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Silencing FOXA1 gene regulates liver cancer cell apoptosis and cell proliferation

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Abstract. – OBJECTIVE: Liver cancer emerged as a major health problem, and it accounts for leading cancer-related death worldwide. Due to recurrence and metastatic behavior, it is challenging to be controlled and managed. Understanding the regulative role of different proteins, which regulates liver cancer in various pathological stages, is essential to be investigated. In this study, we analyzed the correlation between Foxa1 suppression along with apoptosis and cancer stem cell proliferation.

MATERIALS AND METHODS: CD133+ cells were used to induce the initial and advanced stage of liver cancer. Histology was used to study and confirm the tissue complications associated with initial, advanced and Foxa1 silenced liver cancer tissues. Immunohistochemistry and Western blotting were used to quantify Foxa1, CD133 expression. TUNEL assay was performed to study apoptosis.

RESULTS: Initially using CD133+ cells, we successfully developed a mouse model with the initial and advanced stage of liver cancer upon 4 and 8 weeks incubation. Histologically, as the tumor progress, it shows more proliferative cells with disorganized tissue structure. Foxa1 silencing aids in recovering from initial liver cancer, but it has only limited effects with advanced liver cancer. The apoptosis process is enhanced in initial liver cancer, and Foxa1 silenced tissue when compared with the advanced stage of liver cancer. Foxa1 silencing also suppresses the cancer stem cell proliferation.

CONCLUSIONS: Overall, our results reveal the critical role of Foxa1 in regulating apoptosis and liver cancer stem cells.

Key Words:
Foxa1, CD133, Cancer stem cells, TUNEL assay, BrdU.

Introduction

Liver cancer is one of the leading forms of cancer that occurs worldwide with a high mortality rate[1,2]. The complex nature of liver cancer is closely associated with metastasis which makes it difficult to manage[3]. In the advanced stages of liver cancer, the patients were mostly recommended with surgery but still, in many cases, it results with recurrence and metastasis as it is difficult to prognosis in early stages of liver cancer[4]. Accumulating evidence states that the cancer stem cells play a critical role in cancer invasion, metastasis, and drug resistance[5]. Many proteins that are irregularly expressed regulate the cancer stem cells in abnormal fashions and directly targeting them may play a potential role in developing suitable therapeutic targets. Therefore, an in-depth knowledge and mechanism that is associated with cancer stem cells are essential to understanding, which helps to manage them effectively. Recent studies[6-8] indicate the importance of small non-coding RNAs that play a vital role in regulating the liver cancer in each subsequent step, up to the metastatic level. The characterization of non-coding RNAs (ribonucleic acids), particularly at the junction point of epithelial and parenchymal cell layers of the liver, is more significant because it is mostly involved in early cancer metastasis[9]. The small interfering RNA (siRNA) is effectively used in the therapeutic approach to knock down the desired gene of interest[10]. To improve the efficiency of knockdown, one should target right dose and target the particular organ are more critical[11]. Usually the systemic delivery of siRNA results in accumulation in liver and spleen as part of reticular endothelial system[12]. Forkhead box A (Foxa) is a gene member of transcriptional factors that encodes for three members namely Foxa1, Foxa2, and Foxa3[13]. Among those, Foxa1 and Foxa2 have a role in regulating liver metabolism and development[14,15]. Functional studies correlated with Foxa1 are carried out in different cancer cell lines[16,17] and it reveals that Foxa1 also

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had a role in regulating the cell cycle in various tumor-derived cells[18,19]. Also, Foxa1 can bind with packed genomic DNA (deoxy-ribonucleic acid) and make them unwind for the operational role of other transcriptional factors[20]. A recent work confirms that the targeted suppression of Foxa1 with MiR (microRNA)-212 shows tumor growth suppression in hepatic cancer[21] and another study reveals the role of MiR-370 in targeting Fox03a to suppress liver cancer by inducing apoptosis[22], but still the mechanical role of Foxa1 and related protein with liver cancers are needed to be evaluated in depth. In this current work using siRNA for Foxa1, we assessed their regulatory role in different pathological stages of liver cancer.

Materials and Methods

Experimental Animals with Hepatocellular Carcinoma (HCC)

To initiate HCC in an animal model, we used athymic male BALB/c mouse strain. The six weeks old male mice were purchased from Jackson Laboratory, and they were maintained in laboratory conditions for two weeks for stabilization. Then, the mice were injected with CD133 positive cells, an aggressive liver cancer cells in the dose range of 10^6 CD133 cells/20 µl. The injection was made directly into the mouse liver by making a small cut in the left abdomen region. Following that the mice were carefully monitored and looked for liver palpation after 4 weeks. One group of mice was sacrificed at the time interval of 4 weeks and another set after 8^{th} weeks of CD133 specific cell injection. The experimental animals and the protocol followed were approved by the Animal Ethics Committee of the host institution.

Delivering Foxa1 Gene siRNA in Rat Liver

To suppress the Foxa1 expression in rat liver, commercial forms of siRNA against the Foxa1 were obtained from Dharmacon (M-010319) and corresponding control are purchased from Qiagen (1027280). The dosage of siRNA used for injection is 20 µM which was resuspended using 1X siRNA buffer. The siRNA was directly injected into the rat liver by making a small incision which was healed quickly. Following injection, the knockdown effects of siRNA were assessed using Western blotting.

Western Blot Analysis

From the cell lysate of the normal, primary and metastatic form of HCC tissue the protein samples were prepared. The protein samples were loaded into the well and resolved in 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred to the membrane by semi-dry method. The membrane was blocked with 5% skimmed milk and incubated overnight with primary antibody (Anti-Fox1 antibody, Abcam (ab83574, Cambridge, MA, USA) or with an Anti-CD133 antibody, Abcam (ab19898, Cambridge, MA, USA) with a dilution ratio of 1:300. After incubation, the membrane was washed with 1X phosphate-buffered saline (PBS) for three times to remove non-specific binding of the antibody. The membrane is further incubated for 2 h at room temperature (RT) with secondary antibody (anti-Rabbit IgG, conjugated with horseradishp (HRP); Abcam, ab6721, Cambridge, MA, USA) with a dilution ratio of 1:5000. Before developing the membrane, they were once again washed with 1X PBS for three times and later developed with diaminobenzidine (DAB) Sigma-Aldrich (St. Louis, MO, USA) (D7304; Hong Kong, China) kit.

Immunohistochemistry

The liver samples from control, initial and aggressive stage of liver cancer mice were dissected and subjected to formalin fixation. The tissue samples were then subjected to gradual dehydration using ethanol. After clearing with xylene, the tissue was paraffin fixed and subjected to thin sectioning (6 µm size). The sectioned ribbons in the slide were dewaxed and incubated with 10% H_2O_2 to block the endogenous peroxidase activity. Protein cross-links were breakdown using trypsin treatment, which helps to unmask the antigen. Following trypsinization, the sections were treated with primary antibody (Anti-Fox1 antibody, Abcam (ab83574, Cambridge, MA, USA) or with an Anti-CD133 antibody, Abcam (ab19898, Cambridge, MA; USA), along with dilution buffer at 4°C for 8 h. The slides with tissue sections were after washed with 1X PBS for 3X times, which helps to remove nonspecific binding of the primary antibody. After washing, the sections were treated with secondary antibody (anti-Rabbit IgG (HRP) Abcam, ab6721, Cambridge, MA, USA) for 30 min at room temperature. After thorough washing the slides with sections, the primary antibody that binds to specific protein was detected using the DAB Kit (Sigma-Aldrich, St. Louis, MO, USA) D7304, as a chromogen.

Terminal Deoxynucleotidyl Transferase dUTP Nick-end Labeling (TUNEL) Assay

Apoptotic cells show a DNA damage and fragmentation which is crucial in analyzing the cancer tissue using TUNEL assay[23]. The nick of the DNA fragments was end-labeled by incorporating 5-bromo-2'-deoxyuridine (BrdU), a synthetic nucleoside analog of thymidine residues to the 3'-ends of each DNA fragment. The apoptotic cells, intake more BrdU and incorporated into their DNA, which latter were identified using a specific antibody against BrdU (Anti-BrdU antibody; Abcam, ab8152, Cambridge, MA, USA; 1:100 dilution). The primary antibody that binds with specific BrdU was detected using HRP conjugated secondary antibody (anti-Mouse IgG (HRP); Abcam, ab6728, Cambridge, MA, USA) and later developed with diaminobenzidine (DAB) Kit.

Statistical Analysis

To attain the statistical significance, the experiments that are carried out were repeated for three times and using student's *t*-test the differences among the data were calculated. The calculation for statistical analyses was shown as mean ± SEM (standard error of the mean). The significant level was considered when the calculated p-values < 0.05.

Results

Induction of Liver Cancer Using CD133+ Cells in a Mouse Model

To induce liver cancer, the BALB/c mouse strain was injected with $CD133^+$ cells as described in materials and methods section. The $CD133^+$ cells have a role in maintaining the stemness properties in liver cancer stem cells[24], and it is used to induce liver cancer by specifically regulating aggressive nature of cancer stem cells. The injected $CD133^+$ cells trigger tumor initiation in 4 weeks of post-injection, and it develops aggressive stage of liver cancer after 8 weeks' time of initial $CD133^+$ cells injection. The degree of tumor initiation and their development were assessed using histological staining using eosin and hematoxylin. The control liver tissues show an extension of hexagonal cells, which are arranged in a uniform pattern by occupying more

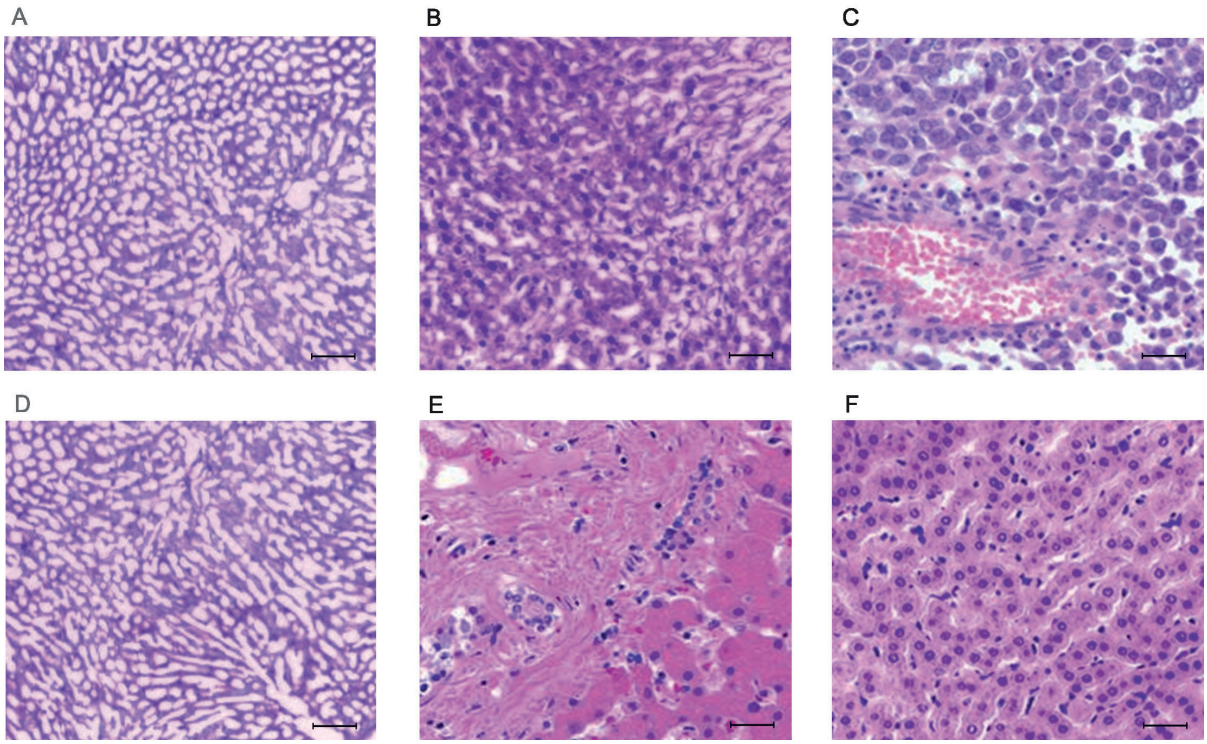

Figure 1. Observation of different pathological stage of liver cancer using histological sectioning. ***A,*** Histological image of control liver with undisturbed layers of tissue. ***B,*** Initial liver cancer tissue section is showing disorganized tissue layers with the enlarged nucleus. ***C,*** Advanced stage of a liver cancer tissue section with metastatic cells. ***D,*** Histological image of control liver with Foxa1 silenced tissue with undisturbed layers. ***E,*** Initial liver cancer tissue section with Foxa1 silenced showing a reorganization of tissue layers. ***F,*** Advanced stage of liver cancer with Foxa1 silenced tissue section without metastatic cells. Scale Bar – 50 µm size.

space between cells (Figure 1A). In response to $CD133^+$ cells injection, the mice developed initial liver cancer on the 4^{th} week, and we found disruption in the tissue arrangement with enlarged nuclei (Figure 1B). On further incubation for up to 8 weeks after $CD133^+$ cells injection, the liver shows a disorganized tissue structure with enlarged nuclei along with cellular migration which represents an advanced stage of liver cancer (Figure 1C). Silencing Foxa1 expression shows only mild variation in control liver tissue (Figure 1D) but it shows control over cellular proliferation in the initial stage of liver cancer (Figure 1E). The effect of Foxa1 silencing has only little effect in advance stage with minimal control over the migrating cells, but the cellular abnormalities continue with enlarged nuclei (Figure 1F).

Analyzing Foxa1 Expression in Foxa1 Silenced Liver Tissue

The Foxa1 shows upregulated expression as a tumor develops to next advanced levels, which was confirmed by western blotting analysis. When compared with the control tissue, the initial stage of liver cancer shows double the expression of Foxa1 (Figure 2, lane 1 and 2) and its expression further increased, as the tumor advanced to the complex stage as of 8 weeks (Figure 2, lane 3). The siRNA injection against Foxa1 shows their reduced expression in control liver and initial liver cancer tissue (Figure 2C, lane 1 and 2) but it has only minimal downregulation as in case of advanced liver cancer (Figure 2C, lane 3).

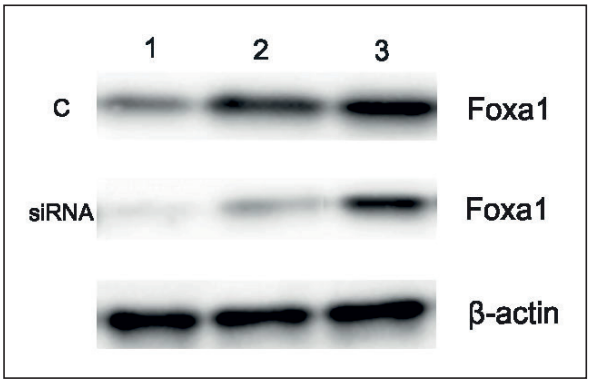

Figure 2. Western blotting analysis. First row – Lane 1-3: expression of Foxa1 in the order of control, initial and advanced stages of liver cancer. Second row – Lane 1-3: expression profile of Foxa1 protein in Foxa1 silenced tissue is represented in lane 1-3 as samples from control, initial and advanced stages of liver cancer. For loading control, β-actin was used.

To explore their expression in liver tissue, immunohistochemistry was performed against the Foxa1 protein, and we observed that Foxa1 expression became elevated as tumor proceeds to the next level (Figure 3A-C). Similarly, their downregulation responds well in the initial stage of liver cancer, and it is associated with Foxa1 signal reduction with cellular pattern improvement (Figure 3D and 3E). Foxa1 signals were not showing marked variation in the advanced stage of liver cancer (Figure 3F).

Link Between Apoptosis and Foxa1 Down Expression

The apoptotic cells were assessed using TUNEL assay, which helps in tracking the apoptosis cells in different pathological stages of liver cancer. The control liver tissue shows no signal for apoptosis (Figure 4A), but increased expression of apoptotic signals was observed in initial liver cancer tissue (Figure 4B). Moreover, in the advanced stage of liver cancer, the apoptotic signals dramatically reduced (Figure 4C). Interestingly, we observed in Foxa1 downregulated tissue the apoptotic signals were enhanced minimally in control liver tissue (Figure 4D) but more upregulated in initial and advanced stage liver cancer tissue (Figure 4E-F).

Foxa1 Silencing Regulates Liver Cancer Stem Cells

The liver cancer stem cell plays a significant role in cancer development, and here we tried to understand their role associated with Foxa1 expression. Typically the control liver tissue shows less or no liver cancer stem cells (Figure 5A), and it also has a less role in initial liver cancer with minimal expression of CD133 (Figure 5B). But its expression, upregulated upon tumor develops into an advanced stage (Figure 5C). Foxa1 silencing has control over cancer stem cells, which were examined in control (Figure 5D), initial (Figure 5E) and advanced stage of liver cancer (Figure 5F).

Discussion

The liver cancer occurs due to changes that occur in genetic level, which is induced by many factors like family history, environmental risks, hepatitis B virus (HBV) infection[25]. Different proteins are regulating liver cancer, and recent studies show molecular level treatment using

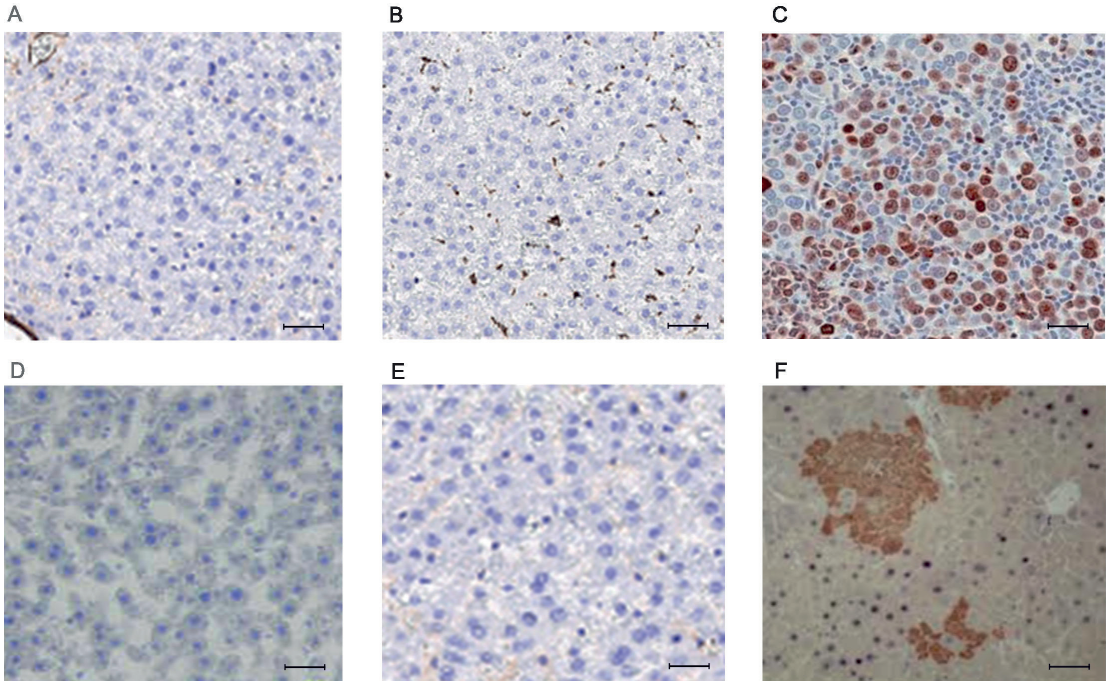

Figure 3. Normal expression of Foxa1 and their silenced expression in initial and advanced liver cancer. ***A,*** Immunohistological image of control liver with minimal Foxa1 expression. ***B,*** Initial liver cancer tissue section is showing optimal expression of Foxa1. ***C,*** Advanced stage of liver cancer showing upregulated expression of Foxa1. ***D,*** Immunohistological image of control liver with Foxa1 silenced tissue without Foxa1 expression. ***E,*** Initial liver cancer tissue section with Foxa1 silenced showing minimal expression of Foxa1. ***F,*** Advanced stage of liver cancer with Foxa1 silenced tissue showing upregulated expression of Foxa1. Scale Bar – 50 µm size.

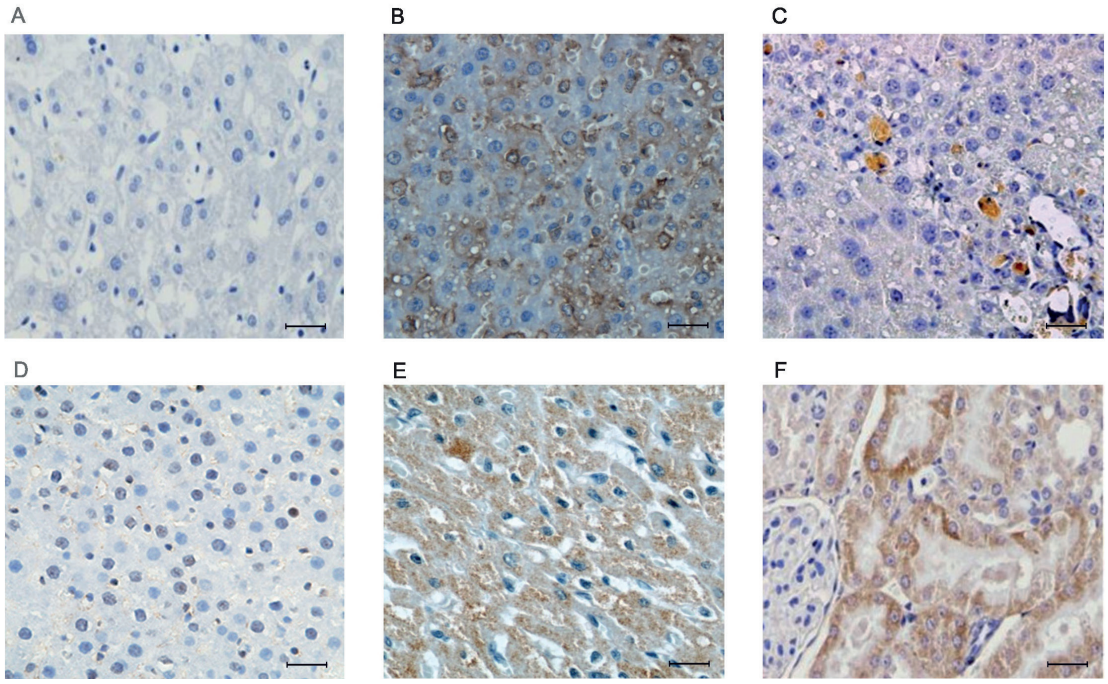

Figure 4. The link between Foxa1 and apoptotic protein. ***A,*** Immunohistological image of control liver with minimal apoptotic protein expression. ***B,*** Initial liver cancer tissue section is showing enhanced expression of an apoptotic protein. ***C,*** Advanced stage of liver cancer showing optimal expression of an apoptotic protein. ***D,*** Immunohistological image of control liver with Foxa1 silenced tissue with less apoptotic protein expression. ***E,*** Initial liver cancer tissue section with Foxa1 silenced showing upregulated expression of an apoptotic protein. ***F,*** Advanced stage of liver cancer with Foxa1 silenced tissue showing optimal upregulated expression of an apoptotic protein. Scale Bar – 50 µm size.

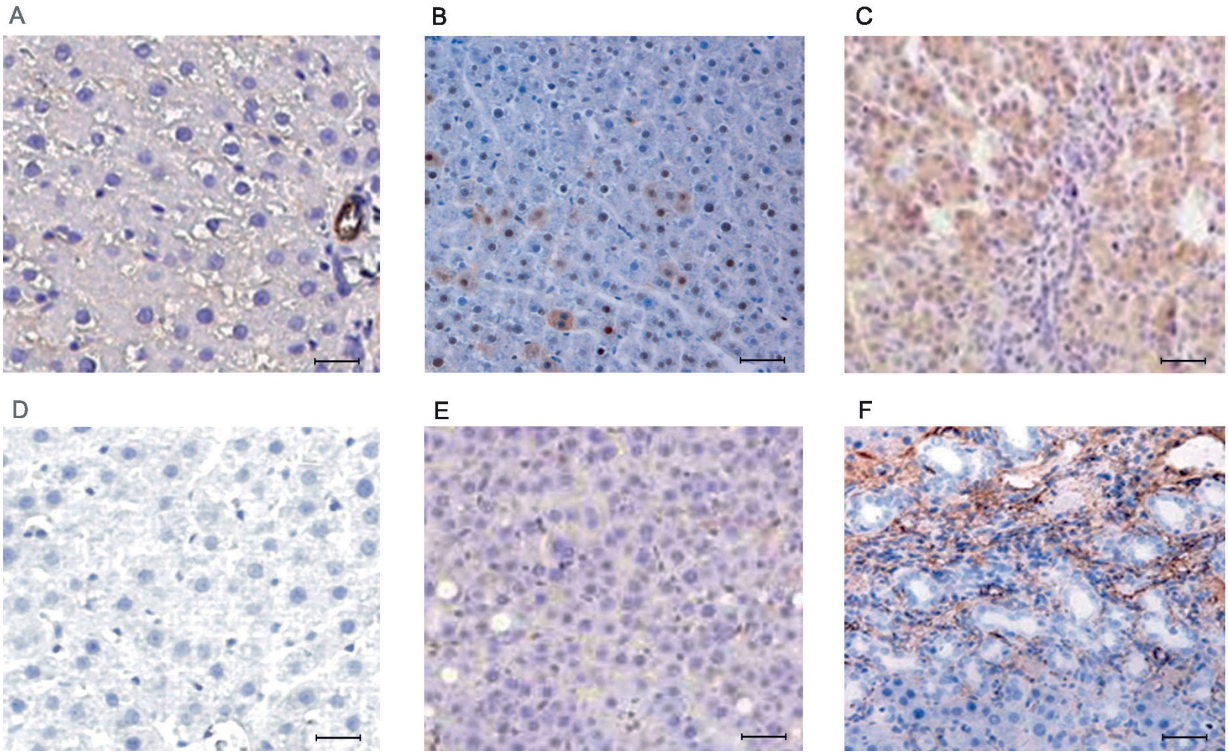

Figure 5. Correlation between Foxa1 and cancer stem cell. ***A,*** Immunohistological image of control liver with minimal CD133 expression. ***B,*** Initial liver cancer tissue section is showing optimal expression of CD133. ***C,*** Advanced stage of liver cancer showing upregulated expression of the CD133 protein. ***D,*** Immunohistochemistry of control liver with Foxa1 silenced tissue with less CD133 protein expression. ***E,*** Initial liver cancer tissue section with Foxa1 silenced showing controlled expression of the CD133 protein. ***F,*** Advanced stage of liver cancer with Foxa1 silenced tissue showing optimal expression of the CD133 protein. Scale Bar – 50 μm size.

Sir-related enzymes 4 (sirtuin 4) which induce aging in liver cancer cells[26]. Relatively, one such protein that regulates cancer stem cells is necessary to investigate. In this present investigation, we analyzed the role of Foxa1 in the context of liver cancer that is successfully induced in a mouse model using $CD133^+$ cells. Mouse models extensively utilized in the field of liver cancer to determine the critical action of particular genes[27]. In our study, we induced liver cancer by injecting $CD133^+$ cells, and it responded naturally as compared with the induction of liver cancer using various chemicals along with similar changes in histological and morphological features[28]. The mice with liver cancer show disorganized tissue structures together with metastatic cells in the advanced stage of liver cancer when compared with the control tissue (Figure 1A-C). Notably, we observed significant recovery of tissue pattern and cell proliferation arrest in initial liver cancer in Foxa1 silenced tissue when compared with the advanced stage of liver cancer (Figure 1D-F). Our results correlate with the recent investigation that Foxa1 promote cell proliferation in endometrial cancer[29]. The siRNA used against Foxa1 works efficiently and suppress their expression well in initial liver cancer than in advanced stage of liver cancer (Figures 2-3). Our finding, clearly defines that Foxa1 silencing shows a significant tumor reversion in the initial stage when compared with advanced liver cancer. The TUNEL assay shows remarkable apoptosis event on initial cancer stage, and its further enhancement in Foxa1 silenced initial liver cancer tissue when compared with advanced stages of liver cancer (Figure 4A-F). Foxa1 a transcriptional factor that regulates many proteins like Bcl-2 (B-cell lymphoma 2)[30] and in our investigation, we found out that it directly regulates the apoptotic mechanism. Also, we concluded that the suppression of Foxa1 had control over cancer stem cells (Figure 5A-F).

Conclusions

Overall, our results imply that Foxa1 suppression has a well-controlled mechanism over initial liver cancer than in advanced stage. Also, we

conclude that Foxa1 suppression relaxed their control over apoptotic protein and had control over cancer stem cell proliferation.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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