapeutics for liver cancer.

Conclusions

Immunohistochemistry and immunoblotting experiments confirm the ELF expression in the CD13 positive liver cancer stem cells along with stem cell markers such as Oct-4 and STAT3, respectively. The finding concludes that an association of ELF expression was noted in liver cancer patients. Hence, ELF may have value as prognostic indicators and may facilitate the development of novel therapeutics for liver cancer.

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Conflict of Interest

The Authors declare that they have no conflict of interest.

Figure 2. Immunoblotting analysis of biopsy samples with Anti-CD13, Anti-Oct-4, Anti-ELF, Anti-STAT3, and Anti-β-actin antibodies. Lane 1 and 2 represent liver cancer tissue samples and Lane 3 and 4 represent control, respectively.

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