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UNDERSTANDING THE MECHANISM OF DRUG-RESISTANT AND TUMOR RECURRENCE IN LIVER CANCER

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ABSTRACT

Chemo-resistant and tumor recurrence are the major hurdle to overcome the cancer patients. Especially in hepatocellular carcinoma (HCC) is notoriously refractory to chemotherapy because of its tendency to develop multi-drug resistance (MDR), through various mechanisms. Aim: The current research is focussed on understanding the mechanism involved in chemo-resistant and tumor recurrence in liver cancer. Methods: Human hepatocellular carcinoma cell line (Huh7) was used entire study. Huh7 cells were cultured with known chemotherapeutic drugs such as 5-FU, Paclitaxel and Cisplatin-based on their Cmax concentration, and then these drug-treated cells were examined for chemoresistant and tumor recurrence properties through flow cytometry analysis, spheroid formation assay, and morphological analysis. Results: In morphological analysis confirm these all the chemo drugs were shown more cytotoxic effete than control, even though there were few viable cells noticed in cisplatin treatment. In flow cytometry analysis cisplatin pre-treated cells were well expressed LCSC marker such as CD133 and stem cell transduction factors like Oct-4 & Nanog than control. In addition to this, all the CD133 expressed cells also expressed to EpCAM. In spheroid formation assay, cisplatin pre-treated cells shown well-defined spheroid than control. Conclusion: LCSC plays a major role in chemoresistant and tumor recurrence through PI3K/Akt/mTOR, wnt-β catenin signaling, NF-kB signaling. So, targeting LCSC through EpCAM targeted therapy along with chemotherapy might be the better option for enhanced prognosis.

Keywords: LCSC, Chemoresistant, Tumor recurrence, Hepatocellular carcinoma.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world. Major etiologic factors for HCC are chronic viral infections such as hepatitis B & C, factors like chronic alcoholism and metabolic disorders also modestly involved in HCC [1]. Localized hepatocellular carcinoma patients have an excellent survival rate with standard treatment options like surgery, chemotherapy, and radiotherapy [2]. However in advance and metastatic disease are associated with poor prognosis, and the patients will suffer from chemo-resistant and tumor relapse because of numerous reason [3].

Chemoresistance is a complex mechanism, involving various biological pathways. Abundant studies have reported that multi-drug resistant is associated with over-expression of ATP binding cassette drug efflux [4], DNA damage repair [5-7], the hypoxia-inducible factor1-α (Hif1-α) [8], epithelial-mesenchymal transition (EMT) [9,10], Calcium signalling [11], autophagy induction [12], epigenetic regulation [13], Cancer stem cell [14], miRNAs [15]

and immunosuppressive microenvironment [16], have also been concerned in that multi-drug resistant.

Tumour relapse is believed to be a major hurdle for cancer treatment. Tumor relapse or cancer recurrence arises from incomplete eradication of tumor cells after the standard treatment such as surgery, chemotherapy, and radiotherapy. There are three important factors believed for tumor relapse such as cancer stem cells (CSCs), neosis, and a phoenix rising [17].

Since the new concept of cancer stem cells (CSCs) was introduced in the late 1990s, it has gradually gained worldwide acceptance and influenced all approaches to cancer research and therapy. The CSC, which are also accurately called 'tumor-initiating cells', represent a small population of cancer cells, sharing common properties with normal stem cells (SCs), that can initiate new tumors following injection into animal models, while the majority of other cancer cells cannot [18]. The reported fractions of CSCs in tumors vary from 0.1 to 30% depending on the type and the advancement of cancer [19].

Signaling pathway such as PI3K/Akt/mTOR, wnt-β catenin signaling, NF-kB signaling, Hotch signaling are actively involving in cancer stem cell activation. Especially the activation of wnt-β catenin signaling pathway has been observed 1/3 in Hepatocellular carcinoma. wnt-β catenin signaling pathway plays a major role in tumor initiation, activation, invasion and metastasis in hepatocellular carcinoma [20].

This current study was designed to understand the mechanism involved in chemo-resistance and tumor recurrence using well known chemotherapeutic drugs such as cisplatin, 5flurouracil, and paclitaxel treatment.

MATERIALS AND METHODS

Reagents:

Cisplatin (Cat#1550), and Paclitaxel (cat #1567), purchased from Bio vision, and 5-fluorouracil (cat #F6627) purchased from Sigma. HepG2 cell lines were purchased through National Centre for Cell Science (NCCS), Pune. Fetal bovine serum (Cat#11573397) purchased from Gibco. Antibiotic-Antimycotic (cat#15240062) purchased from Gibco. ITS (Cat#41400045) purchased from Invitrogen. FITC-conjugated CD133 monoclonal antibody (clone # EMK08, Cat# 11-1339-41) was purchased from eBioscience. PE-conjugated EpCAM monoclonal antibody (clone # EBA-1, Cat# 347198), PE-conjugated Nanog monoclonal antibody (clone # N31-355, Cat# 561300), FITC-conjugated Oct-4 monoclonal antibody (clone # 40/oct-3, Cat# 560253) was purchased from BD Bioscience.

Cell culture /Drug sensitivity assay

Huh7 cells were obtained from NCCS, Pune. The cells were cultured in complete RPMI 1640 medium supplemented with 10% FBS, ITS, antibacterial and anti antifungal up to 3 passages to get enough cells, then these cells were seeded in 6 well plates, incubated at least for 24 hours, once reached above 80% confluence, these cells were treated with half the value of Cmax concentration of Cisplatin (1.655 µg/ml), 5-FU (8.3 µg/ml), and Paclitaxel (1.595 µg/ml) for 3 days at 37oC in 5% CO2 Incubator. Drug medium was changed at every alternative day. The image was captured under the inverted microscope.

Flow cytometry

The pre-treated Huh7 cells were dissociated with 0.25 % trypsin-EDTA (1 mM) (Invitrogen) for 3 min and washed with Calcium and magnesium-free Dulbecco phosphate buffered saline solution by spinning at 300g for 7 minutes 4 °C. Then these cells were diluted in 100 µl FACS buffer (PBS containing 1 % fetal bovine serum) and then incubated for 1 h at 4 °C in FACS buffer with the corresponding mAb: anti-CD133- FITC and anti-EpCAM-PE. After incubation washes the cells by spinning at 300g for 7 minutes at 4°C, discard the supernatant and add 200 µl of FACS buffer for analysis. Flow cytometry analysis was performed with a BD FACSCanto II flow cytometer (BD Biosciences).

Huh7 cells and Pre drug-treated Huh7 cells were dissociated with 0.25 % trypsin-EDTA (1 mM) (Invitrogen) for 3 min and washed with Calcium and magnesium-free Dulbecco phosphate buffered saline solution by spinning at 300g for 7 minutes 4 °C. Then these cells were fixed and permeabilized by BD Transcription factor buffer (Fix/perm) for 30 minutes at 4°C. Then wash the cells by spinning at 300g for 7 minutes at4 °C. Discard the supernatant and add 100 µl of BD Transcription factor buffer (perm/wash) to the cells and then incubated for 45 minutes at 4 °C with the corresponding mAb: anti-oct-4 FITC and anti-Nanog-PE. After incubation washes the cells by spinning at 300g for 7 minutes at 4°C, discard the supernatant and add 200 µl of FACS buffer for analysis. Flow cytometry analysis was performed with a BD FACSCanto II flow cytometer (BD Biosciences).

Spheroid formation assay

3D cell culture reagent, Matrigel (Cat.no: 354230) was obtained from BD Biosciences and was used to culture liver spheroids. 5 mg/ ml concentration of matrigel was prepared and used for culturing spheroids. HepG2 cells were treated with low dose cisplatin, paclitaxel, 5-FU for 3 days respectively. Then these cells were harvested and 1000 Cells/96 well plate were incubated at 37 °C degrees with 5 % CO2 and culture to get the optimal spheroid size. Culture medium was refreshed every 2-3days up to 9 days.

RESULTS

Morphological analysis

The morphology of control cells remains the same after cultured 3 days also, whereas in drug-treated cells were shrinking and lost their morphology. The cell death was observed equally in every concentration of drug-treated cells and also there is no much difference shown in cisplatin, 5-FU, paclitaxel-treated cells. It's clearly evident that these drugs have shown their cytotoxic effects in maximum levels and whatever leftover viable cells were capable of resisting the chemotherapy.

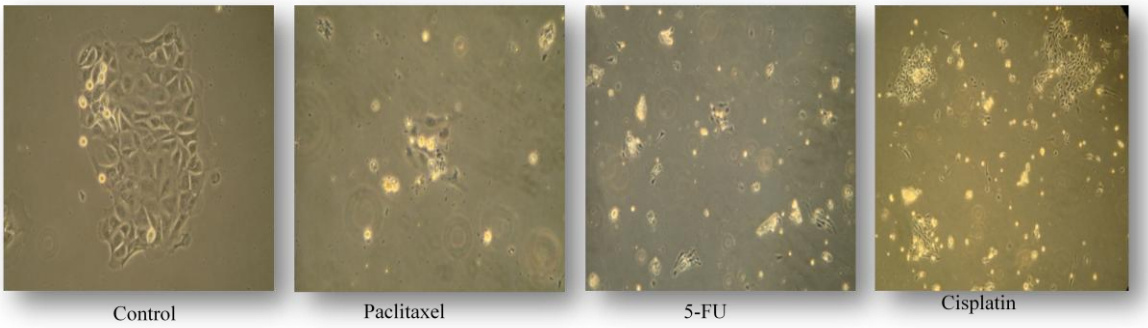


Figure 1: Microscopic examination of drug-treated Huh7 cells. Paclitaxel, 5-FU & cisplatin treated cells shows increased cell death when compared to control cells.

Screening of conventional chemotherapy which promotes more cancer stem cells in liver cancer?

Part of low dose chemo drugs treated cells were stained for cancer stem cell marker CD133 and analyzed in flow cytometry. CD133, a well-studied cancer stem cell marker expression was highly enriched at 5.6% in low dose cisplatin-treated cells and subsequently, the expression pattern was decreased in low dose paclitaxel 3.45% and low dose 5-FU 1.98%. Even though low dose paclitaxel and low dose5-fu treated cells were significant expresses the CSC marker it's not as good as low dose cisplatin treated cell expression. It means all the three drugs were promoting cancer stem cells but only low dose cisplatin drug is capable for far above the ground level of chemoresistant and cancer relapse than other two drugs. In clonogenic assay draw a parallel result of the flow cytometry. In cisplatin pretreated cell were able to form decent colonies, but paclitaxel and 5-FU pre-treated cells were unable to form as well as cisplatin pre-treated.

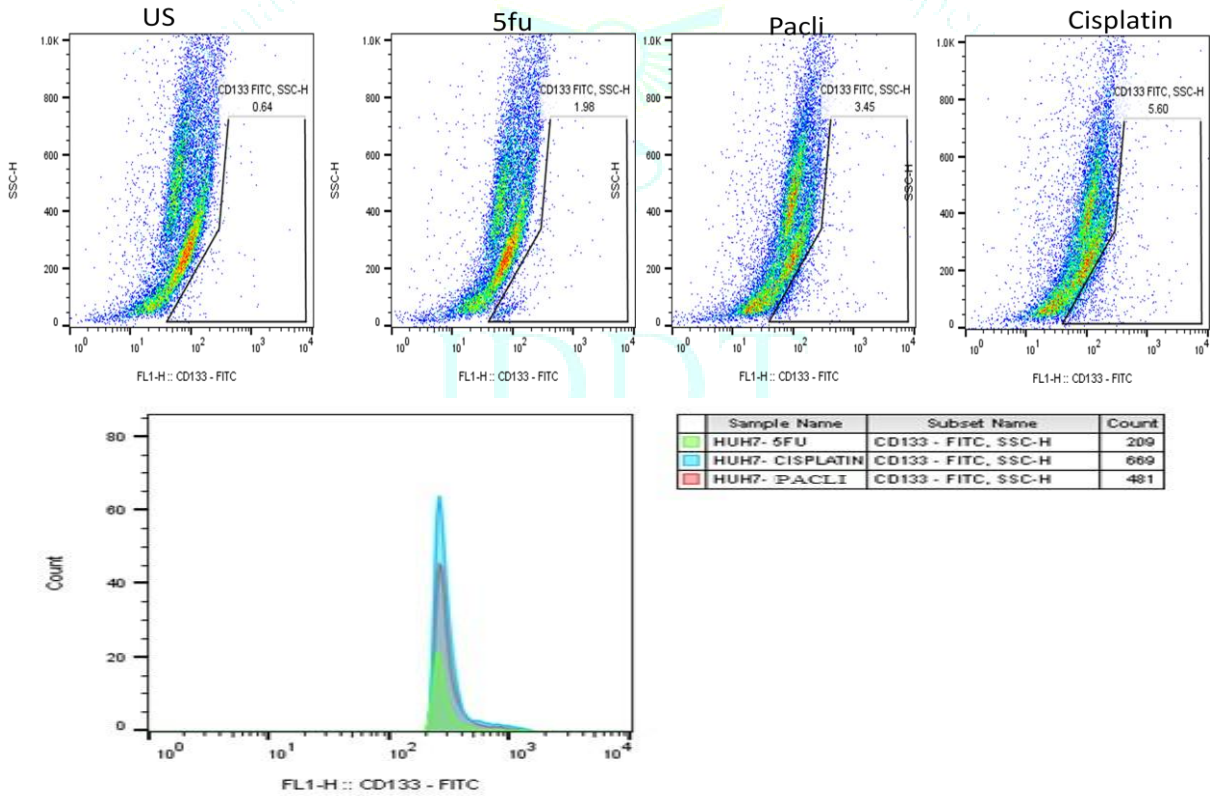


Figure 2: Flow cytometry analysis, LCSC expression was enriched in cisplatin-pretreated cells and followed by 5-FU and paclitaxel pretreated cells.

Analysis of the tumor recurrence properties using spheroid formation assay

Tumor recurrence or Tumor relapse is a major obstacle in cancer therapy. We evaluate the tumor recurrence properties in ex vivo by using spheroid formation assay. 5-FU, Paclitaxel, Cisplatin pre-treated Huh7 cells along with control Huh7 cells were subjected to spheroid formation assay. Interestingly we observed distinct spheroids in Cisplatin pre-treated cells and undersized spheroids in 5-FU and Paclitaxel pre-treated cells. There was no spheroid observed in control Huh7 cells even after 9 days of culture. These findings confirm that LCSC plays the major role in tumor relapse.

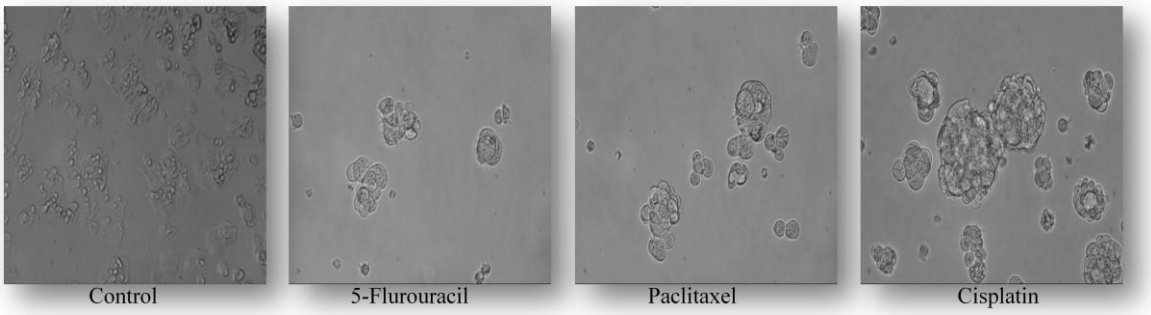
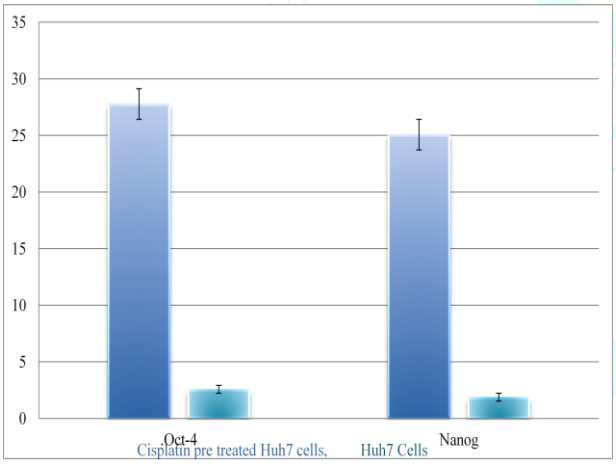


Figure 3: Spheroid formation assay experiment shows well-defined spheroid formation in cisplatin pre-treated cells when compared to other drug-treated cells.

Transduction Factors as CSC Markers involved in liver cancer Chemo-resistance

Stem cell transcription factors like Octamer 4 (Oct4), Nanog expression levels were dramatically increased in chemoresistant cancer cells due to DNA demethylation regulation of Oct4 and Nanog. Flow cytometry analysis, we observed the enhanced expression of Oct4 and Nanog in Huh7 cell line whereas in normal liver cell line expression was very low. It is well evident that the Oct4-TCL1-AKT pathway and Nanog pathway acts on embryonic stem cells and cancer stem cells in cell proliferation through inhibition of apoptosis.



Graph 1: The stem cell transduction factors marker such as Oct-4 & Nanog expressions are drastically increased in cisplatin pre-treated cells than control.

CSC associated signaling pathway in liver cancer Chemo-resistance

Notch, wnt-β-catenin, PI3/AKT & NF-kB pathways are mainly involved in liver cancer stem cells but most researchers studied EpCAM wnt-βcatenin pathway to understanding cancer stem cell and chemoresistant of various cancer. The EpCAM signaling pathway can be activated by intramembrane proteolysis and shedding of the extracellular domain of EpCAM. EpCAM was sequentially cleaved by two important proteins named as tumor necrosis factor-alpha converting enzyme (TACE) and presenilin 2(PS-2) as EpEX and EpICD. EpEX is released out of the cell, whereas EpICD is released into the cytoplasm. Four and one-half LIM domain protein 2 (FHL2) is a protein which contains two binding sites such as EpCAM and β-Catenin. FHL2 is identified as a cytosolic interaction partner for EpICD, and also it regulates the TACE and PS-2 protein activities. Simultaneously the Wnt signaling pathway activated by the binding of Wnt ligand with its receptor such as frizzled and LRP 5/6, recruits disheveled and induce β-Catenin degradation complex (AXIN, APC, GSK3). This complex inhibits the phosphorylation of β-Catenin. Therefore the β-Catenin gets accumulated in the cytoplasm. This accumulated β-Catenin binds with FHL2 and EpICD complex and translocates into the nucleus. The large nuclear complex proteins regulate gene transcription and activate the EpCAM target genes such as c-myc cyclins, and TCF1. So, targeting wnt-βcatenin signaling may help in an inhibition of cancer stem cells in liver cancer.

EpCAM positive cells are the key regulators of LCSC and chemo-resistant

In the morphological examination, we noticed even after 5 days of cisplatin treatment the Huh7 cells were not undergone cell death completely. It's so, part of the cancer tissue have the potential to resist the chemo drugs. To further confirm this correlation between chemoresistant and cancer stem cells, these cells were studied the cancer stem cell markers such as EpCAM and CD133 using flow cytometry. FACS analysis results indicate that a majority of EpCAM+ cells express CD133 in HuH7 cells, which prompted us to compare the tumorigenic capacity of EpCAM+ and CD133+ cells in these cell lines. Noticeably, EpCAM+ HuH7 cells showed marked tumor-initiating capacity compared with CD133+ HuH7 cells, whereas EpCAM+ and CD133+ cells had the similar tumorigenic ability in HuH7 cells. This data confirms the direct association between CSCs and chemoresistant and further convince that EpCAM is one of the important key markers in regulating CSCs.

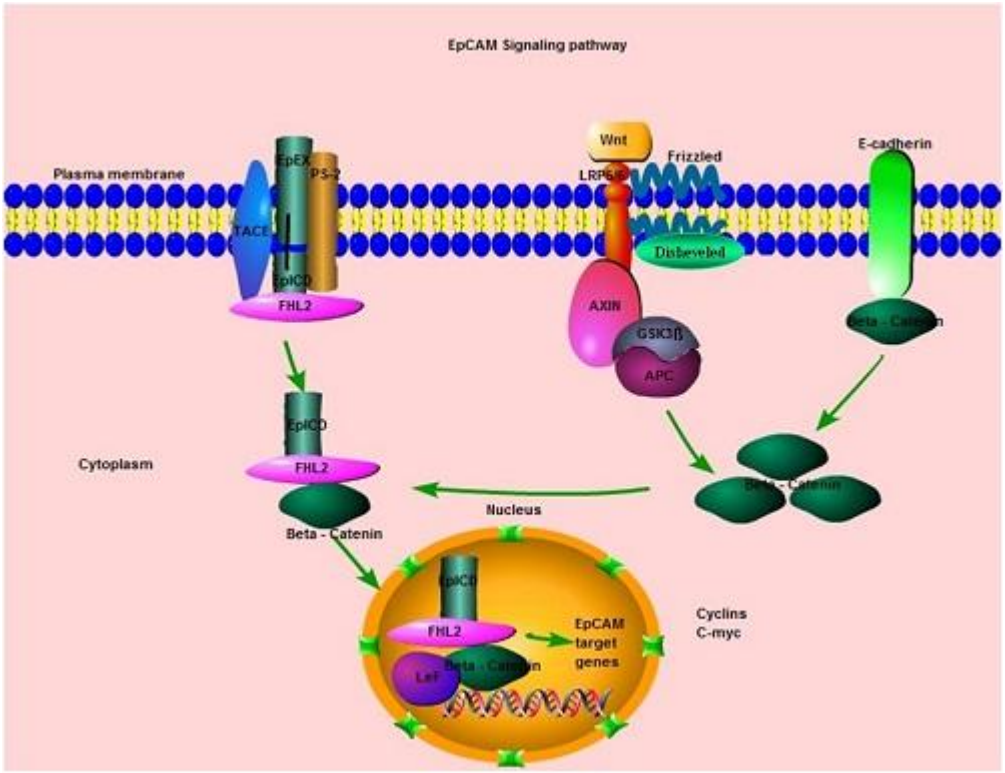


Figure 4: Schematic representation of EpCAM signaling pathway: Intracellular domain of EpCAM (EpICD) cleaved by TACE and PS-2 enzymes and translocate into the cytoplasm. Meanwhile, ß- Catenin accumulates in the cytoplasm due to the inhibition of ß-Catenin degradation complex (AXIN, GSK3 ß, APC) in Wnt - ß Catenin pathway. With help of FHL2, EpICD and ß Catenin enters into the nucleus. These nuclear complex proteins regulate gene transcription and activate the EpCAM target gene such as Cyclins and C-myc.

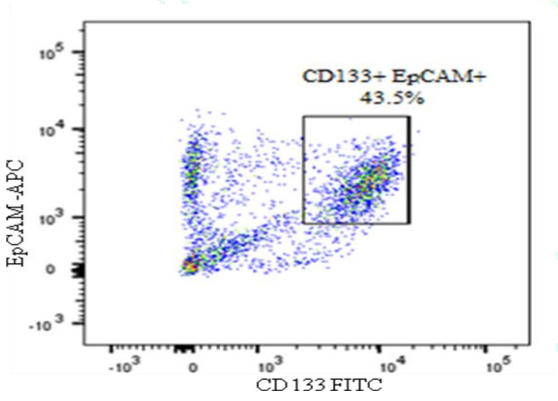


Figure 5: Flow cytometry analysis of LCSC marker CD133 & EpCAM.

DISCUSSION AND CONCLUSION

Yi Chen et al 2012 reported that CD133+EpCAM+ cells have capable of initiating tumour cells in Huh7 cells compared with CD133+EpCAM-, CD133-EpCAM+, CD133-EpCAM- cells, including enrichment in side population cells, higher differentiation capacity, increased colony-formation ability, preferential expression of stem cell-related genes, appearance of drug-resistant to some chemotherapeutics, more spheroid formation of culture cells and stronger tumorigenicity in NOD/SCID mice [21] Taro Yamashita et al 2009 confirmed that EpCAM+ AFP+ HCC cells are shown hepatic stem/progenitor cells features and these cells were capable of initiating highly invasive HCC in NOD/SCID mice. Also they found that aberrant Wnt/β-catenin signalling activation in EpCAM positive cells than EpCAM negative cells and blockage of EpCAM resulted in decrease Wnt/β-catenin signalling activation [22]. Gedaly R et al claim that arresting the Wnt/b-catenin pathway could inhibit the LCSC activation in HCC, and this was correlated with a decrease cells proliferation in S phase [20]. Chemoresistance and tumor recurrence is a major hurdle in therapeutics in many cancers including liver cancer. Our findings confirm that Cisplatin chemo drugs unable to kill Huh7 cells completely. Further analysis of this resistance cells, we observed liver cancer stem cell marker like CD133 expression was elevated in cisplatin-pretreated cells. Interestingly CD133 positive cells also express EpCAM. These results were reconfirmed by analyzing stem cells transduction factors such as Oct4 and Nanog in Cisplatin pretreated cells and control. Interestingly Oct4 and Nanog expression was increased in cisplatin pre-treated cells than control. Cisplatin pre-treated cells were able to form well distinct spheroids than other chemo drug pretreated cells, and no spheroid formation in control even after 9 days of culture. This finding suggests that tumor recurrence propertied in ex vivo. Based on all the results it's confirming that cancer stem cell plays a major role in chemoresistant and tumor recurrence so, targeting liver CSC through EpCAM targeted therapy might be a better choice to an enhanced prognosis of hepatocellular carcinoma.

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Conflict of Interest: The authors declare that there is no conflict of interests regarding the publication of this paper.

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