

ORIGINAL ARTICLE

Roles of ZEB2 and RBM38 in liver cancer stem cell proliferation

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Summary

Purpose: Liver cancer stem cells are associated with tumor progression, metastasis, and resistance to chemotherapy. Therefore, it is important to understand the proteins that support the tumor microenvironment. The suppression of ZEB2 results from inactivation of the Wnt/ β catenin pathway. Like RBM38, it suppresses tumor outgrowth and helps increase the survival of cancer patients. However, no studies have examined the direct roles of ZEB2 and RBM38 in the tumor microenvironment.

Methods: We developed an early/advanced stage liver cancer mouse model using CD133⁺ cell injection that mimics liver cancer in all ways. Histology, Western blotting, and immunohistochemistry analyses were used to examine cancer progression.

Results: Histologically, the early liver cancer showed micro-foci structures; the advanced cancer showed distinct morphological changes with enlarged nucleoli and cell clumping. Immunohistochemical and Western blotting analyses of CD133 and ZEB2 proteins showed similar upregulated expression as the tumor progressed. However, RBM38 expression increased dramatically in early liver cancer but was downregulated in advanced liver cancer.

Conclusions: ZEB2 favors a tumor microenvironment that supports liver cancer stem cell proliferation, while RBM38 expression negatively affects the tumor microenvironment and restricts liver cancer stem cell proliferation.

Key words: Liver cancer, CD133, ZEB2, RBM38, tumor microenvironment, cancer stem cell

Introduction

Liver cancer is the fifth most common cancer worldwide and the third leading cause of cancer mortality [1]. Despite advances in diagnosis and treatment, the 5-year overall survival rate of advanced liver cancer is less than 5% [2]. The poor outcome is associated with tumor recurrence and metastasis [3]. Risk factors for developing liver cancer are chronic hepatitis B or C virus infection, cirrhosis, and metabolic liver disease [4]. The occurrence of hepatocellular carcinoma is more complicated, as it results from gene mutations [5], and epigenetic changes in many oncogenes and

tumor-suppressor genes [6], and the deregulatory mechanisms of non-coding or coding genes [7].

Liver cancer stem cells play roles in tumor initiation, recurrence, metastasis, relapse, and chemoresistance [8,9]. Therefore, targeting liver cancer stem cells directly is an emerging strategy for treating this disease. However, it is still necessary to understand the microenvironment that supports liver cancer stem cells. Zinc finger E box binding homeobox 2 (ZEB2) is a transcription factor that plays a critical role in epithelial mesenchymal transition (EMT)-induced processes [10].

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Recent studies have reported that miR-498 targets ZEB2 and facilitates inactivation of the Wnt/ β catenin and TGF- β /SMAD pathways [2]. However, their regulatory roles in liver cancer stem cells have not been explored. RNA binding motif protein 38 (RBM38) regulates biological functions such as cell proliferation, cell cycle arrest, and myogenic differentiation [11,12]. Recently, the tumor suppressor role of RBM38 in liver cancer was identified; it works by stabilizing the p53–mdm2 interaction [13]. However, there are no reports linking RBM38 to liver cancer stem cells. Here, we examined the roles of ZEB2 and RBM38 proteins in supporting the liver cancer stem cell microenvironment.

Methods

Mice with hepatocellular carcinoma

To establish hepatocellular carcinoma, athymic 5-week-old, male BALB/c mice (n=15) were purchased from Laboratory Mice and Rats, Labome, and given 2 weeks to acclimatize to the laboratory. The experimental protocol was approved by the institutional ethics committee. After 2 weeks, the mice were injected with aggressive CD133 liver cancer cells (10^8 CD133 cells/30 μ L) in the left abdomen via a small incision. The animals were allowed food and water *ad libitum* and maintained on a 12 h light/dark cycle. Following injection, the mice were monitored each day and the liver was palpated 4 weeks post injection. At this time, the first five mice were sacrificed and the liver was dissected. Similarly, 8 weeks after CD133 cell injection, the second group of mice was sacrificed. Five mice not injected with CD33 cells served as a control.

Histology

Immediately after sacrifice, the liver was carefully removed and fixed in 10% paraformaldehyde at room temperature for 24 h. Then, the tissue was dehydrated in a gradually increasing isopropyl alcohol series. Xylene was used as a clearing agent for 50 min and then the tissue was immediately transferred to paraffin wax for

tissue embedding and block formation. The paraffin-embedded tissue was sectioned at 6 μ m and deparaffinized using xylene. The tissue sections were rehydrated on slides using isopropyl alcohol and stained with hematoxylin and eosin. The sections were mounted with DPX and visualized after 1 day under a light microscope.

Immunohistochemistry

The paraffin-infiltrated tissue samples were embedded in wax and sectioned at 6 μ m. Following deparaffinization with xylene, the slides were immersed in isopropyl alcohol, rehydrated with water, and washed with 1 \times phosphate-buffered saline (PBS). Antigens were retrieved by boiling in citrate buffer for 10 min, which helps unmask antigens and improve antibody detection. The slides were again washed with 1 \times PBS and blocked with 10% bovine serum albumin (BSA) solution for 2 h at room temperature. Then, the slides were incubated with suitable primary antibody (anti-ZEB2, anti-RBM38, or anti CD133) at 4°C for 6 h. Following incubation, the nonspecifically bound antibody was washed away three times with 1 \times PBS. Then, the slides were incubated with appropriate horseradish peroxidase (HRP) conjugated secondary antibody for 2 h at room temperature. Finally, after washing, DAB solution was used to reveal the specific signals.

Western blotting

After dissecting the livers from control and experimental mice, the tissue samples were placed in microcentrifuge tubes and homogenized in ice cold protein RIPA lysate buffer. The tube was kept in boiling water for 10 min, cooled, and stored at -80°C. The protein samples were loaded in 12% SDS-PAGE gels with at 70 μ g sample per well. The proteins were separated in the gel and transferred to PVDF membranes using a semi-dry method. Following transfer, the membrane was incubated with 4% BSA to block nonspecific sites on the membrane. Then, the membrane was incubated with primary antibody (anti-ZEB2, anti RBM38, or anti-CD133) at 4°C for 6 h and then with HRP-conjugated secondary antibody. Following repeated washes with 1 \times PBS, the membrane was developed with DAB to help detect the chromogenic signals.

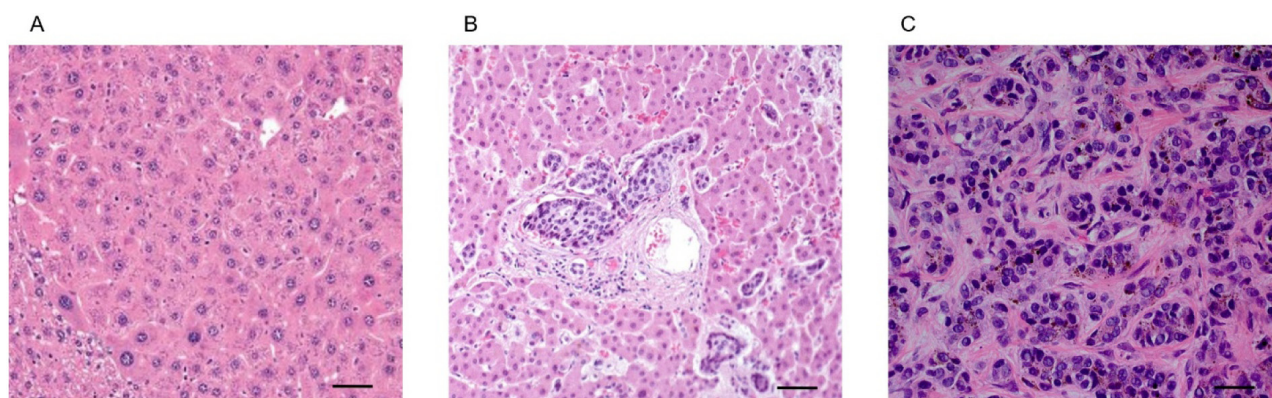


Figure 1. Histology of liver cancer development. **A:** Histological sections of normal liver tissue with no abnormal morphological structures. **B:** Liver tissue with microfoci structures. **C:** Liver cancer tissue with more proliferative cells and cell clumps. Hematoxylin and eosin stain. Scale bar= 20 μ m.

Statistics

The groups were compared using the Student's *t*-test (SPSS Statistics v13.0) and the values are expressed as the mean \pm SD. A *p* value <0.05 was considered significant.

Results

Mice injected with CD133⁺ cells develop early and advanced liver cancer

In the mouse abdomen, CD133⁺ cells have a role in maintaining stemness and regulate the aggressive nature of liver cancer stem cells [14]. Fol-

lowing CD133⁺ cell injection, early liver cancer was seen by 4 weeks and advanced liver cancer was observed by 8 weeks. The pathological changes in the early and advanced liver cancer were confirmed histologically (Figure 1). In the control tissue, the cells were uniform morphologically (Figure 1A), while in the early cancer, transitional cells formed and aggregated in different sites (Figure 1B). In the advanced stage, the tissue morphology was completely changed because of the high transition frequency of cells (Figure 1C). The cells in advanced liver cancer formed different isolated foci that were very close to each other (Figure 1C).

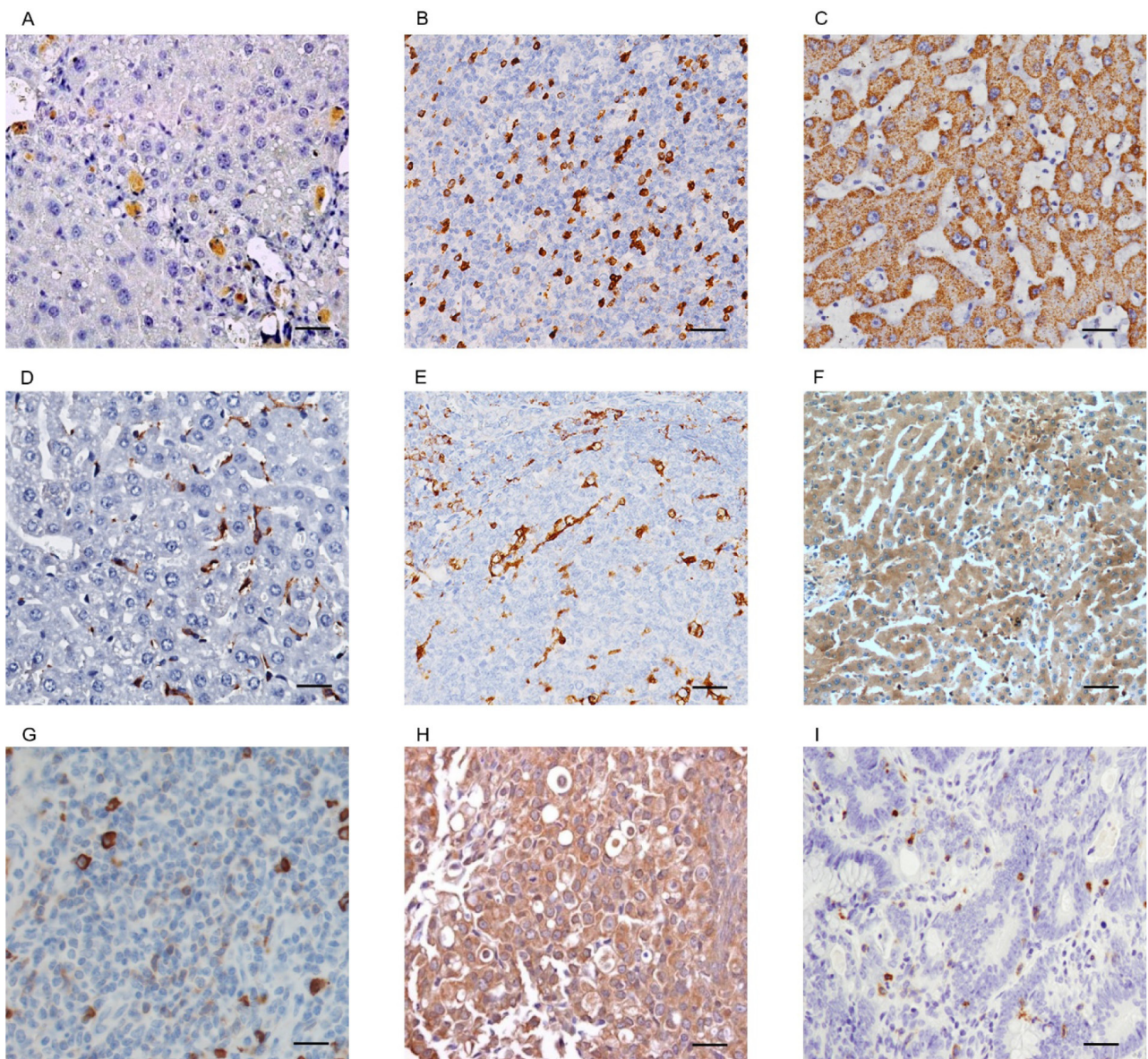


Figure 2. Immunohistochemical expression of CD133, ZEB2, and RBM38 in liver cancer. **A:** Normal liver tissue with restricted expression of CD133. **B:** Early liver cancer with slightly upregulated CD133 expression. **C:** Advanced liver cancer with enhanced CD133 expression. **D:** Normal liver tissue with limited expression of ZEB2. **E:** Early liver cancer tissue with marginally upregulated ZEB2 expression. **F:** Advanced liver cancer showing enhanced ZEB2 expression. **G:** Normal liver tissue with limited RBM38 expression. **H:** High RBM38 expression in early liver tissue. **I:** Downregulated RBM38 expression in advanced liver cancer. Scale bar= 20 \times .

Expression of CD133 protein during liver cancer progression

To validate the different pathological stage of liver cancer and to understand the liver cancer stem cells that regulate cancer stages, we performed immunohistochemistry of CD133 protein (Figure 2). In control samples, the expression of CD133 was limited, indicating that there were few abnormal liver cancer stem cells. Mouse liver tissue showing early liver cancer started to produce more CD133-positive cells. In advanced liver cancer, the expression of CD133 was increased many times and most cells expressed CD133.

Link between liver cancer stem cells and ZEB2 expression

Cancer upregulation and accelerated stem cell proliferation were clearly observed. To examine the link with ZEB2 expression, we analyzed the expression of ZEB2 in early and advanced liver cancer tissue (Figure 2). Its expression was very weak in control tissue and much higher in early liver cancer tissue. Ultimately, the ZEB2 signal was seen in the majority of the tissue, reflecting the proliferative behavior of liver cancer stem cells. The cellular localizations of CD133 and ZEB2 were similar at the different stages of liver cancer.

RBM38 is not a tumor suppressor in advanced liver cancer

The effects of RBM38 on restricting the tumor aggressiveness were analyzed immunohisto-

chemically (Figure 2). In controls, the expression of RBM38 was limited, but it increased significantly in the initial stage of liver cancer reflecting its tumor-suppressive nature. However, it was down-regulated in advanced liver cancer, and no longer regulated liver cancer stem cells.

Understanding the synergic effects of CD133, ZEB2, and RBM38

To confirm the immunohistochemistry results, Western blotting was performed using anti-CD133, anti-ZEB2, and anti-RBM38 antibodies (Figure 3). In control liver tissue, all three antibodies showed limited expression. However, in early liver cancer, the respective expression of CD133 and ZEB2 was 1.8- and 2.2-times higher than in controls. Interestingly, RBM38 expression was 3.8 times higher than in controls. By contrast, in advanced liver cancer, CD133, ZEB2, and RBM38 expression were 3.6-, 3.4-, and only 1.6-times their respective controls.

Discussion

Most researchers believe that liver cancer originates from the abnormal differentiation and proliferation of stem cells [15]. Carcinogenic factors such as fatty hepatitis, viral hepatitis, and liver-associated metabolic diseases trigger malignant changes in liver stem cells [16]. Many studies support the link between CD133 and liver cancer stem cells and CD133 is a useful marker for detecting characteristic liver cancer stem cells [17,18].

Here, we successfully established an early/advanced stage liver cancer model using CD133⁺ cell injection and tumor generation similar to previous experiments [19]. We used slightly different CD133⁺ cells doses, mouse strain, and aged mice, which did not affect the overall outcome. Tumor development using CD133⁺ cells injection shows similar morphological and histological variation to that seen with the chemical induction of liver cancer [20].

Microscopic foci in the histological sections of the liver showed early hepatocellular carcinoma [21,22]. In the advanced stage, there were cell aggregates with eosinophilic cytoplasm and well-organized enlarged nucleoli [23]. Recent studies of colorectal cancer have demonstrated the role of ZEB2 in promoting a tumor microenvironment that supports cancer stem cell proliferation [24]. One study suppressed ZEB2 with miR-498, directly demonstrating its role in tumor growth and metastasis [2]. We found that ZEB2 and CD133 had similar expression patterns, clearly defining its role in supporting a microenvironment that favors liver cancer stem cell proliferation.

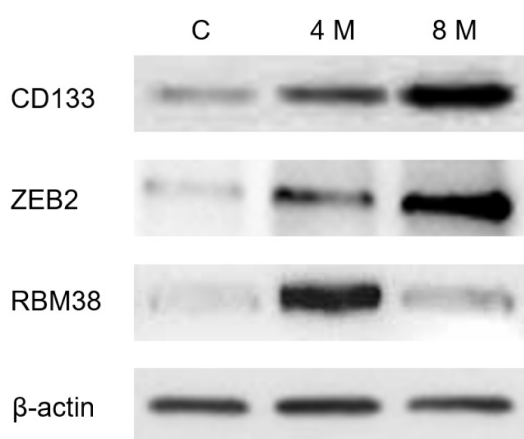


Figure 3. Immunoblotting with anti-CD133, anti-ZEB2, and anti-RBM38 antibodies in early and advanced liver cancer. **Lane 1:** Control liver tissue showing the expression of CD133, ZEB2, RBM38, and β -actin. **Lane 2:** Early liver cancer showing limited upregulation of CD133 and ZEB2 protein, but marked RBM38 expression. **Lane 3:** Advanced liver cancer showing increased upregulation of CD133 and ZEB proteins, but downregulated RBM38 expression. As a loading control, β -actin was used.

Null mice deficient in RBM38 develop spontaneous tumors and premature ageing [25], and RBM38 helps improve the longevity of liver cancer patients [26]. Here, we observed greater RBM38 expression in early liver cancer, which might reflect its protective role in suppressing tumor activity. In advanced liver cancer, however, RBM38 expression was downregulated; its absence appears to favor a microenvironment that supports liver cancer stem cell proliferation.

In conclusion, CD133⁺ cell injection results in a model of early/advanced liver cancer. CD133 and

ZEB2 were expressed similarly in the tumor development stages, confirming that ZEB2 positively regulates the liver cancer stem cell microenvironment. By contrast, more RBM38 was expressed in the initial stage and it was subsequently downregulated in advanced liver cancer, negatively regulating liver cancer stem cells.

Conflict of interests

The authors declare no conflict of interests.

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