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ORIGINAL ARTICLE

Aberrant expression of CD13 and stem cell markers in CD133-induced liver cancer in mice

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Summary

Purpose: *The purpose of the study is to identify the Cancer Stem Cells (CSCs) and to determine their expression profiles in different pathological stages of liver cancer by using multiple markers.*

Methods: *In this study, the expression profiles of CD133 and CD13, along with those of stem cell markers Oct4 and SOX2, were analyzed using immunohistochemistry and immunoblotting to clarify the character of CSCs in different stages of liver cancer.*

Results: *CD133 liver cancer cells were injected into mice, and the tissues were processed for histology. The histological data revealed the progression of liver cancer. Immunohistochemical analysis showed the strong expression of CD133 in metastatic cancer. In contrast, the expression of CD13 in both primary and metastatic liver cancer was found to be very strong. Interestingly, the expression levels of Oct4 and SOX2 were found to be upregulated in primary tumors, but, in the metastatic stage, their expression was downregulated. The immunoblot analysis also confirmed the same result.*

Conclusions: *Our findings demonstrate that tumor-suppressor proteins Oct4 and SOX2 have a prominent expression profile in the primary stage of cancer, but, in the metastatic stage, their expression is downregulated, leading to the failure to prevent metastatic cancer.*

Key words: *cancer stem cell, CD133, CD13, Oct4, SOX2, metastatic*

Introduction

The liver, which is an important organ in the human system, participates in numerous chemical events responsible for the survival of the body. It plays a vital role in the storage of nutrients and is involved in several processes, including digestion, metabolism, and detoxification [1]. Diseases in the liver can arise from viral infections, alcohol consumption, and other phenomena that result in injury to this organ [2]. Liver cancer is the most commonly diagnosed tumor, and it is the fifth most common cancer worldwide [3]. Many reports have shown that men are more susceptible to liver cancer than women [4].

During the early stages of liver cancer, patients may undergo liver transplantation or surgical resection. Many reports show that it is complicate to treat liver cancer surgically, as most liver cancers are diagnosed at advanced stages and recurrence is highly prevalent. Similar to other types of cancers, liver cancer involves cancer stem cells (CSCs), which are primarily responsible for recurrence [5].

CSCs have the capability of self-renewal and differentiation and are often more resistant than other cell types to radiation and anti-cancer drugs [6,7]. It has been demonstrated that CSCs are pre-

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sent in various tumors, such as those in the prostate, brain, breast, colon, and pancreas [5,8,9]. Although the existence of CSCs was hypothesized 50 years ago, their occurrence, differentiation, and molecular activities were illustrated only during recent decades [10,11].

It is essential to differentiate CSCs from stem cells to comprehend the development and progression of the disease [12]. At present, there is a lack of specificity in identifying the markers related to the CSCs associated with liver cancer [13]. Recently, it has been hypothesized that CSCs can be identified using multiple markers, and these may help to differentiate CSCs from stem cells [14]. Hence, this is promising approach to clarifying the prognosis of liver cancer.

One such marker is CD133, a glycoprotein belonging to the 5-transmembrane domain family, which was identified as a cell surface marker for both stem cells and CSCs [15]. As a CSCs marker, CD133 has been recognized in the colon [16,17], brain [18,19] and prostate [20]. Apart from this, CD133 was also observed in endothelial progenitor cells of hepatocellular carcinoma [21]. Another marker, CD13, an aminopeptidase N glycoprotein, was also identified as a cell surface marker, but its expression was detected even in the semi-quiescent state of CSCs in human liver cancer cells [22]. In this context, it is clear that different markers are necessary for the specific identification of CSCs.

The TGF-beta receptor type II (TBRII), OCT4, and SOX2 genes play vital roles in maintaining the pluripotency and determining the fate of CSCs [23-25]. Hence, we used the aberrant expression of CD13, along with stem cell markers, such as OCT4 and SOX2, to identify the CSCs of liver cancer by inducing CD133 in a mouse model.

Methods

Animal model of liver cancer

The experiments carried out using the mouse model were approved by the Institutional Animal and Ethical Committee Boards. This experiment used the female athymic BALB/c mouse strain. Liver cancer cells with CD133 were injected into the mice to generate cancer. CD133 cells were sorted by flow cytometer. The animals were anesthetized, and the liver was located by making small incision at the right side of the abdomen. CD133-positive liver cancer cells (10^4 cells/20µl) were injected into the liver, and the cavity was closed after injection using stitches. After injection, the mice were monitored for the formation of liver palpation. During the 2nd week, one set of mice was sacrificed and observed for primary tumor formation, whereas the second set was incubated for up to 7 weeks so that metastatic liver cancer could develop.

Immunohistochemistry

The liver tissues were dissected, fixed in 10% formalin and processed further for paraffin embedding using a standard protocol. Thin tissue sections (4 µm) were deparaffinized using xylene and hydrated; 10% H2O2 was used to block the endogenous peroxidase activity. The tissue sections were blocked with serum to stop non-specific staining. The processed sections were incubated separately with primary antibodies, such as anti-CD133, anti-CD13, anti-Oct4, and anti-SOX at 4°C overnight. Then, the sections were washed using phosphate buffered saline (PBS); this was followed by incubation using a secondary antibody. The slides were then developed using DAB (3,3'-diaminobenzidine) and observed under a microscope.

Immunoblot analysis

Tissue lysate was prepared from normal mouse which was not infected with liver cancer cells (control) as well as from both the primary and metastatic forms of the liver cancer tissues. The proteins were determined

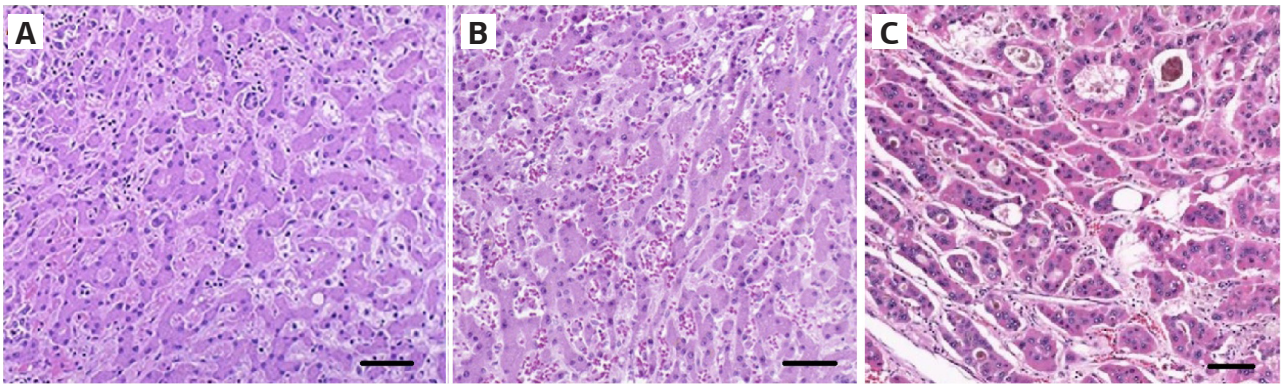

Figure 1. Histological analysis of normal and liver cancer tissues. **A:** Histology of normal liver section of mice showing an identical pattern of cells. **B:** Primary liver cancer tissue sections illustrating an irregular pattern of cell arrangement. **C:** Metastatic liver cancer tissue sections with abnormal proliferation of cells. Hematoxylin and eosin staining, original magnification x4.

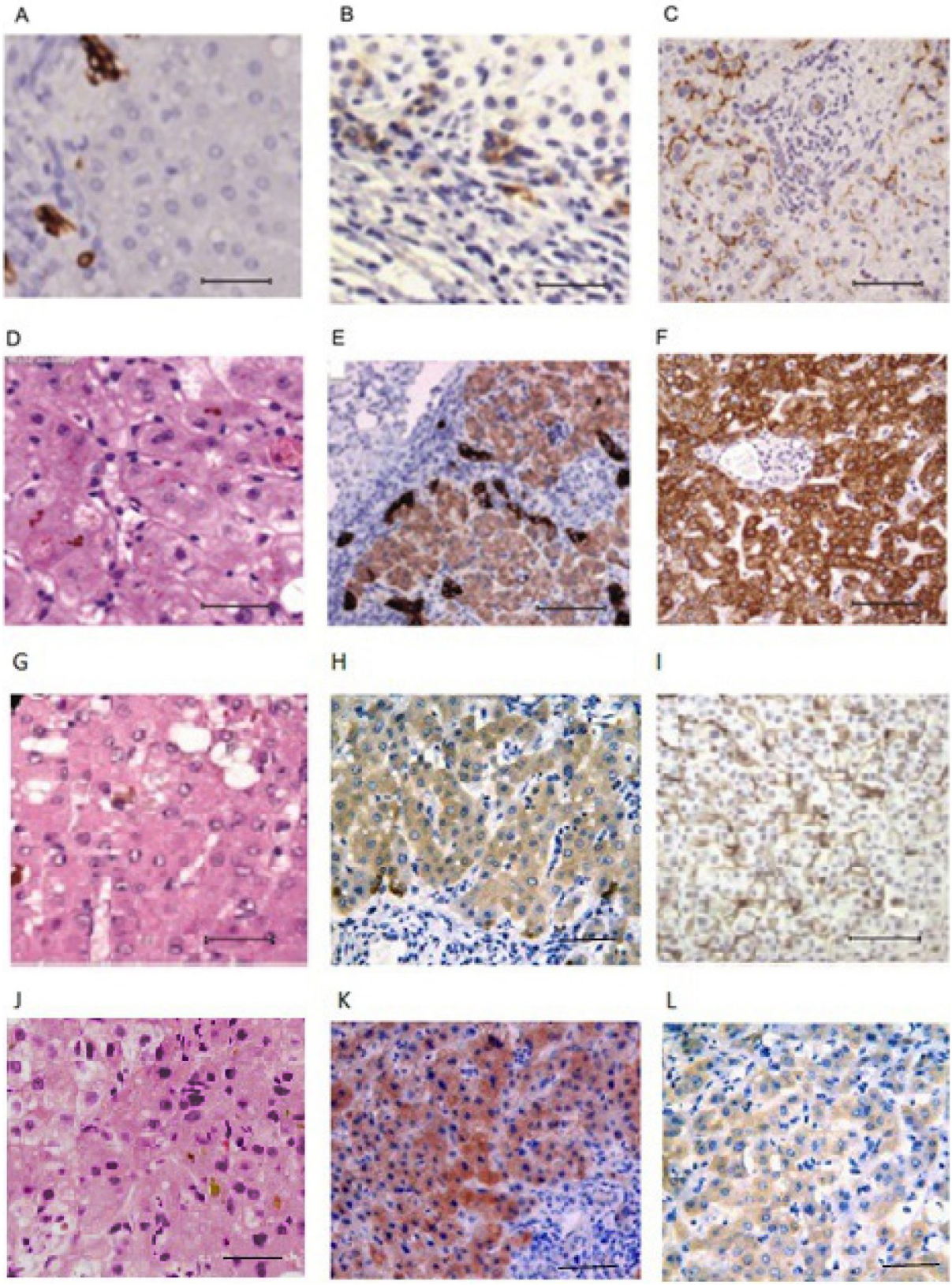

Figure 2. Expression profile of CD133, CD13, Oct4 and SOX2 in normal and liver cancer tissues using immunohistochemistry. **A:** Expression of CD133 in normal liver tissues of mice; **B:** CD133 expression in primary liver cancer tissues of mice; **C:** CD133 expression in metastatic liver cancer tissues of mice; **D:** CD13 expression in normal liver tissues of mice. **E:** CD13 expression in primary liver cancer tissues of mice; **F:** CD13 expression in metastatic liver cancer tissues of mice; **G:** Oct4 expression in normal liver tissues of mice; **H:** Oct4 expression in primary liver cancer tissues of mice; **I:** Oct4 expression in metastatic liver cancer tissues of mice; **J:** SOX2 expression in normal liver tissues of mice; **K:** Expression of SOX2 in primary liver cancer tissues of mice; **L:** Expression of SOX2 in metastatic liver cancer tissues of mice. Original magnification x10.

using 12% SDS-PAGE. The gel was then transferred to a nitrocellulose membrane, and this was followed by blocking using 2% bovine serum albumin (BSA) (Cat. No. 05470, Sigma-Aldrich). The membrane was further incubated with anti-CD133 (Cat. No. MAB4310), anti-CD13 (Cat. No. SAB5500037), anti-Oct4 (Cat. No. P0056), and anti-SOX2 (Cat. No. S9072) primary antibodies. The non-specific binding was washed, followed by incubation using a secondary antibody. The slides were developed, and the signals obtained were visualized using DAB (Cat. No. D8001, Sigma-Aldrich). Anti-β actin antibody was used as loading control (Cat. No. A1978, Sigma-Aldrich).

Statistics

Statistical analyses were performed using SPSS for Windows 11.0 (SPSS Inc., Chicago, IL, USA). The experiments were carried out in triplicate. The intensity of the bands, ascertained via immunoblot, was analyzed by scanning in a gel documentation system (Bio-Rad, USA). Statistical significance was evaluated using t-test of the mean intensity of the immunoblot as determined by Bio-Rad imaging software; the level of significance was set at $p<0.05$.

Results

Progression of liver cancer in mice using CD133

In this study, liver cancer was effectively initiated by inducing tumorigenic CD133 in a mouse model. The mice responded well and developed primary tumors in the 2nd week and metastatic liver cancer in the 7th week; these were histologically analyzed and compared to the control (Figure 1). The control sections showed an even cellular arrangement (Figure 1A), whereas the primary and metastatic liver cancer tissues showed an uneven arrangement of cells (Figure 1B,C). Compared to primary liver cancer tissues (Figure 1B), metastatic cancer tissues showed large, proliferative cell masses in a patchy and clumsy arrangement (Figure 1C).

Expression of CD133 and CD13 in primary and metastatic liver cancer

Immunohistochemistry analyses of liver tissues with anti-CD133 and anti-CD13 antibodies are shown in Figure 2A-F. As illustrated in Figure 2A,D both CD133 and CD13 showed low levels of expression in normal tissues. The expression levels of both CD133 and CD13 were upregulated as a function of cancer progression. CD133 was evident in primary liver cancer tissues (Figure 2B), whereas the signals were very strong in metastatic tissues, denoting abnormal expression of CD133 (Figure 2C). On the other hand, the expression of CD13 was strong in primary liver cancer (Figue 2E) compared with that of CD133. In the case of metastatic cancer, aberrant expression with very strong signals was observed from the surroundings of the proliferative cell mass (Figure 2F).

Expression of Oct4 and SOX2 in primary and metastatic liver cancer

The immunohistochemistry analysis of Oct4 and SOX2 genes is illustrated in Figure 2G-L. Both OCT4 and SOX2 were dispersed in the sections of tissue from control mice (Figure 2G,J). In the case of primary liver cancer, the expression levels of Oct4 and SOX2 were high (Figure 2H,K). This implies

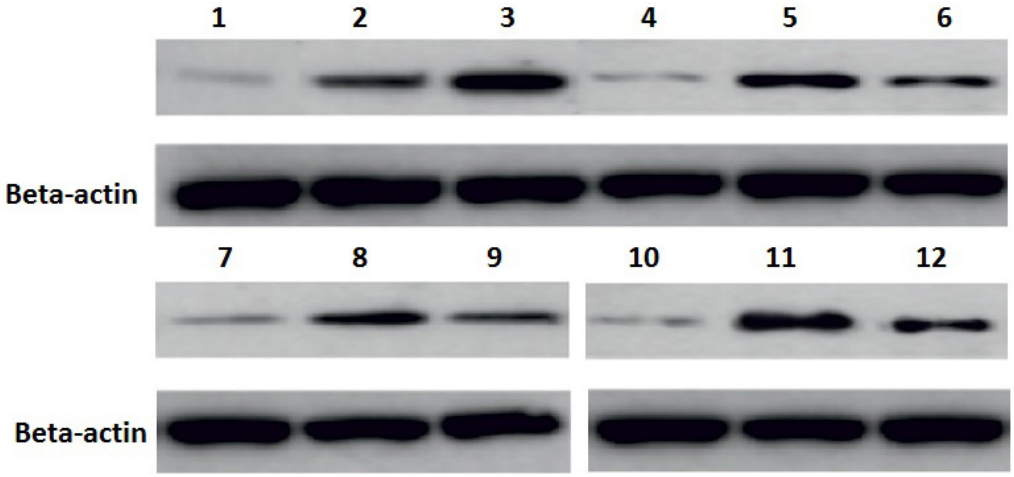

Figure 3. Immunoblot analysis. Lane 1. Expression of CD133 in normal liver tissues; Lane 2. CD133 expression in primary liver cancer tissues; Lane 3. CD133 expression in metastatic liver cancer tissues; Lane 4. CD13 expression in normal liver tissues; Lane 5. CD13 expression in primary liver cancer tissues; Lane 6. CD13 expression in metastatic liver cancer tissues; Lane 7. Expression of Oct4 in normal liver tissues; Lane 8. Oct4 expression in primary liver cancer tissues; Lane 9. Oct4 expression in metastatic liver cancer tissues; Lane 10. SOX2 expression in normal liver tissues; Lane 11. SOX2 expression in primary liver cancer tissues; Lane 12. SOX2 expression in metastatic liver cancer tissues. β actin – loading control.

that both these stem cell markers activated the repair process once the cancer progression started. Unfortunately, in metastatic liver cancer, the expression levels of both Oct4 and SOX2 were down regulated (Figure 2I,L), which indicated that the CSC marker CD133 was expressed and proliferated vigorously, thereby suppressing the expression of stem cell markers Oct4 and SOX2.

Immunoblot analysis

To confirm the expression of CD133, CD13, OCT4, and SOX2, an immunoblot analysis was performed, and these data are presented in Figure 3. The expression levels of CD13 and CD133 were high in primary and metastatic liver cancer compared with normal tissues. However, the expression levels of OCT4 and SOX2 were high in primary liver cancer but downregulated in metastatic cancer. Both the immunohistochemistry and the immunoblot analysis confirmed the aberrant expression of the CSC markers CD13 and CD133, which, in turn downregulates the stem cell markers Oct4 and SOX2.

Discussion

Liver cancer has a high mortality rate; hence, it is essential to understand the pathological nature of this disease [26]. High mortality and recurrence rates are also associated with the difficulty in diagnosing liver cancer in its early stages [27]. In this study, CD133 cells were successfully injected into mice, and we performed a histological analysis of affected tissues to determine its morphology, which resembles that of humans [28]. The injected mice developed both primary and metastatic cancer characterized by anomalous masses of cells.

The expression profiles of OCT4 and SOX2 were analyzed and compared with that of CD133. This analysis elucidated the characteristic features of CSCs that help understand how Oct4 and SOX2 control liver cancer. As soon as the tumor formed, the stem cell markers OCT4 and SOX2 were steeply elevated to try to safeguard the tissues from cancer progression by repairing them. However, the expression levels of CSC markers CD13 and CD133 were abnormal and, because of the proliferation and expression of Oct4 and SOX2, were downregulated, leading to the failure to control the metastatic cancer.

This study determined that CD13 and CD133 are responsible for metastatic cancer and recurrence and indicated that they can be used as prognostic tools for diagnosing liver cancer. Our results also coincide with the findings of Dong and Jiao, who found that the expression levels of stem cell markers TBRII and ELF were elevated in the primary tumor but downregulated in the metastatic stage [23]. The molecular mechanism of Oct4 and SOX2 has been elucidated, and these two genes have specific effects on transcription factors and are associated with apoptosis [24,25]. In contrast, the functions of CD133 and CD13 were similar: their expression triggered the proliferative ability of CSCs, thereby inducing cancer [8,15]. However, the expression of CD13 was slightly different from that of CD133. Indeed, CD13 was strongly expressed in semi-quiescent CSCs [22] and, hence, the expression of CD13 was high compared with that of CD133, even in the primary stage of liver cancer. It is well known that CSCs remain dormant in the quiescent stage, but the CD13 interrupts such quiescence and thereby paves the way for cancer.

This study clarified the roles of OCT4 and SOX2 in preventing liver cancer in the primary stage; however, as the cancer progressed, CD133 and CD13 showed abnormal expression by downregulating Oct4 and SOX2, leading to the formation of liver cancer. However, the molecular mechanisms underpinning this expression profile should be studied in detail. Our results will provide the foundation for identifying prognostic markers for use in the treatment of liver cancer.

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Conflict of interests

The authors declare no conflict of interests.

References

1. Gao B, Jeong WI, Tian Z. Liver: an organ with predominant innate immunity. Hepatol 2008;47:729-36.
2. Farazi PA, DePinho RA. Hepatocellular carcinoma pathogenesis: from genes to environment. Nat Rev Cancer 2006;6:674.

3. Bosch FX, Ribes J, Díaz M, Cléries R. Primary liver cancer: worldwide incidence and trends. Gastroenterology 2004;127:S5-S16.
4. Bruix J, Boix L, Sala M, Llovet JM. Focus on hepatocellular carcinoma. Cancer Cell 2004;5: 215-9.
5. Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer 2008;8:755.
6. Ma S, Lee T, Zheng B, Chan K, Guan X. CD133+ HCC cancer stem cells confer chemoresistance by preferential expression of the Akt/PKB survival pathway. Oncogene 2008;27:1749.
7. Chen Y, Yu D, Zhang H et al. CD133+ EpCAM+ phenotype possesses more characteristics of tumor initiating cells in hepatocellular carcinoma Huh7 Cells. Int J Biol Sci 2012;8:992.
8. Zhu Z, Hao X, Yan M et al. Cancer stem/progenitor cells are highly enriched in CD133+CD44+ population in hepatocellular carcinoma. Int J Cancer 2010;126:2067-78.
9. Ma S, Chan KW, Hu L et al. Identification and characterization of tumorigenic liver cancer stem/progenitor cells. Gastroenterology 2007;132:2542-56.
10. Salsbury A. The significance of the circulating cancer cell. Cancer Treat Rev 1975:2:55-72.
11. Morrison SJ, Kimble J. Asymmetric and symmetric stem-cell divisions in development and cancer. Nature 2006;441:1068.
12. Walkup MH, Gerber DA. Hepatic stem cells: in search of. Stem Cells 2006;24:1833-40.
13. Ludwig JA, Weinstein JN. Biomarkers in cancer staging, prognosis and treatment selection. Nat Rev Cancer 2005;5:845.
14. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 2003;100:3983-8.
15. Yin AH, Miraglia S, Zanjani ED et al. AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood 1997;90:5002-12.
16. O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 2007;445:106.
17. Ricci-Vitiani L, Lombardi DG, Pilozzi E et al. Identification and expansion of human colon-cancer-initiating cells. Nature 2007;445:111.
18. Singh SK, Clarke ID, Terasaki M et al. Identification of a cancer stem cell in human brain tumors. Cancer Res 2003;63:5821-8.
19. Hemmati HD, Nakano I, Lazareff JA et al. Cancerous stem cells can arise from pediatric brain tumors. Proc Natl Acad Sci USA 2003;100:15178-83.
20. Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res 2005;65:10946-51.
21. Ho JW, Pang RW, Lau C et al. Significance of circulating endothelial progenitor cells in hepatocellular carcinoma. Hepatology 2006;44:836-43.
22. Haraguchi N, Ishii H, Mimori K et al. CD13 is a therapeutic target in human liver cancer stem cells. J Clin Investig 2010;120:3326-39.
23. Dong Y, Jiao S. CD133 induced tumorigenicity mice express TGF-beta receptor type II (TBRII) and embryonic liver fodrin (ELF) along with stem cell markers. Eur Rev Med Pharmacol Sci 2016; 20: 2296-2301.
24. Karwacki-Neisius V, Göke J, Osorno R et al. Reduced Oct4 expression directs a robust pluripotent state with distinct signaling activity and increased enhancer occupancy by Oct4 and Nanog. Cell Stem Cell 2013; 12: 531-45.
25. Jeong CH, Cho YY, Kim MO et al. Phosphorylation of Sox2 cooperates in reprogramming to pluripotent stem cells. Stem cells 2010;28:2141-50.
26. Leenders MW, Nijkamp MW, Rinkes IHB. Mouse models in liver cancer research: a review of current literature. World J Gastroenterol 2008;14:6915.
27. Heindryckx F, Colle I, Van Vlierberghe H. Experimental mouse models for hepatocellular carcinoma research. Int J Clin Exp Pathol 2009;90:367-86.
28. Kelland L. "Of mice and men": values and liabilities of the athymic nude mouse model in anticancer drug development. Eur J Cancer 2004;40:827-36.