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# Hepatitis-C Virus Increases Tumorigenic Potential of Hepatocellular Carcinoma (HCC) Cells by Expanding an Aldehyde Dehydrogenase 1 (ALDH1) Positive Stem Cell Like Population

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**Abstract**

Hepatitis C virus (HCV) is a major risk factor for chronic liver disease and hepatocellular carcinoma (HCC), the third-deadliest cancer. Despite extensive research efforts, the relationship between HCV and liver cancer stem cells (LCSCs) is still largely unknown. Our current study demonstrates that the continued expression of HCV significantly increased the expression of aldehyde dehydrogenase 1 (ALDH1) as well as the percent of ALDH1 positive liver cells (ALDH1+ cells), which is associated with increased steady-state level of CSC-like population. Furthermore, this continued expression of HCV in ALDH1+ cells induces the enhancement of cancer cell growth in vitro and is accompanied by a significant increase in xenograft tumor growth *in vivo*. Indeed, the ALDH1+ cells with continued expression of HCV were less vulnerable to treatment than normal ALDH1+ cells. Our results showed that HCV is capable of enhancing stem cell–like signatures and suggested the existence of a hierarchical organization in liver cell lines bearing tumorigenic potential in the order of HCV+/ALDH+> HCV-/ALDH+.

**Keywords:** HCV; ALDH1; Liver Cancer Stem Cells (LCSCs); Hepatocellular carcinoma; ALDH1

## Introduction

Hepatitis C virus (HCV) infects approximately 71 million people worldwide, and chronic HCV infection often leads to fibrosis, cirrhosis and enhances the probability of developing hepatocellular carcinoma (HCC) [1-3].

HCV infection may increase the induction of reactive oxygen species (ROS) by modulating mitochondrial functions, leading to DNA damage [4] and contributes to genomic instability and HCC. These oncogenic events may occur simultaneously in various populations of hepatocytes and hepatic stem/progenitor cells. Moreover, HCV genomic RNA encodes for a large polyprotein precursor of approximately 3100 amino acids that is post-translationally cleaved by cellular and viral proteases into the mature structural (core, E1, E2) and p7 proteins as well as non-structural proteins (NS) (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [5,6]. These HCV proteins disturb intracellular signal transduction, leading to malignant transformation of infected cells. Moreover, recent study suggested that HCV could replicate in human fetal hepatocytes [7,8]. More recently, sustained expression of HCV was shown to induce stem cell–like signatures [9], however its impact on liver cancer stem cells (LCSCs) is largely unknown.

HCC is an aggressive disease with a poor outcome. Several hepatic stem/progenitor markers such as aldehyde dehydrogenase 1 (ALDH1), CD133, AFP, CK19, and c-Myc are useful for isolation of LCSCs [10], which are known to be responsible for metastasis, tumor relapse, and chemotherapy resistance. ALDH1 is one enzyme of ALDH family containing twelve ALDH enzymes primarily located in liver cells but also can be found in other cells [11-13]. These enzymes play a major role in the metabolism of many molecules such as amino acids, carbohydrates, lipids, vitamins and steroid [14] and are implicated in oxidative stress-associated diseases including cancer, diabetes and chronic alcohol exposure [15]. ALDH1 has shown to preferentially characterize the CD133 liver cancer stem cells [16] and its activity is up-regulated as a part of the oxidative stress response in various tumors [17].

Although LCSCs have been recently identified as contributors to HCC development [18] and a positive correlation between the levels of HCV replication and expression of doublecortin-like kinase 1 (DCLK1), a CSC-associated marker has been reported [19,20]. Recent investigations indicated that HCV could replicate in human fetal hepatocytes [7]. Moreover, continued expression of HCV was shown to induce stem cell–like properties [9], the relationship between HCV and LCSCs is still unknown. In the current study, we investigated the possible relationship between HCV and the CSCs associated marker ALDH1 and the potential gain of CSC properties in HCV positive cells.

## Materials and Methods

### Antibodies and reagents

Anti-mouse core and anti-mouse Alexa Fluor 546 were purchased from Abcam. (Cambridge, UK) while anti-goat Alexa Fluor 488 from

Thermo Fisher (Waltham, MA, USA). Nuclei were stained with Hoechst stains (Invitrogen, Waltham, MA, USA). Goat anti-human ALDH1 and goat anti-human β-actin were from Santa Cruz Biotechnology (Dallas, TX, USA). ALDEFLUORTM reagent was from Stem Cell Technologie (Vancouver, BC, Canada). Sorafenib tosylate (Nexavar®, [N-(3-trifluoromethyl-4-chlorophenyl)-N-(4-(2-methylcarbamoylyridin-4-yl) oxyphenyl) urea]) was provided from Bayer Schering Phama (Leverkusen, Germany) or Sigma, St Louis, MO, USA), dissolved in 100% dimethyl sulfoxide (DMSO; Sigma) and diluted with Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) to the desired concentrations.

### Cell culture and drug treatment

The human hepatocellular carcinoma cells Huh7.5 were maintained in Dulbecco's modified Eagle's Medium (DMEM; Invitrogen) containing 4.5 g/l glucose supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 1% nonessential amino acids (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) at 37°C with 5% $CO_2$. 24 h after sorafenib treatment, the media was replaced with fresh media, and the plates were incubated for another 48 h at 37°C.

### Immunoblot

Cells were collected on ice, washed and lysed in 20 mM Tris HCl, 100 mM NaCl, 1% Triton X100 and 10 mM EDTA (ethylenediaminetetraacetic acid) (all from Sigma) at pH-7.4 containing protease and phosphatase inhibitor cocktail (Roche diagnostics, Basel, Switzerland), The proteins were separated on SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), blotted onto a PVDF membrane (polyvinylidene difluoride) (from Merck Millipore, Billerica, MA, USA) and visualized with chemiluminescence reagent (Amersham, Little Chalfont, Buckinghamshire, UK).

### Immunofluorescence staining

Cells were grown on glass coverslips in 12-well plates, treated according to the experiments, fixed with a 3.7% paraformaldehyde (Sigma), permeabilized and saturated with PBS (phosphate-buffered saline) supplemented with 0.7% fish gelatin (Sigma) and 0.025% saponin (Sigma). Primary antibodies were diluted with permeabilization solution and incubated with cells for 1 hour at room temperature. After washing with PBS, fluorescent secondary antibodies were added and Hoechst-stained cells were observed with a Leica TCS SP5 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). Images generated were then processed and analyzed by ImageJ.

### Flow cytometry and cell sorting

ALDEFLUOR reagent (Stem Cell Technologies, Vancouver, Canada) was used for the immunofluorescent detection of intracellular ALDH1 enzyme activity according to the manufacturer's protocol. The stained cells were analyzed using the FACS Aria II (BD Biosciences, San Jose, CA, USA) and sorted into the ALDH1+ cells, which were detected on the green fluorescence channel (515–545 nm).

### Mice

Xenograft tumors were generated in nude mice and allowed to grow for up to four weeks. Subsequently, the mice were handled in accordance with UPSUD guidelines. Five mice were used in each study. Tumor formation was assayed after subcutaneous injection of equal numbers of Huh7.5/ALDH+ and Huh7.5 expressing HCV sub-genomic replicon in 100 µl of culture medium into 9 week female old nude mice. $10^6$ cells were subcutaneously injected into the right flanks of mice. Tumor growth was observed for up to 4 weeks. The tumor volume was calculated as $V = (1/2)\ xy^2$, where x is the longest diameter and y is the shortest diameter of the tumor.

### Colony forming assay

Cells were trypsinized and seeded in 6-well plates (500 cells/well) to form colonies in 2-3 weeks. The plates were incubated at 37°C and the media was replaced with fresh media 3 times per week. 24 h after sorafenib treatment, the media was replaced with fresh media, and the plates were incubated at 37°C. 4 days later, the cells were fixed and stained with a crystal violet solution (Sigma). (Je n'ai pas compris comment tu as fait).

### Statistical analyses

Statistical analysis was performed using GraphPad Prism 5.03 software. Data were analyzed using unpaired Student's t test. Statistical significance was determined as follows: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

## Results and Discussion

### Up-regulation of ALDH1 expression in hepatocytes expressing HCV sub-genomic replicon

We examined the expression of ALDH1 in Huh7.5 cells and Huh7.5 cells expressing HCV sub-genomic replicon (Huh7.5R). Immunoblot analysis indicated an increase of ALDH1 expression in Huh7.5R (Figure 1A). The ratios for the relative band intensity of ALDH1 after normalization with actin are shown in Figure 1B. Furthermore, Huh7.5R cells were analyzed by confocal microscopy and ALDH1 was found mainly low in the cytoplasm of Huh7.5 cells, whereas the signal increased dramatically in the cytoplasm of Huh7.5R cells (Figure 1C). These results suggested that HCV could up-regulate the expression of ALDH1. Data from confocal pictures were analyzed using ImageJ software and were presented as graph representing the signal intensity of ALDH1 and core. in normal cells (Huh7.5), the line profile plot of ALDH1 was very low, while a large peak of ALDH1-green signal was observed and was superimposable with the line profile plot of core (red signal).

### These results suggested a positive correlation between HCV and ALDH1

ALDH1 is a stem cell marker expressed in primary tumor specimens and detected in limited cellular sub-population of human primary tumor cells [17]. ALDH1+ cells from Huh7.5 and Huh7.R were sorted using specific ALDH1 antibody (Figure 1D), we therefore determined the percentage of ALDH+ population from Huh7.5 and Huh7.5R cells by performing fluorescence-activated cell sorting (FACS) analysis (Figure 1D).

FACS analysis revealed significantly an increase in ALDH1+ cell population in Huh7.5 expressing HCV sub-genomic replicon. the line profile of ALDH1+ population in Huh7.5 cells was very low (14.2%), while a large peak of ALDH1+ population in Huh7.5R cells was observed (30.9%).

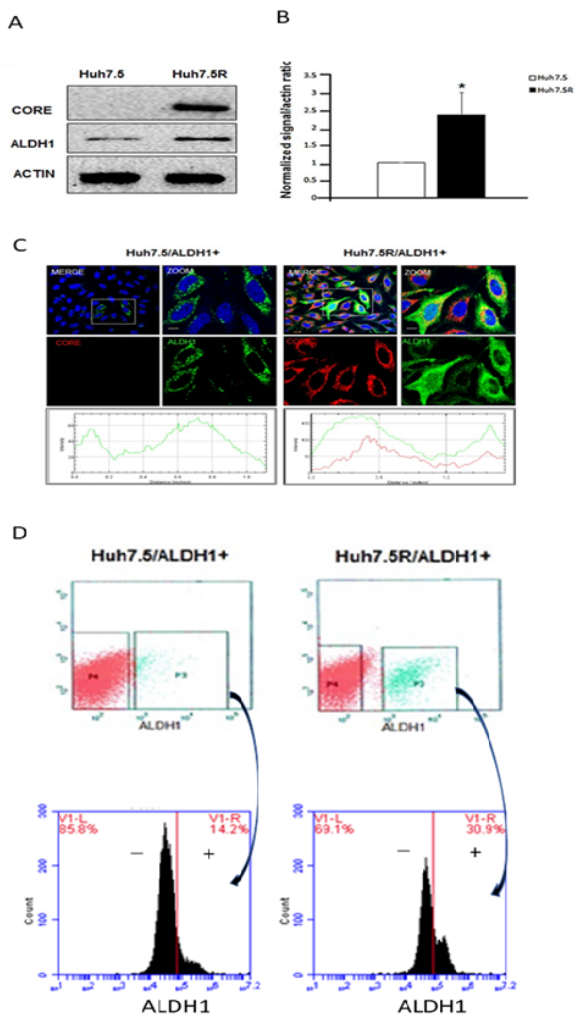


**Figure 1:** Analysis of ALDH1 in hepatoma cellular carcinoma (HCC) cells Huh7.5 cells and Huh7.5R expressing HCV sub-genomic replicon. (A, B) Immunoblot analysis and quantification of ALDH1 and HCV core in lysates of Huh7.5 and Huh7.5R. Actin was used as a loading control. The bar graph presents the densitometry analysis of the immunoblots from three independent experiments. Values are means ± SD or S.E.M. Unpaired Student's t test was used. *p<0.05. (C) Immunofluorescence analysis of ALDH1 and HCV core in Huh7.5 and Huh7.5R cells. Huh7 and Huh7R cells were stained for ALDH1 (green), and HCV core (red). Nuclei were stained with Hoechst solution (blue). White dot squares indicate the area shown in higher magnification. Scale bars (10 µm). (D) Isolation and analysis of ALDH+ cells from hepatoma cell lines by fluorescence-activated cell sorting using specific ALDH1 antibody.

## HCV sub-genomic replicon enhanced proliferation and chemo-resistance of ALDH1+ cell line

In the current study, cell proliferation of ALDH1+ sorted cells was assessed with the Cell Titer-Glo according to the manufacturer's protocol. Moreover, we observed that ALDH1+ cells with sustained HCV expression showed highest cell proliferation rate than normal ALDH1+ cells (Figure 2A), indicating that HCV+/ALDH1+ promoted cancer cell proliferation.

One of the characteristics of cancer stem cells is the chemo-resistance to anti-tumor drugs, we therefore assayed the viability of ALDH1+ sorted cells after treatment with increasing doses of sorafenib. Huh7.5/ ALDH1+ and Huh7.5R/ ALDH1+ cells were seeded onto cell culture plates, cultured for 24 h, and various concentrations of sorafenib (2.5, 5, 10 µg/ml) were added (Figure 2B). After 24 h, the viability of cells was assessed with the Cell Titer-Glo according to the manufacturer's protocol. The comparison of Huh7.5/ ALDH1+ cells to Huh7.5R/ ALDH1+ cells in resistance against sorafenib revealed that ALDH1+ cells with sustained HCV expression were less vulnerable to sorafenib than the normal ALDH1+ cells (Figure 2B). In addition, colony forming assay was done to compare the colony-forming ability of Huh7.5/ALDH+ and Huh7.5R/ ALDH+ cells. The results summarized in Figures 2C and 2D showed that Huh7.5R/ ALDH+ yielded the highest number of colonies than Huh7.5/ALDH+ cells. Furthermore, the colony-forming ability of cell was reduced after exposure to 10 µg/ml of sorafenib (Figures 2C and 2D). However, the increase of resistance to sorafenib was more remarkable in ALDH+ cell population expressing HCV sub-genomic replicon. These results indicated that HCV contributed to the increase of cell resistance to the suppressive effect of sorafenib on proliferation.

## ALDH+ cells expressing HCV sub-genomic replicon are more tumorigenic *in vivo*

As known, CSCs are able to initiate tumors with limited numbers of cells. We next tested the cancer initiating capability of normal ALDH+ cell (Huh7.5/ ALDH+) population and ALDH+ cells with continued expression of HCV (Huh7.5R/ ALDH+) into athymic nu/nu mice. We injected equal numbers of (Huh7.5/ ALDH+) and (Huh7.5R/ ALDH+) into mice and monitored tumor growth for up to four weeks. 106 cells were subcutaneously injected into the right flanks of mice. Figure 2E illustrated the substantial differences in tumor size induced by normal ALDH+ cell line and ALDH+ cells expressing HCV sub-genomic replicon. After 4 weeks of growth, the differences in tumor volume were quantified. Tumor monitoring analysis indicated that Huh7.5R/ ALDH+ cells generated larger tumors than Huh7.5/ ALDH+ cells (Figures 2E and 2F), indicating an increase in tumor growth and progression.

## Positive correlation between HCV and ALDH1

HCV replication can be inhibited by interferon alpha (IFN-α) [21,22], to assess the possible correlation of HCV with ALDH1 expression. We treated cells expressing HCV sub-genomic replicon with IFN-α and evaluated the expression of ALDH1. Immunoblot analysis indicated that the inhibitory effect of IFN-α on HCV replication caused a reduction in ALDH1 expression (Figure 3A). The ratios for the relative band intensity of ALDH1 and core after normalization with actin are shown in Figure 3A, lower anel. Furthermore, Huh7.5 cells expressing HCV sub-genomic replicon were plated and exposed to IFN-α (1000 IU/ml) for 48 h, then fixed, permeabilized, and labeled for nuclei, HCV core and ALDH1. Data showed that the fluorescence intensity of core and ALDH1 were notably lower in-treated cells compared to non-treated cells (Figure 3B). Indeed, cells expressing HCV sub-genomic replicon were treated with IFN-α (1000 IU/ml) for 48h and harvested for flow cytometry analysis. Cells were fixed and stained using specific HCV core and

ALDH1 antibodies. Flow cytometric analysis of HCV sub-genomic replicon populations, gated on ALDH+/HCV CORE+ cells revealed decreased Huh7R/ALDH1+ cells (Figure 3C) with decreased CORE and ALDH1 expression (Figure 3D and 3E). These results suggested a positive correlation between HCV and ALDH1.

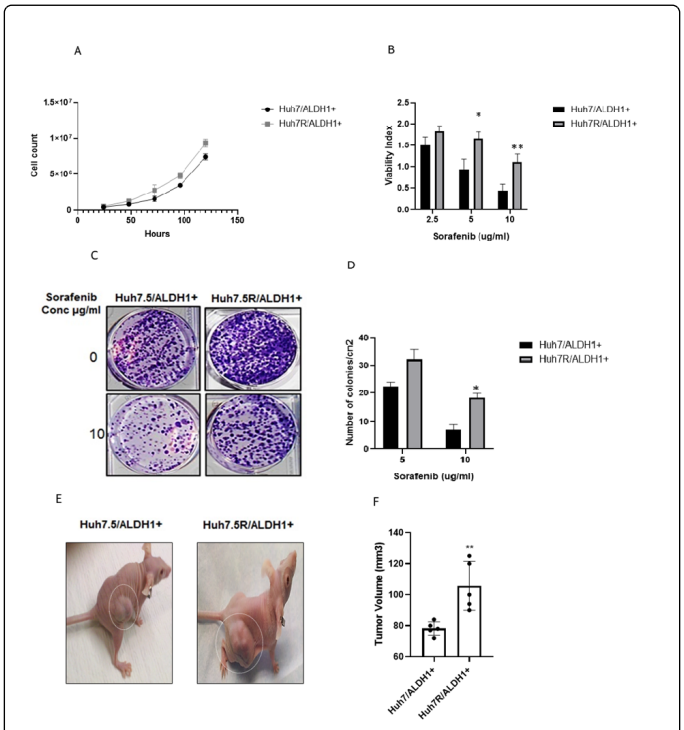


**Figure 2:** Sustained HCV expression increases ALDH1+ cells and enhances their tumorigenicity and chemoresistance (A) Continued expression of HCV increases the ALDH1+ cell population in hepatoma cellular carcinoma cells. (B). HCV contributed to the increase of cell resistance and to the suppressive effect of sorafenib on proliferation. Huh7.5/ ALDH1+ and Huh7.5R/ ALDH1+ cells were seeded onto cell culture plates, cultured for 24 h, and various concentrations of sorafenib (2.5, 5, 10 ug⁄mL) were added. After 24 h, the viability of cells was assessed with the Cell Titer-Glo according to the manufacturer's protocol. (C) Colony formation assay. The ALDH1+ cells expressing HCV formed significantly more colonies and were more resistant to sorafenib than the normal ALDH1+cells. (D). The number of colonies that were macroscopically visible, derived from the Huh7.5/ALDH1+ and Huh7.5R/ALDH1+ cells. (E) Huh7.5 cells and Huh7.5R cells were subcutaneously injected into the right flanks of 9 weeks old athymic nu/ nu mice. (F) Tumor volume analysis in nude mice carrying tumors of Huh7.5/ ALDH1+ and Huh7.5R/ ALDH1+ was monitored and measured for 4 weeks after subcutaneous injection.

We have used Huh7.5 cells expressing HCV sub-genomic replicon to investigate the association between HCV and cancer stem cells. It has been reported that ALDH1 is a cancer stem cell marker. ALDH1 is a cytosolic enzyme that highly expressed in Normal and Malignant Human Stem Cells [17,18]. More importantly, ALDH1 was found to be associated with chemo-resistance [19,20] in CSCs. In this view, we choose to pursue ALDH1 expression because high expression of ALDH1 is thought to provide a survival advantage in a toxic cellular microenvironment. In this study, the pluripotent stem cell marker ALDH1 was up-regulated in HCV-expressing cells. The basal expression level of ALDH1 was found to be low in normal hepatocytes (Huh7.5). However, active HCV replication contributed to its elevated expression. Visualization of ALDH1 by confocal microscopy suggested that this protein was highly enriched in HCV-expressing cells (Huh7.5R). Moreover, treatment of HCV replicon cells with INFα leaded to marked reductions in the level of HCV core, in addition, we demonstrated that the curing of the HCV replicon led to reduction in the level of ALDH1 [21,22].

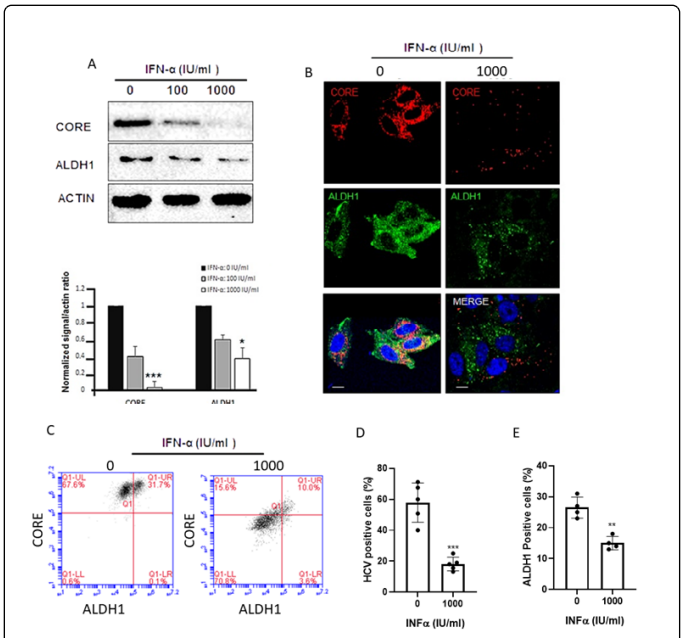


**Figure 3:** Positive correlation between HCV and ALDH1(A) Immunoblot analysis and quantification of ALDH1 and HCV core in lysate of Huh7.5R treated with IFN-α (100 and 1000 IU/ml) and incubated for 48 hours. Actin was used as a loading control. The bar graph presents the densitometry analysis of the immunoblots from three independent experiments. Values are means ± SD or S.E.M. Unpaired Student's t test was used. $^{*}$P<0.05, $^{***}$ P<0.0001. (B) Immunofluorescence analysis of Huh7.5R. Huh7.5R cells were plated and exposed to IFN-α (1000 IU/ml) for 48 hours, then fixed, permeabilized, and labeled for ALDH1 (green), HCV core (red) and nuclei were stained in blue. Scale bars (10 µm). C, D, E. flow cytometry analysis of Huh7.5R after treatment with IFN-α (1000 IU/ml) for 48 hours. Cells were fixed, permeabilized, and labeled for ALDH1 and HCV core.

ALDH1 was up-regulated as a part of the oxidative stress response in various tumors [23]. Recent study demonstrated that up-regulation of ALDH1 was a stress response in bacteria [24]. Moreover, HCV infection has been associated with increased concentrations of reactive oxygen species (ROS), Indeed, HCV core protein, NS5A and NS3 proteins have been found to increase the oxidative stress in liver [25-28]. Our result suggested that ALDH1 up-regulation could be a stress response in HCV infection. Isolation of ALDH+ cells from hepatoma cell lines by fluorescence-activated cell sorting (FACS) revealed that the percentage of ALDH1+ cell population was significantly increased in HCV-expressing cells. In addition, purified

ALDH+ cell population from HCV-expressing cells were found to be more tumorigenic than normal ALDH+ cells in both *in vitro* and *in vivo*, in that they were able to proliferate significantly faster and also to induce greater number of tumor colonies. Indeed, ALDH+ cells - expressing HCV also showed enhanced colony-forming capability in in vitro and were more resistant to cancer drug treatment (sorafenib). Recently, Lauren and collaborators demonstrated that retroviral-infection increases tumorigenic potential of breast carcinoma cells by increasing ALDH1 positive cancer stem-cell population [29]. Moreover, the up-regulation of ALDH1 in tumors led to chemo-resistance during cancer chemotherapy [30-32]. Several lines of evidence presented here implicated HCV in the enhanced expression of ALDH1, leading to enhanced resistance of cells against sorafenib. Tsamandas and collaborators demonstrated that hepatic progenitor cells are frequently present in liver tissues of hepatitis C patients. Furthermore, it was showed that patients who do not respond to the standard HCC treatment showed higher expression of cancer stem cell markers [33]. The hepatoma cells used in this report are expected to contain a subpopulation of CSCs. We showed that both normal Huh7.5 cells and HCV-expressing cells were able to form solid tumors in nude mice. However, tumor monitoring analysis indicated that tumor growth rate was increased in Huh7.5R/ ALDH+ mice indicating that ALDH+/HCV-expressing cells increased tumor growth and progression. Hepatitis C virus (HCV) is a major risk factor for the development of chronic liver disease and hepatocellular carcinoma (HCC). Its role in liver CSCs is largely unknown but recent study suggested that HCV could replicate in human fetal hepatocytes [7]. Moreover, continued expression of HCV was shown to induce Cancer Stem Cell-Like properties in vitro and in murine tumor xenograft [9]. Indeed, HCV NS5A transgenic mice following alcohol exposure showed activation of cancer stem cell properties [34].

## Conclusion

In conclusion, the present report has provided further evidence that HCV infection predispose cells toward the path of acquiring cancer stem cell properties by inducing ALDH1 expression. The analyses of our data revealed an HCV-ALDH1-CSC axis in the hepatoma cell line. Using both *in vitro* and *in vivo* model, our data provided evidence that this collaboration may possibly promote HCV-induced hepatocarcinogenesis during chronic infection and chemo-resistance to anti-tumor drugs during chemotherapy. An in-depth understanding of the underlying molecular mechanisms regulating /HCV-induced gain of CSC properities is essential for the development of improved therapeutics.

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