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CD133 induced tumorigenicity mice express TGF-beta receptor type II (TBRII) and embryonic liver fodrin (ELF) along with stem cell markers

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Abstract. – OBJECTIVE: Cancer stem cells play a major role in developing hepatocellular carcinoma (HCC), and they are not well identified in in vivo settings. Also, their occurrence and specificity are not well defined in different pathological stages of the tumor. CD133 is a cancer stem cell specific marker which also lacks to specify all the cancer stem cell population and similarly different markers fails to identify cancer stem cells. To overcome this multi marker helps to identify the cancer stem cells with maximum coverage and aids to understand their expression in different pathological stages of HCC.

MATERIALS AND METHODS: In this study, we compared the expression of CD133 along with TBRII and ELF using Immunohistochemistry and western blotting technique that probably helps to reveal the nature of cancer stem cells in different pathological stages of HCC.

RESULTS: We initially develop CD133 induced tumorigenicity mice with HCC and compared their tissue morphology using histology. The histological data reveal that as tumor progress, the cell proliferative ability increase together with a large nucleus. The Immunohistochemical data against CD133 shows a prominent increase in their expression as tumor progress, but we found out that the tumor suppressor related proteins, TBRII and ELF shows increased expression in primary tumor stages to prevent tumor initiation, but neither of them shows prominent up-regulation in metastasis stages of the tumor.

CONCLUSIONS: Our results conclude that the tumor suppressor proteins TBRII and ELF shows elevated expression pattern in the primary stage, but in advance stages its expression gets down-regulated and that fails to control metastasis stage.

Key Words:
Hepatocellular carcinoma, CD133, TBRII, ELF.

Introduction

Among the different organ system of human, liver is the largest organ system next to skin[1]. It weighs around 3 pounds and plays many vital functions of body mechanism that are associated with metabolism, digestion, detoxification, immunity and in the storage of different nutrient. Liver disease occurs due to different agents like poisoning, alcohol consumption or due to viral infections which ultimately results in liver injury[2]. The carcinoma of liver especially, Hepatocellular carcinoma (HCC) is the fifth most abundant tumor in a world which accounts for 3rd highest mortality rate[3]. The highest prevalence of HCC is mostly related to viral infections of hepatitis B and C[4,5]. As with other types of cancer, HCC also possesses cancer stem cells, which are responsible for tumor initiation, self-renewal, chemo resistance, recurrence and metastasis[6].

The occurrence of cancer stem cells was hypothesized more than 40 years back[7,8] but their existence was studied extensively only in the last decades[9]. It is important to differentiate and identify cancer stem cells from the stem cells so that we can understand the disease progression[10]. The markers to identify cancer stem cells related to HCC lacks specificity and at present we are able to identify only 60%-70% of cancer stem cells[11]. In generally the identification of cancer stem cells in a specific way, with maximum coverage are improved using multimarker hypothesis[12]. CD133 is such a marker to identify cancer stem cells, but it lacks specificity because it was also identified in circulating endothelial progenitor cells of HCC[13]. Under this condition simply using a single marker alone cannot help to improve the specificity and sensitivity of identification, but accessing them with different markers helps with maximum identification.

TGF-beta receptor type II (TBRII) and embryonic liver fodrin (ELF) are used here as markers to identify cancer stem cells of HCC. Here, we overall compared the different cancer stem

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cell marker to analyze them in a better way by inducing tumorigenicity using CD133 in a mouse model.

Materials and Methods

Experimental Animals with HCC

The animal used together with protocol to be carried out was approved by the institutional animal Ethical Committee. For developing HCC mouse model, aggressive Liver cancer cells with CD133 were used. The CD133 specific cells were sorted out using a flow cytometer. The experiments are carried out in female athymic BALB/c mouse strain by injecting CD133 positive (10^4 cells/20 µl) liver cancer cells directly into the liver. Initially, the liver was located by making a small incision in the left side of the abdomen and, after the injection, the cavity was closed with stitches. Following injection, the mice were observed for liver palpation and one set of mice were sacrificed on 3rd week to obtain primary tumor and another set of mice were incubated for up to 8 weeks to obtain a metastatic form of HCC. Immunohistochemistry

For immunohistochemistry, the dissected tissues were initially fixed in 10% formalin solution and processed to get paraffin embedded tissue. The microtome was set with 6-µm size and the sections obtained are fixed in the microscopic glass slide. Following de-paraffinition with xylene, they are hydrated with water. To block the endogenous peroxidase activity, the sections are immersing in freshly prepared 10% H_2O_2 and 10% Methanol in 1X PBS for 20 min. After washing with 1X PBS, the sections were treated with 0.1% trypsin in 0.1% $CaCl_2$ at 37° C for 5 minutes. In order to facilitate binding of specific antibody, the sections were incubated with an Anti-TBRII antibody (Abcam, Cambridge, MA, USA) (ab61213) or with an Anti-SPTBN1 antibody (Abcam, ab124888), for overnight at 4° C. After wash out the non-specific binding of primary antibody, they are then incubated with suitable secondary antibody for 30 minutes at room temperature. After throughout washing the sections, the primary antibody is detected using DAB (diaminobenzidine) Kit as a chromogen.

Western Blot Analysis

The cell lysate was prepared from normal tissue as well as from primary and metastatic form of HCC tissue. The proteins are isolated and resolved in 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. They are then transferred to the membrane and incubated with primary antibody (Anti-TBRII antibody, Abcam (ab61213) or with an Anti-SPTBN1 antibody (Abcam, ab124888) in the dilution ratio of 1:500. The non-specific binding of primary antibody is washed out and further incubated with the suitable secondary antibody of dilution concentration (1:3000). Later the signals obtained are visualized using DAB kit.

Results

Effect of CD133 on Developing HCC Mouse Model

In the present study, a mouse model of HCC was successfully initiated by injecting liver tumorigenic CD133 cells. The experimental mice respond well to injected CD133 cells, which are examined through histological observation by comparing with the control mice (Figure 1A-1C). Following injection, the mouse is established with the primary tumor, which is histologically visualized on 3rd week after CD133 cells injection (Figure 1B). Similarly, the metastatic condition of the HCC was achieved on the 8th week after post injection of CD133 cells (Figure 1C). When compared with the control, which has an even arrangement of the cellular pattern (Figure 1A) the primary and metastatic Osteosarcoma cells are large with deeply stained together with the disorderly arrangement of cells (Figure 1B-1C). The one unique feature of the metastatic form (Figure 1C) is that it has an enlarged proliferative mass of cells with an irregular pattern of tissue arrangement than primary HCC (Figure 1B). Also, some of the cells are displaced so that it is able to visible the gap between the clumps of cells (Figure 1C).

CD133 Expression in Primary and Metastatic HCC

The immunohistochemistry of normal liver tissue with anti-CD133 antibody shows a minimal expression as shown in the Figure 2A. But in response to HCC progression their expression gets upregulated (Figure 2B-C). In the case of primary HCC patches of cells are showing signals for CD133 (Figure 2B) whereas, in the metastatic form of HCC, strong signals are visualized from the background of the higher proliferative mass of cells (Figure 2C).

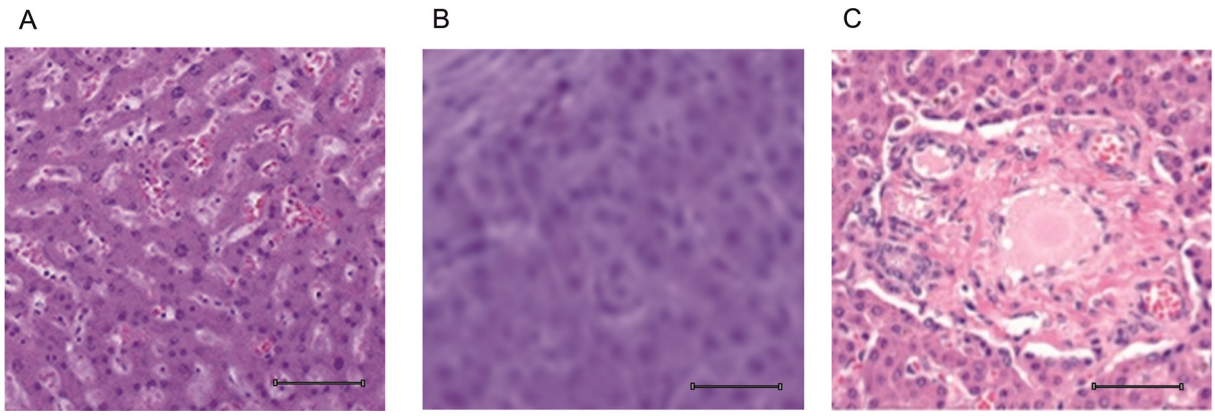

Figure 1. Histological characteristic of normal and HCC tissues. ***A,*** Histological section of normal liver tissue of mouse showing the uniform arrangement of tissue pattern. ***B,*** Sections of primary HCC tissue with an abnormal mass of cells. ***C,*** Metastatic HCC with deeply stained cells with a higher proliferative mass of cells. Scale bar = 100 μm.

TBRII is Highly Expressed in Primary and Metastatic HCC

TBRII act as a tumor suppressor gene that plays a major role in self-renewal and in maintaining stem cell population[14]. It also has a role in maturation as well as function as an inhibitor to prevent tumor formation[15]. The bifunctional role of TBRII expression is distinct in HCC and that their suppression results with tumor formation[16]. Still the role of TBRII in cancer development is not well studied and only little is known about the expression pattern of the TBRII in experimental animals that are developed with HCC.

In order to understand the TBRII expression pattern in different pathological stages of HCC, Immunohistochemistry was performed using anti-TBRII antibody to track the stem cells. The control mice show the remarkable presence of TBRII signals that are distributed in their sections (Figure 2D). The mice developed with HCC shows proliferated expression of TBRII protein, which in counterpart implies the supportive trigger of the repair process after developing initiative HCC (Figure 2E). On the other hand the mice with metastasis form of HCC exhibit restricted expression of TBRII (Figure 2F) which implies the cell's inability to reduce proliferation.

Expression Studies of ELF in Primary and Metastatic HCC

There is an interaction between TBRII pathway and ELF signaling in that ELF, a β spectrin act as an adaptor molecule that regulates TBRII[17,18]. To understand the HCC progression and to cross check the results further, the expression pattern of ELF was studied using Immunohistochemistry. The control liver tissue has a limited ELF protein expression (Figure 2G) but upon HCC development, especially in the primary stages of osteosarcoma, the ELF protein shows increased expression as shown in the Figure 2H. But the advanced metastatic form of HCC shows only a restricted expression of ELF with very mild signals (Figure 2I).

Western Blotting to analysis CD133, TBRII and ELF Expression

The results obtained using immunohistochemistry was further validated using Western blotting by comparing the expression of CD133, TBRII and ELF. Through which it is possible to precisely determine the HCC regulating proteins in different tissue. The protein samples from normal liver tissue, primary and metastatic HCC tissue are subjected to Western blotting analysis using an anti-CD133 antibody, anti- TBRII antibody and Anti-SPTBN1 antibody (Figure 3). Through experiment, we observed that the CD133, TBRII and ELF are linked with HCC progression. The result with ELF shows that it has an increased expression in primary HCC when compared with the normal tissue. But its expression shows a rapid decline in metastatic HCC.

Discussion

Liver cancer still remains a lethal malignant form of cancer worldwide, therefore understanding the different pathological condition of HCC is particularly important[19]. Also, the high death rate is due to poor prognosis in early stages of

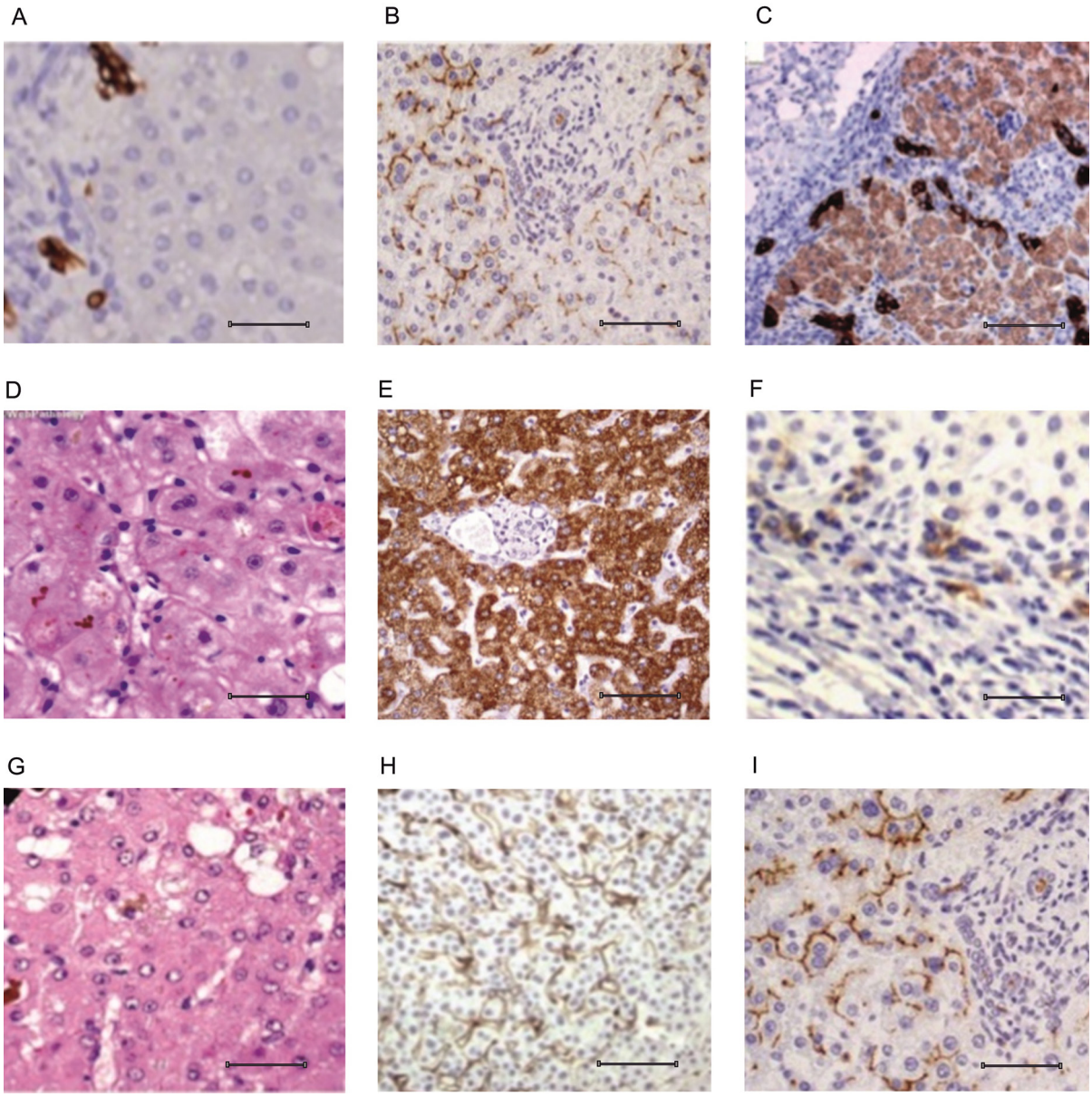

Figure 2. Analysis of CD133, TBRII & ELF expression in normal and HCC tissues. ***A,*** CD133 expression in the normal liver tissue of the mouse. ***B,*** Primary HCC shows CD133 expression. ***C,*** Metastatic HCC exhibit CD133 expression. ***D,*** TBRII expression in the normal liver tissue of the mouse. ***E,*** Primary HCC showing increased TBRII expression. F. Metastatic HCC with down regulated TBRII expression. ***G,*** ELF expression in the normal liver tissue of the mouse. ***H,*** Primary HCC shows increased ELF expression. ***I,*** Metastatic HCC with a decreased ELF expression. Scale bar = 100 µm.

HCC[20]. In the present study, the injected CD133 cells work effectively in a BALB/c strain of mouse and their histological tissues morphology and nature resembles human[21,22]. The experimental success of developing primary and metastatic Osteosarcoma is visualized with an abnormal proliferative mass of cells together with deeply stained clustering of cells (Figure 1B-1C).

To understand the role of TBRII and ELF in HCC their expression was analyzed (Figure 2D-I) and compared with CD133 (Figure 2A-C). The comparative analysis helps to characterize the nature of cancer stem cell pattern which also helps to understand the role of TBRII and ELF in controlling HCC. The molecular role of TBRII was identified to have an effect on many transcription

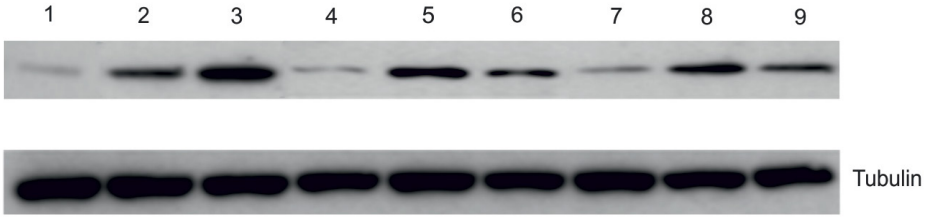

Figure 3. Western blotting analysis. The protein lysate prepared from control as well as from HCC tissue samples are subjected to Western blotting analysis using an anti-CD133 antibody, anti-TBRII antibody and anti-ELF antibody. Lane 1 denotes a CD133 expression in the normal liver tissue of the mice. Lane 2 represents a CD133 expression in the primary HCC tissue. Lane 3 specifies a CD133 expression in the metastatic HCC tissue. Lane 4 denotes a TBRII expression in the normal liver tissue of the mice. Lane 5 represents an increased TBRII expression in the primary HCC tissue. Lane 6 specifies down-regulated TBRII expression in the metastatic HCC tissue. Lane 7 denotes an ELF expression in the normal liver tissue of the mice. Lane 8 represents an increased ELF expression in the primary HCC tissue. Lane 9 specifies down-regulated ELF expression in the metastatic HCC tissue. Tubulin was used as a loading control.

factors and genes associated with growth and apoptosis[23].

Other than that the function of CD133 was also well defined in HCC, that its expression is associated with triggering the proliferative ability of liver cells and knock-out of CD133 leads to have a control over their proliferation ability[24]. The present study revealed the role of TBRII and ELF in controlling the HCC in the primary stage of cancer by having a higher expression pattern. As the HCC progress, the CD133 also shows over expression (Figure 2A-C); it implies that the developmental of HCC is associated with CD133 expression. Similarly, our study reveals the TBRII and ELF down-regulation is associated with HCC progression (Figures 2 and 3).

Conclusions

We effectively established mice model with HCC by injecting CD133 cells. The histological data shows complicated cell morphology as the HCC progress. The results with CD133 expression shows progressive expression as tumor advances. But TBRII and ELF shows up-regulated pattern of expression in the primary stage and as in metastasis stages, it down-regulated that pays ways for cancer development.

Conflicts of interest

The authors declare no conflicts of interest.

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