

Upregulated CD133 expression in tumorigenesis of colon cancer cells

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Author contributions: Yang ZL analyzed the CD133 expression in a panel of colon cancer cell lines and spheroid culture and drafted the manuscript; Zheng Q, Yan J and Pan Y participated in the study design and performed the RT-qPCR analysis; Wang ZG conceived the study and revised the manuscript.

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Received: August 7, 2010 Revised: November 12, 2010

Accepted: November 19, 2010

Published online: February 21, 2011

Abstract

AIM: To analyze the upregulated CD133 expression in tumorigenesis of primary colon cancer cells.

METHODS: Upregulated CD133 expression in tumorigenesis of colorectal cancer cell lines (Lovo, Colo205, Caco-2, HCT116 and SW620) was analyzed by flow cytometry. Human colon cancer tissue samples were stained with anti-human CD133. SW620 cells were sorted according to the CD133 expression level measured by fluorescence-activated cell sorting. Spheroids of colorectal cancer cells were cultured with the hanging drop. Expression of CD133 and Lgr5 in spheroids of colorectal cancer cells and monolayer culture was detected by RT-qPCR. Spheroids of colorectal cancer cells were analyzed using anti-human CD133 with immunohistochemical staining.

RESULTS: CD133 antigen was expressed in colorectal cancer cell lines (Lovo, Colo205, Caco-2, HCT116 and SW620) as well as in primary and metastatic human colon cancer tissues. However, the CD133 was differently expressed in these cell lines and tissues. The expression levels of CD133 and Lgr5 were significantly

higher in spheroids of parental, CD133^{hi} and CD133⁺ cells than in their monolayer culture at the mRNA level ($P < 0.05$). Immunohistochemical staining of spheroids of CD133⁺ cells showed that CD133 was highly expressed in colorectal cancer cell lines.

CONCLUSION: Upregulated CD133 expression plays a role in tumorigenesis colorectal cancer cells, which may promote the expression of other critical genes that can drive tumorigenesis.

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Key words: CD133; Colon cancer cells; Tumorigenesis; Cancer stem cells

Peer reviewer: Ioannis Kanellos, Professor, 4th Surgical Department, Aristotle University of Thessaloniki, Antheon 1, Pano-rama, Thessaloniki 55236, Greece

Yang ZL, Zheng Q, Yan J, Pan Y, Wang ZG. Upregulated CD133 expression in tumorigenesis of colon cancer cells. *World J Gastroenterol* 2011; 17(7): 932-937 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i7/932.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i7.932>

INTRODUCTION

CD133, also known as prominin-1, a transmembrane pentaspan protein, is originally described as a surface antigen specific for human hematopoietic stem and progenitor cells^[1,2]. Later, CD133 is recognized as a stem cell marker for other normal tissues of brain^[3], kidney^[4], prostate^[5], liver^[6], pancreas^[7], and skin^[8]. It has been increasingly reported that CD133 is a marker of putative cancer stem cells (CSC) in brain tumor^[9,10], prostate cancer^[11], colon cancer^[12-14], lung cancer^[15], hepatocellular carcinoma^[16], melanoma^[17], ovarian cancer^[18], and pancreatic cancer^[19]. Accordingly, CD133 has been referred to as "the molecule of the moment"^[20].

It has been recently shown that CD133 expression is broadly distributed in primary colon cancer cells including cancer stem cells, both CD133⁺ and CD133⁻ metastatic colon cancer cells initiate tumors^[21-23]. However, whether CD133 expression plays a role in tumorigenesis of colorectal cancer cells is unknown.

In the present study, upregulated CD133 expression in several colorectal cancer cell lines as well as in human primary and metastatic colon cancer tissue samples was analyzed. SW620 cell line was sorted using CD133 antigen. Spheroids of parental, CD133⁻ and CD133^{hi} cells were cultured with the hanging drop. Expressions of CD133 and Lgr5 were detected in spheroids of colorectal cancer cells. CD133 was widely expressed in human colorectal cancer cell lines as well as in primary and metastatic colon cancer tissues and upregulated CD133 expression was detected in spheroids of colorectal cancer cells, indicating that upregulated CD133 expression may promote the expression of other critical genes that can drive tumorigenesis.

MATERIALS AND METHODS

Cell lines and cell culture and tissue samples

Human colorectal cancer cell lines (Lovo, Colo205, Caco-2, HCT116 and SW620) were cultured in RPMI1640 medium containing 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 10 μ mol/L thioglycerol, 12.5 U insulin, 0.5 mg hydrocortisone, and 30mg penicillin G/0.05 g streptomycin. Colorectal cancer cells were cultured at 37°C in a humidified atmosphere containing 10% CO₂. CD133 expression was detected in formalin-fixed, paraffin-embedded primary and metastatic colorectal cancer tissue samples from Affiliated Sixth People's Hospital of Shanghai Jiaotong University. The study was approved by the Ethics Committee of Affiliated Sixth People's Hospital of Shanghai Jiaotong University.

Fluorescence-activated cell sorting

Single-cell suspensions were stained with antibodies against human CD133 (AC133, 1:40) and human CD133/1 and CD133/2(1:10, APC conjugated, Miltenyi Biotech, Germany). Dead cells, cell debris, doublets and aggregates were excluded by forward and side scattering and pulse-width gating. Colorectal cancer cells (1×10^5) were stained in an eppendorf tube. Primary antibody was incubated for 45 min on ice and second antibody (anti-mouse Alexa488, 1:400) was incubated for 30 min on ice in the dark. Flow cytometry analysis was carried out on a fluorescence-activated cell sorting (FACS) caliber (BD). Colorectal cancer cells (1×10^6) were prepared for sorting, stained with human CD133/1 (1:10, APC conjugated, Miltenyi Biotech) and 1 μ g/mL propidium iodide (PI) to exclude dead cells during sorting. The cells were sorted using FACS Aria (BD). Matched isotype antibodies were applied in parallel as controls.

Colon spheroids were culture with hanging drop

SW620 colorectal cancer cells and their sorted CD133⁻ and CD133^{hi} cells were prepared as a single cell suspension. The cells were counted and diluted in RPMI1640

containing 20% FBS and antibiotics to a concentration of 500 cells per 20 μ L/drop in a sterile basin. The lid was lifted, inverted and placed on top of the dish containing 10 mL PBS. An 8-channel pipette was used to make rows of 20 μ L drops on the up-turned inner surface of the tissue culture dish lid. The drops were incubated at 37°C in an atmosphere containing 10% CO₂ for 10 d.

Immunohistochemistry

Frozen sections of the spheroids of colorectal cancer cells were fixed in acetone at -20°C for 10 min and rehydrated in PBS. Endogenous peroxidase was inactivated by immersing the sections in 0.3% hydrogen peroxide for 20 min. The primary antibody for frozen sections of the spheroids of colorectal cancer cells and paraffin-embedded sections of colorectal cancer tissue samples was a mouse anti-human monoclonal CD133/2 (1:40, Miltenyi Biotech, Germany) and a rabbit anti-human polyclonal CD133 (1:100, Abcam, England), respectively. The sections were incubated overnight at 4°C in a humidified chamber, then with biotinylated secondary antibody (VECTASTAIN ABC kit, Vector Laboratories) for 30 min at room temperature. Each section was incubated with the VECTASTAIN ABC reagent for 30 min at room temperature. The sections were developed using the DAB (Vector Laboratories) as the substrate and then counterstained with hematoxylin. The negative control was performed by incubating samples with PBS.

Quantification of CD133 expression by quantitative polymerase chain reaction

Total RNA was isolated from cultured colorectal cancer cells and their spheroids using the RNeasy extraction kit (GE Healthcare) and reverse transcribed using high-capacity cDNA reverse transcription kit (Applied Biosystems) according to their manufacturer's instructions, respectively. Relative quantitative polymerase chain reaction (PCR) was performed on a 7300 fast real-time PCR system (Applied Biosystems) using SYBR green PCR master mix (Applied Biosystems). The human-specific intron spanning primer pairs for CD133 were provided by QIAGEN (Catalog number: QT00075586). The sequences of primer pairs used for GAPDH and Lgr5 are CAATGACCCCTTCATTGACC (forward) and TGATGACAAGCTTCCCGTTC (reverse), and CTTTCCCGCAACCTCAGCGTCTTC (forward) and TTTCCCGCAAGACGTA ACTC (reverse), respectively. PCR was performed for 1 cycle at 50°C for 2 min and 1 cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Specificity of PCR products was tested according to the dissociation curves. Relative values of transcripts were calculated using the equation: $2^{-\Delta\Delta Ct}$, where ΔCt is equal to the difference in threshold cycles for target and reference.

Statistical analysis

Results were expressed as mean \pm SD for three repeated individual experiments in each group. Statistical analyses were conducted using the SPSS software (version 10.0).

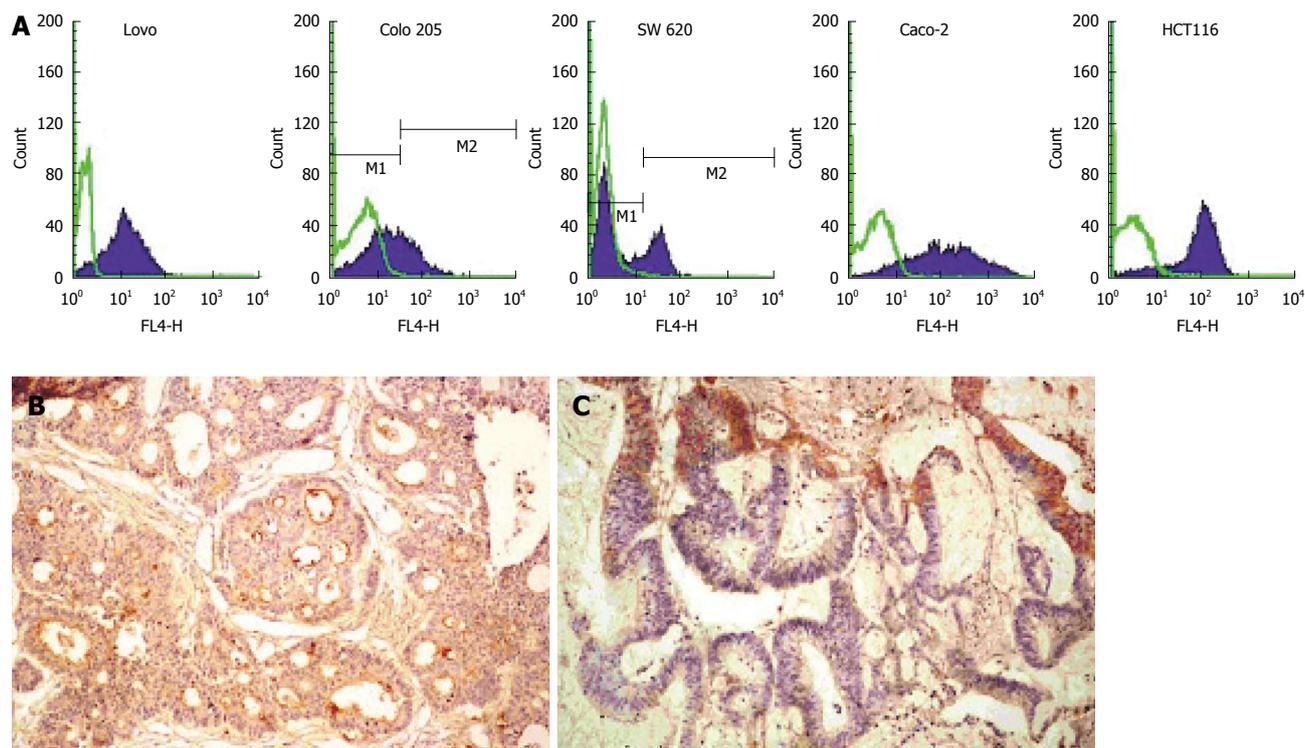


Figure 1 Fluorescence-activated cell sorting showing CD133 expression in different colorectal cancer cell lines (A), CD133 staining of human primary colorectal cancer tissue (B) and metastatic colorectal cancer tissue (C) (Original magnification $\times 100$). Brown indicates positive staining.

Correlation between sample groups and molecular variables was assayed with paired *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

CD133 expression in colon cancer cell lines and human colon cancer tissues

CD133 antigen was expressed in all colorectal cancer cell lines with a difference of 30%-95% (Figure 1A). CD133 in human colorectal cancer tissue samples was stained with polyclonal antibody. CD133 expression was detected in 18 of the 20 primary cancer tissue samples, exclusively on the membrane of the vast majority of colorectal cancer gland cells (Figure 1B), and in 9 of the 10 metastatic colorectal cancer tissue samples with positive staining in cytoplasm of cancer cells (Figure 1C).

CD133 expression in spheroids of sorted colorectal cancer cell subpopulations

To minimize the contamination between the sorted CD133⁺ and CD133⁻ cells, a high CD133 expression cell subpopulation (CD133^{hi}) and a CD133⁻ cell subpopulation sorted from the SW620 cells could be persistently passed. CD133 antigen was stably expressed in the monolayer culture (Figure 2A). To mimic the tumorigenesis of colorectal cancer cells *in vivo*, spheroids of the sorted cells were cultured with hanging drop. The parental, CD133^{hi} and CD133⁻ cells could grow into spheroids. CD133 expression was upregulated in spheroids of CD133⁻ cells. Although the CD133 expression rate was not changed,

the mean fluorescence intensity (MFI) was significantly increased in spheroids of CD133^{hi} cells, and the CD133 expression rate and MFI were significantly increased in spheroids of parental cells detected by FACS assay (Figure 2B). Immunohistochemical staining of CD133 antigen was observed in spheroids of CD133⁻ cells (Figure 2C). The CD133 gene expression level was significantly higher in spheroids of SW620, CD133^{hi} and CD133⁻ cells than in their monolayer culture at the mRNA level (4.224 ± 0.063 vs 2.680 ± 0.117 , 3.653 ± 0.061 vs 1.325 ± 0.044 , 8.746 ± 0.029 vs 3.761 ± 0.065 , $P < 0.05$) (Figure 2D).

Lgr5 expression in spheroids of sorted colorectal cancer cell subpopulations

Lgr5 expression was analyzed by RT-qPCR in order to observe the role of the expression of other colon stem cell genes in tumorigenesis of colorectal cancer cells. The results showed that the Lgr5 expression level was significantly higher in spheroids of parental, CD133^{hi} and CD133⁻ cells than in their monolayer cells (5.942 ± 0.091 vs 4.003 ± 0.039 , 6.611 ± 0.214 vs 3.645 ± 0.046 , 5.910 ± 0.035 vs 3.903 ± 0.083 , $P < 0.05$) (Figure 3).

DISCUSSION

Whether CD133 antigen can be used as a marker of colorectal cancer stem cells is still controversial. The focus is that CD133 expression is not restricted to just a small number of colorectal cancer cells. In this study, the CD133 expression was upregulated in colorectal cancer cell lines and primary or metastatic colorectal cancer tissue

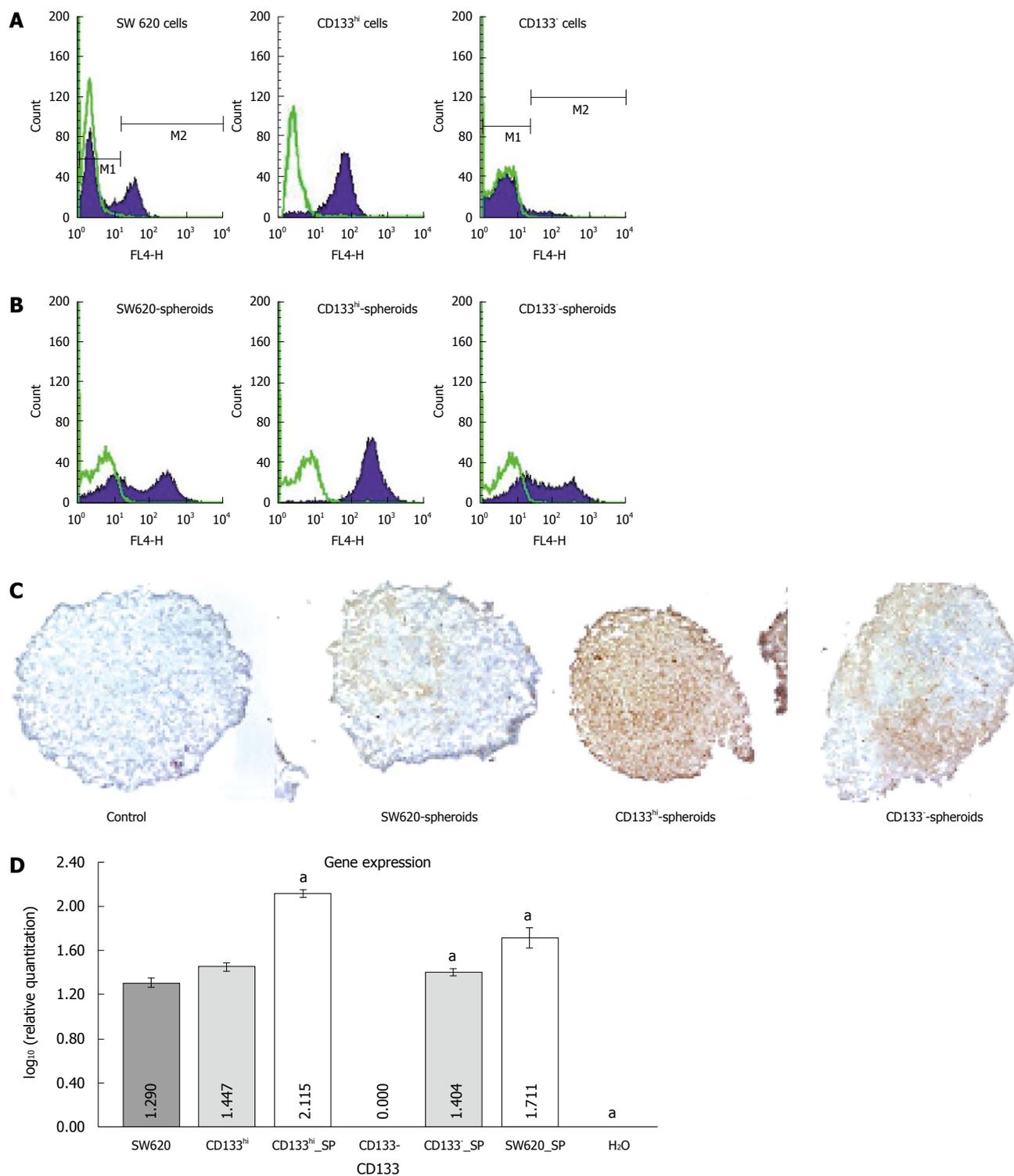


Figure 2 Fluorescence-activated cell sorting showing CD133 expression in SW620, CD133⁻ and CD133^{hi} cells (A) and in their spheroids (B), CD133 staining in spheroids of SW620, CD133⁻ and CD133^{hi} cells (original magnification × 100, brown indicates positive staining) (C), and reverse transcription-polymerase chain reaction showing CD133 expression in SW620, CD133⁻ and CD133^{hi} cells and their spheroids. ^aP < 0.05 vs monolayer cells. SP: Spheroid.

samples, showing that CD133 antigen can be expressed in colorectal cancer cell lines with a difference of 30%-95%. CD133 expression was detected in 18 of the 20 primary colorectal cancer tissue samples, exclusively on the membrane of a large number of colorectal cancer gland cells, and in 9 of the 10 metastatic colorectal cancer tissue samples with a positive staining in cytoplasm of colorec-

tal cancer cells, which is consistent with the reported findings^[21-23]. The different CD133 expression levels in colorectal cancer cell lines may be related to the different glycosylation to the mask specific epitopes of CD133 antigen in colorectal cancer cell differentiation^[24]. Therefore, our data indicate that CD133 is commonly expressed in colorectal cancer cells.

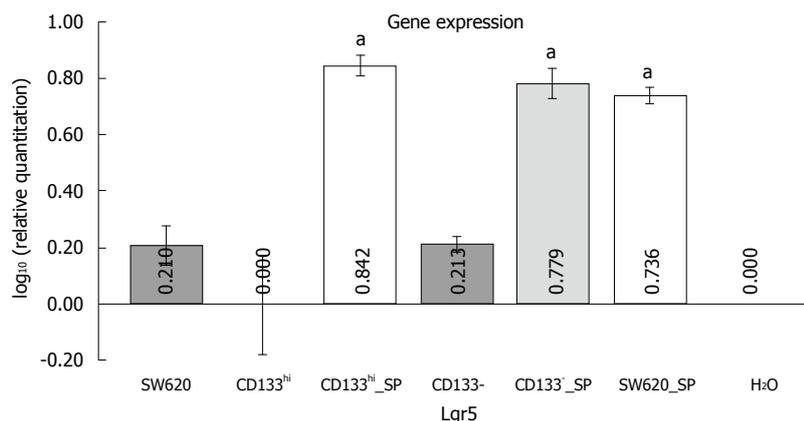


Figure 3 Quantitative reverse transcription-polymerase chain reaction showing Lgr5 expression in SW620, CD133^{hi} and CD133^{hi} cells and their spheroids. ^aP < 0.05 vs monolayer cells. SP: Spheroid.

To investigate whether the upregulated CD133 expression plays a role in tumorigenesis of colorectal cancer cells, SW620 cell line containing two cell subpopulations (CD133^{hi}, CD133⁻) was selected and sorted using CD133 antigen, the spheroids of parental, CD133^{hi} and CD133⁻ cells were cultured with the hanging drop *in vitro*, which is based on the natural disposition of cells to aggregate without the need for polymer scaffolds such as matrigel, polyglycolic acid or microporous supports to achieve homogeneous multicellular tumor spheroids^[25]. The spheroids represent a popular *in vitro* 3D tissue structure that mimics *in vivo* tumor tissue organization and microenvironment^[26,27]. In the present study, CD133^{hi} and CD133⁻ cells could be cultured into their spheroids, CD133 expression was upregulated in spheroids of CD133⁻ cells. Although the CD133 expression was not changed, the mean fluorescence intensity (MFI) was significantly increased in spheroids of CD133^{hi} cells as detected by FACS assay. Immunohistochemical staining of CD133 antigen was observed in spheroids of CD133⁻ cells, indicating that CD133 antigen expression is upregulated in spheroids of CD133⁻ and CD133^{hi} cells. Further analysis revealed that the CD133 gene expression level was significantly higher in spheroids of SW620, CD133^{hi} and CD133⁻ cells than in their monolayer culture at the mRNA level, suggesting that the upregulated expression of CD133 including protein and gene plays a role in tumorigenesis of colorectal cancer cells.

Since the upregulated CD133 expression plays a role in tumorigenesis of colorectal cancer cells, whether CD133 protein supports the growth of colorectal cancer is a subject that should be actively studied. As CD133 by itself may lack of a functional role in initiation of tumors and metastasis of human colorectal cancer^[28,29], it has an impact on the survival of colorectal cancer patients^[22,29]. It has been recently demonstrated that prominin 1 (also called CD133)-marked mouse intestinal stem cells are susceptible to neoplastic transformation^[30], possibly due to the fact that upregulated CD133 expression may promote the expression of other critical genes that can drive tumorigenesis of colorectal cancer cells. In this study, the expression level of Lgr5 (leucine-rich-repeat-containing G-protein-coupled receptor 5), also known as Gpr49, a colon stem cell marker

gene^[31], was significantly higher in spheroids of parental, CD133^{hi} and CD133⁻ cells than in their monolayer cells.

In conclusion, the upregulated CD133 expression plays a role in tumorigenesis of colorectal cancer cells, which may be related to the expression of other critical genes that can drive tumorigenesis of colorectal cancer cells. Further study is needed to confirm the present results *in vivo*.

ACKNOWLEDGMENTS

The authors thank Francesca Walker and Hui-Hua Zhang (Ludwig Institute, Melbourne) for their assistance to cell experiments and FACS studies, and You-Fang Zhang (Ludwig Institute, Melbourne) for his support in immunohistochemical studies.

COMMENTS

Background

It has been recently shown that CD133 expression is broadly distributed in primary colorectal cancer cells, and not restricted to cancer stem cells. Whether the upregulated CD133 expression plays a role in tumorigenesis of colorectal cancer cells is unknown.

Research frontiers

It has been increasingly reported that CD133 is a marker of putative cancer stem cells (CSC) in some cancers. However, it has been recently shown that CD133 expression is broadly distributed in primary colon cancer cells and not restricted to cancer stem cells, and both CD133⁻ and CD133 metastatic colorectal cancer cells initiate tumors. Whether the upregulated CD133 expression plays a role in tumorigenesis of colorectal cancer cells is unknown. In this study, the upregulated CD133 expression was found to play a role in tumorigenesis of colorectal cancer cells.

Innovations and breakthroughs

Recent reports have shown that whether CD133 antigen can be used as a marker of colorectal cancer stem cells is controversial. This is the first study to report the role of upregulated CD133 expression in tumorigenesis of colorectal cancer cells. Furthermore, our *in vitro* studies suggested that the upregulated CD133 expression may promote the expression of other critical genes that can drive tumorigenesis of colorectal cancer cells.

Applications

Whether the upregulated CD133 expression plays a role in tumorigenesis of colorectal cancer cells was studied, the results may help to solve the controversy on CD133 antigen as a marker of colorectal cancer stem cells.

Terminology

CD133, also known as prominin-1, a transmembrane pentaspan protein, is originally described as a surface antigen specific for human hematopoietic stem

and progenitor cells. Lgr5 (leucine-rich-repeat-containing G-protein-coupled receptor 5), also known as Gpr49, is a colon stem cell marker gene.

Peer review

The authors detected the expression of CD133 in a panel of colorectal cancer cell lines and human colorectal cancer tissue samples. The expression of CD133 and Lgr5 in spheroids of the sorted colorectal cancer cell subpopulations suggests that the upregulated expression plays a role in tumorigenesis of colorectal cancer cells, which may promote the expression of other critical genes that can drive tumorigenesis. The results are interesting.

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S- Editor Sun H L- Editor Wang XL E- Editor Ma WH