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Research Paper

Isolation and characterization of cancer stem-like cells from MHCC97H Cell Lines

Shanyong Yi[a], Kejun Nan[a,*], Aihua Yuan[b], Chuangxin Lu[a]

[a]*Cancer Center of the First Affiliated Hospital, College of Medicine of Xi' an Jiaotong University, Xi' an,710061, China;*

[b]*Department of Cancer Center of the Nanjing First Hospital, Affiliated to Nanjing Medical University, Nanjing, 210006, China.*

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Abstract

Objective: To identify and isolate CD133 positive cancer stem-like cells ($CD133^+$ cells) from the highly invasive human hepatocellular carcinoma cell line(MHCC97H), and examine their potential for clonogenicity and tumorigenicity. **Methods:** $CD133^+$ and $CD133^-$ cells were isolated from MHCC97H cell line by magnetic bead cell sorting(MACS), and the potentials of $CD133^+$ cells for colony formation and tumorigenicity were evaluated by soft agar cloning and tumor formation following nude mice inoculation. **Results:**$CD133^+$ cells represent a minority(0.5-2.0%) of the tumor cell population with a greater colony-forming efficiency and greater tumor production ability. The colony-forming efficiency of $CD133^+$ cells in soft agar was significantly higher than $CD133^-$ cells(36.8 ± 1.4 vs 12.9 ± 0.8, $P < 0.05$). After 6 weeks, 3/5 mice inoculated with 1×10^3 $CD133^+$ cells, 4/5 with 1×10^4 $CD133^+$ cells and 5/5 with 1×10^5 $CD133^+$ cells developed detectable tumors at the injection site, while only one tumor was found in mice treated with same numbers of $CD133^-$ cells. **Conclusion:** CD133 may be a hallmark of liver cancer stem cells (CSC) in human hepatocellular carcinoma(HCC), because the $CD133^+$ cells identified and isolated with anti-CD133 labeled magnetic beads from MHCC97H cell line exhibit high potentials for clonogenicity and tumorigenicity. These $CD133^+$ cells might contribute to hepatocarcinogenesis, as well as the growth and recurrence of human HCC, and therefore may be a useful target for anti-cancer therapy.

Keywords: hepatocellular carcinoma; CD133; cancer stem cell; tumorigenicity

INTRODUCTION

Cancer stem cells(CSC) are a small cluster of tumor cells in a tumor cell population that have a high potential for self-renovation and multidirectional differentiation. Increasing evidence suggests that tumors harbor a small population of CSC that contributable not only to the tumor bulk, but also to the tumorigenesis and recurrence. The importance of CSC in human tumors indicates they might be useful targets in the diagnosis and treatment of cancers. More and more investigators have prospectively identified CSC in human solid tumors based on a set of cell surface markers[1-12]. However, to date, no study has convincingly put this proposition into practice. The first obstacle is that CSC are only a small subpopulation of tumorigenic cells and most probably are present in a heterogeneous mixture with differentiated progenies, while undifferentiated CSC(also called true CSC) remain hidden among them. The second obstacle is that there is a shortage of reliable markers for identifying CSC in solid tumors.

Hepatocellular carcinoma(HCC) is a highly aggressive malignancy with poor prognosis. It is currently the fourth most common cancer and second leading cause of cancer-related death in China[13]. The mechanisms responsible for the development and progression of HCC are poorly understood. It is therefore of great significance to identify, isolate and study CSC in HCC. CD133 is a putative hematopoietic and neuronal stem-cell marker, which has been extensively studied for possible use in identifying CSC in solid tumors. Previous data

*Corresponding author.

E-mail address: nankij@163.com

showed that CD133 is a marker closely correlated with tumorigenicity, since it was selectively expressed in human fetal and repairing liver tissues, and was closely associated with hepatocarcinogenesis. It has been suggested to be a useful tumorigenic marker in brain and prostate[2-4]. CD133$^+$ cells were also found in some human HCC cell lines, such as BEL-7402, SMMC-772, Huh-7, and Hep3B[14-16]. It remains controversial whether CSC are exhibited under culture conditions. The human MHCC97 cell line might contain CSC as it is a highly invasive line isolated from human HCC.[17] In the present study, our goal was to identify and isolate CSC from the MHCC97 cell line, to confirm the previous report that CD133$^+$ cells represent CSC in HCC, and examine their potential for clonogenicity in soft agar and tumorigenicity in nude mice. There is presently no study to clarify such questions.

MATERIALS AND METHODS

Cell culture

Hepatocellular carcinoma cell line MHCC97H was purchased from the Liver Cancer Institute, Zhongshan Hospital, Fudan University(Shanghai, China). The cells were cultured in an exponentially growing culture at 37℃ in a humidified, 5% CO_2 atmosphere in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum(FBS).

MACS and Flow cytometry

MHCC97H cells were labeled with primary CD133/1 antibody(mouse IgG1, Miltenyi Biotec, Shanghai, China, 1 μl per million cells), subsequently magnetically labeled with rat anti-mouse IgG1 Micro beads (Miltenyi Biotec, 20 μl per 10 million cells) and separated on a MACS LS column(Miltenyi Biotec). All the procedures were carried out according to manufacturer's instructions. The purity of sorted cells was evaluated by flow cytometry. Flow cytometric analysis was carried out with an Epics Altra machine (Beckman Coulter, USA), using CD133/1 primary antibody(Miltenyi Biotec) and FITC-conjugated secondary antibody(Sigma Chemical Co, USA).

Colony formation assay

The colony formation assay in soft agar was carried out as described previously. In brief, the base layer was made by mixing 1% soft agar and equivalent 2× medium and prepared in 24-well plate. Then CD133-sorted MHCC97H cells were harvested, suspended in medium containing 0.3% soft agar(Invitrogen, USA), and seeded upon the base layer at a density of 100 cells per well. All experiments were conducted in triplicates. Plates were maintained at 37℃ in a humidified incubator and cells were fed every 5 days with 0.1 ml of medium. After 3 weeks, the numbers of the formed colonies were assessed by counting under a microscope. Representative views were photographed. For the second round of colony formation, some colonies from the first round were picked up and their cells were reseeded in 24-well plates with soft agar following the same procedures as the first cycle. The results of the second colony formation assay were assessed as described above.

Tumor formation in an animal model

Six-to eight-week-old female nude mice were purchased from the Animal Institute of the Chinese Academy of Medical Science(CAMS) and maintained in microisolator cages. All experiments were approved by the Animal Care Committee of CAMS. Freshly sorted CD133$^+$ and CD133$^-$ cells were suspended in 200 μl PBS respectively and bilaterally inoculated into the backs of 15 nude mice, using 1×10^3, 1×10^4 and 1×10^5 cells per inoculum. Tumor formation was monitored from 1 week after inoculation. After 6 weeks, all of the mice were killed, and tumor masses were removed and microscopically examined.

Statistical analysis

All data were expressed as mean ± SD and evaluated with Student's *t*-test. $P < 0.05$ was accepted as statistically significant.

RESULTS

CD133$^+$ cells in the MHCC97H cell line

After screening hepatoma cell lines, we found that the MHCC97H cell line contained CD133$^+$ cells. Although the CD133$^+$ cells represented only a very small subpopulation when analyzed by flow cytometery(***Fig. 1***), they could be sorted out by utilizing MACS. CD133$^+$ cells were again analyzed by flow cytometry after the MACS sorting, and the purity ranged from 71.5% to 92.4%(***Fig. 2***) compared to 0.5-2.0% for unsorted MHCC97H cells.

High clonogenicity of CD133$^+$ cells

CD133$^+$ cells enriched by MACS efficiently formed colonies 3 weeks after growing twice in soft agar. Based on the calculation of the numbers of formed colonies per 100 seeded cells, the values were 36.8 ± 1.4/ 12.9 ±0.8 for CD133$^+$/CD133$^-$ cells in soft agar, respectively (***Table 1***). We further tested the colony formation from cells isolated from primary colonies, and the colony formation efficiency in soft agar in the secondary round of plating was 26.7 ± 1.3/9.5 ± 0.6 for CD133$^+$/CD133$^-$ cells in soft agar, respectively(***Table 1***). These results strongly indicate that the CD133$^+$ cells possessed the capability of maintaining their high clonogenicity.

Tumorigenicity of CD133$^+$ and CD133$^-$ cells *in vivo*

To validate the capacity for tumorigenicity of CD133$^+$ versus CD133$^-$ cells in nude mice, we bilaterally, subcu-

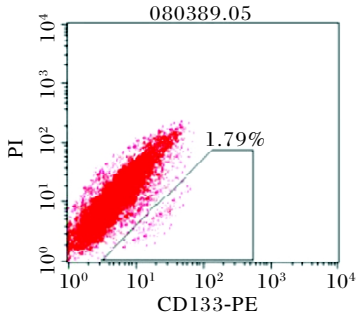

Fig. 1 Purity analysis of MHCC97-H cells by flow cytometry before MACS sorting

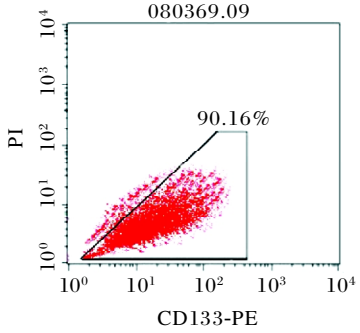

Fig. 2 MACS sorted CD133$^+$ cells collected and checked for purity by flow cytometry

Table 1 **Colony formation efficiency(colony number per 100 seeded cells)**

	CD133$^+$(%)	CD133$^-$(%)
Primary cycle	36.8 ± 1.4	12.9 ± 0.8★
Second cycle	26.7 ± 1.3	9.5 ± 0.6★

Compared to the corresponding CD133$^+$ group, ★ $P < 0.05$.

taneously inoculated 1×10^3, 1×10^4 and 1×10^5 CD133$^+$ and the same numbers of CD133$^-$ MHCC97H cells into the backs of nude mice. As shown in ***Fig. 3***, CD133$^+$ and CD133$^-$ cells displayed significantly different tumorigenesis in these animals 6 weeks after inoculation. In mice inoculated with CD133$^+$ cells, detectable tumor formation was observed in 3/5 mice in the 1×10^3 cells group, 4/5 mice in 1×10^4 cells group and 5/5 mice in 1×10^5 cells group, while only one tumor was detected in all the animals in the CD133$^-$ cells groups(***Table 2***). CD133$^+$ cell injection could initiate significantly larger tumor formation than the CD133$^-$ cells group tumor, with diameters larger than 10 mm($P < 0.001$, ***Fig. 4***). The lesions were confirmed by histopathological examination(***Fig. 5***).

Table 2 **Tumorigenicity of CD133$^+$ and CD133$^-$ hepatoma cells in nude mice**

Cell numbers injected per mouse	CD133$^+$ cells	CD133$^-$ cells
1×10^3	3/5	0/5
1×10^4	4/5	0/5
1×10^5	5/5	1/5

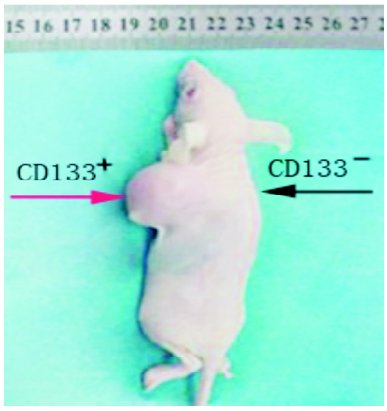

Fig. 3 Photograph of a nude mouse taken 6 weeks after transplantation with CD133$^+$ and CD133$^-$ cells

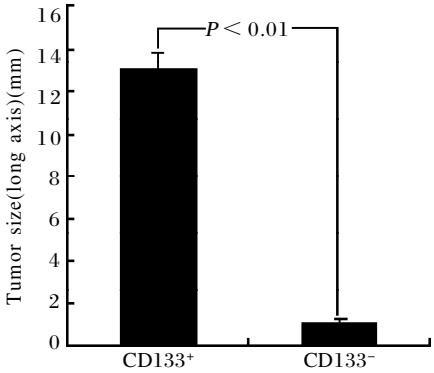

Fig. 4 Tumor size(longer axis) compared between the CD133$^+$ and CD133$^-$ groups at 6 weeks after injection

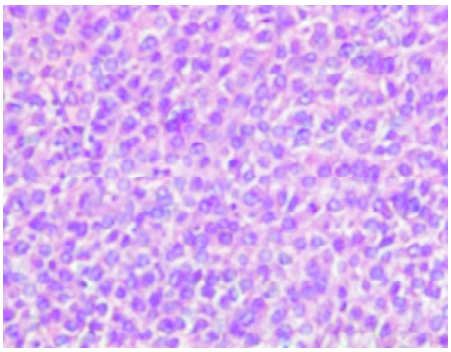

Fig. 5 The grafts of the CD133$^+$ cells excised at 6 weeks after transplantation(HE, × 40)

DISCUSSION

Cancer has long been viewed as a heterogeneous population of cells. In the last few years, a growing body of evidence has been reported supporting the notion that tumors are organized in a hierarchy of heterogeneous cell populations with different biologic properties and that the capability to sustain tumor formation and growth exclusively resides in a small proportion of cells

called CSC[7-11]. This subpopulation of CSC is able to self-renew, differentiate, and regenerate a phenocopy of the original tumor when implanted into the nude mouse. Numerous studies indicate that the growth of liver cancer, and a number of other types of cancer, is initiated and driven by a subpopulation of cancer cells with stem-like characteristics, including self-renewal capacity and the ability to differentiate[14-16]. CD133, a 5 transmembrane domain cell-surface glycoprotein, is regarded as an important marker for the identification and isolation of primitive stem/progenitor cells in both hematopoietic and nonhematopoietic tissues[18]. It was originally found on hematopoietic stem cells and hematopoietic progenitor cells deriving from human fetal liver, bone marrow, and peripheral blood. Recently, CD133 has been widely used as a molecular marker for characterizing normal neuronal, hematopoietic and other stem cells, and some types of cancer stem or stem-like cells in tumors originating from brain, colon and prostate cancer[10,11,19].

In the present study, we demonstrated that CD133 was expressed in MHCC97H cells, and that $CD133^+$ cells represented only a very small subpopulation(0.5%-2.0%) of the unsorted MHCC97H cells. After sorting for purity, $CD133^+$ cells ranged from 71.5% to 92.4%. The result suggests that MACS is an efficient methodology for the enrichment of CSC from the MHCC97H cell line. Our data showed that $CD133^+$ cells have high clonogenicity in vitro and high tumorigenicity *in vivo* compared with $CD133^-$ cells, which suggests that these cells have at least some of the properties of cancer stem or stem-like cells. Our results suggest that within a tumor cell line, committed cells may possess limited tumorigenicity, and tumors formed by these cells exhibit pathological characteristics that differ from those formed by CSC.

In conclusion, our study demonstrated that there exists a small subpopulation of $CD133^+$ cells in HCC cell lines, with higher clonogenicity *in vitro* and potent tumorigenicity in immunodeficient mice. These findings could provide some insight into isolation and characterization of cancer initiating cells. The identification of molecules expressed in the small subpopulation of CSC and the selective targeting of CSC that are pivotal for the growth of the entire tumor mass should not only lead to the more efficient elimination of this crucial population of cancer cells but also open new avenues for the development of more effective cancer therapies[20-22].

References

[1] Kim CF, Jackson EL, Woolfenden AE, Lawrence S, Babar I, Vogel S, *et al.* Identification of bronchioalveolar stem cells in normal lung and lung cancer. *Cell* 2005; 121: 823-35.

[2] Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, *et al.* Identification of human brain tumor initiating cells. *Nature* 2004; 432: 396-401.

[3] Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res* 2005; 65:10946-51.

[4] Patrawala L, Calhoun T, Schneider-Broussard R, Li H, Bhatia B, Tang S, *et al.* Highly purified $CD44^+$ prostate cancer cells from xenograft human tumors are enriched in tumorigenic and metastatic progenitor cells. *Oncogene* 2006; 25:1696-708.

[5] Xu Jian,Wang Shui, Xu Li-sheng, Du Qing,Liu Xiao-an. Preliminary study on cancer stem-like cells in human breast cancer cell lines. *Acta Univ Med Nanjing*(in Chinese) 2007;27: 350-5.

[6] Chiba T, Kita K, Zheng YW, Yokosuka O, Saisho H, Iwama A, *et al.* Side population purified from hepatocellular carcinoma cells harbors cancer stem cell-like properties. *Hepatology* 2006; 44: 240-51.

[7] Yi SY, Nan KJ. Tumor-initiating stem cells in liver cancer, *Cancer Biol Ther* 2008;7:325-30.

[8] Suetsugu A, Nagaki M, Aoki H, Motohashi T, Kunisada T, Moriwaki H. Characterization of $CD133^+$ hepatocellular carcinoma cells as cancer stem/progenitor cells. *Biochem Biophys Res Commun* 2006; 351: 820-4.

[9] Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, *et al.* Identification of pancreatic cancer stem cells. *Cancer Res* 2007; 67: 1030-7.

[10] O' Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumor growth in immunodeficient mice. *Nature* 2007; 445: 106-10.

[11] Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, *et al.* Identification and expansion of human colon-cancer-initiating cells. *Nature* 2007; 445: 111-5.

[12] Prince M E, Sivanandan R, Kaczorowski A, Wolf GT, Kaplan MJ, Dalerba P, *et al.* Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. *Proc Natl Acad Sci USA* 2007; 104:973-8.

[13] Yang Ling, Li Liandi, Chen Yude, *et al.* Cancer Incidence and mortality estimates and prediction for year 2000 and 2005 in China. *Chinese J Health Stats* 2005;22(4):218-21.

[14] Yin S, Li J, Hu C, Chen X, Yao M, Yan M, *et al.* CD133 positive hepatocellular carcinoma cells possess high capacity for tumorigenicity. *Int J Cancer* 2007; 120:1444-50.

[15] Ma S, Chan KW, Hu L, Lee TK, Wo JY, Ng IO, *et al.* Identification and characterization of tumorigenic liver cancer stem/progenitor cells. *Gastroenterol* 2007; 132: 2542-56.

[16] Zen Y, Fujii T, Yoshikawa S, Takamura H, Tani T, Ohta T, *et al.* Histological and culture studies with respect to ABCG2 expression support the existence of a cancer cell hierarchy in human hepatocellular carcinoma. *Am J Pathol* 2007; 170: 1750-62.

[17] Wu FS, Zheng SS, Wu LJ, Teng LS, Ma ZM, Zhao WH, *et al.* Calcitriol inhibits the growth of MHCC97 heptocellular cell lines by down-modulating c-met and ERK expressions. *Liver Int* 2007; 27: 700-7.

[18] Richardson GD, Robson CN, Lang SH, Neal DE, Maitland NJ, Collins AT. CD133, a novel marker for human prostatic epithe-

lial stem cells. *J Cell Sci* 2004;117:3539-45.

[19] Calabrese C, Poppleton H, Kocak M, Hogg TL, Fuller C, Hamner B, *et al.* A perivascular niche for brain tumor stem cells. *Cancer Cell* 2007; 11: 69-82.

[20] Yang ZJ, Wechsler-Reya RJ. Hit′ em where they live: targeting the cancer stem cell niche. *Cancer Cell* 2007; 11: 3-5.

[21] Mar í a PC , Isidro SG. Killing time for cancer stem cells (CSC): Discovery and development of selective CSC inhibitors. *Curr Med Chem* 2006; 13: 1719-25.

[22] Piscaglia AC, Shupe DT, Gasbarrini A, Petersen BE. Microarray RNA/DNA in different stem cell lines. *Curr Pharm Biotechnol* 2007; 8:167-75.