Glucose transporter-1 expression in CD133+ laryngeal carcinoma Hep-2 cells

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Abstract. CD133 is a useful putative marker of cancer stem cells (CSCs) in human laryngeal tumors. Numerous studies have demonstrated that CD133+ CSCs possess higher clonogenicity, invasiveness and tumorigenesis compared with CD133- cells. Recently, interest in the Warburg effect in the microenvironment of CSCs has escalated. The Warburg effect dictates that cancer cells rely on glycolysis rather than oxidative phosphorylation under aerobic conditions. In numerous cancer cells, glucose is used mainly for the glycolytic pathway. Stem cells express high levels of glycolytic enzymes and rely mostly on glycolysis to meet their energy demands. Glucose is transported through cell membranes by glucose transporters (Glut). Studies of Glut-1 expression in CSCs are limited. In the present study, we investigated the proliferation of CD133+ Hep-2 cells and whether Glut-1 is expressed in laryngeal carcinoma CD133+ Hep-2 cells. Real-time reverse transcription-polymerase chain reaction (RT-PCR) demonstrated that the size of the CD133 product was 213 bp. Dissociation curve analysis demonstrated only the expected peaks at 82.1˚C for CD133. The mean ΔCt of CD133 expression was 10.98. Prior to isolation, the CD133+ fraction was 1.2% by fluorescence-activated cell sorting (FACS) analysis. Following isolation, the CD133+ fraction was increased to 76.1%. Successive tests also demonstrated that cells grew well following isolation. The proliferation of CD133+ and CD133- cells was not different during the first 3 days (P>0.05). From day 4, the proliferation capacity of CD133+ cells *in vitro* was higher than that of CD133- cells (P<0.05). The mean ΔCt of Glut-1 mRNA expression was 1.78 for CD133+ cells and 1.00 for CD133- cells (P<0.05). The mean Glut-1 protein values in CD133+ and CD133- Hep-2 cells relative to β-tubulin were 0.48 ± 0.02 and 0.21 ± 0.03 (μg/μl), respectively (P<0.05). In conclusion, CD133+ cells demonstrated higher proliferation. Glut-1 mRNA and protein levels were higher in CD133+ than in CD133- cells. Our results suggest that Glut-1 is important in the energy supply of laryngeal CD133+ Hep-2 cells and Glut-1 may represent a potential therapeutic target for the inhibition of the proliferation of laryngeal CSCs.

Introduction

Cancer stem cells (CSCs) have been demonstrated to play a role in laryngeal carcinoma (1-4). CD133 is a useful putative marker for CSCs in human laryngeal tumors (1-4), as well as in other types of cancer (5-8). Numerous studies have demonstrated that CD133+ CSCs possess higher clonogenicity, invasiveness and tumorigenesis compared with CD133- cells (1-8). CD133+ cells are resistant to standard chemotherapy (1,8) and radiotherapy (5-7). However, whether CD133+ CSCs have distinct metabolic programs from the bulk of tumor cells is not well established. Certain regulatory pathways, including the Wnt (9), Notch (10), Hedgehog (11) and PI3K/Akt pathways (12), have been found to be important in governing cell metabolism and energy sensing of CSCs.

Interest in the Warburg effect has escalated in recent years due to the proven utility of FDG-PET for imaging tumors in cancer patients and may be useful in disease diagnosis, staging, restaging and therapy monitoring in numerous types of cancer (13,14), and cervical metastasis of carcinoma from an unknown primary tumor (14). The Warburg effect dictates that cancer cells rely on glycolysis rather than oxidative phosphorylation under aerobic conditions. In numerous cancer cells, glucose is used mainly for the glycolytic pathway (15). Stem cells have been demonstrated to express high levels of glycolytic enzymes and rely mostly on glycolysis to meet their energy demands (16). Glucose is transported through cell membranes by glucose transporters (Glut). Numerous studies (17-20), including ours (21,22), have revealed that Glut-1 is significant in malignant glucose metabolism and that it may contribute to the increased FDG uptake. However, studies of Glut-1 expression in CSCs are limited (6,23,24). In CD133+ thyroid cancer, infantile hemangioma and embryonal

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neoplasms of the central nervous system, Glut-1 exhibited a higher expression than in CD133- cells. Thus, the role of the Warburg effect and Glut-1 in CSCs requires further study.

In our previous studies, we revealed a high Glut-1 expression in laryngeal carcinoma (25-27). We also demonstrated that antisense Glut-1 may decrease glucose uptake and inhibit the proliferation of Hep-2 cells. In the present study, we investigated the proliferation of CD133+ Hep-2 cells and whether Glut-1 is expressed in laryngeal carcinoma CD133+ Hep-2 cells.

Materials and methods

Cell culture. The laryngeal carcinoma Hep-2 cell line was purchased from the Cell Research Institute of the Chinese Academy of Sciences (Shanghai, China). Hep-2 cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Gaithersburg, MD, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 2 mM L-glutamine, 100 U/ml penicillin and 100 g/ml streptomycin at 37˚C in a 5% CO_2 atmosphere. Cells were trypsinized and harvested after reaching 80-90% confluence. The study was approved by the Ethics Committee of he First Affiliated Hospital, College of Medicine, Zhejiang University.

Detection of CD133 expression in Hep-2 cells by real-time reverse transcription-polymerase chain reaction (RT-PCR). Cells were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was extracted from cells according to the manufacturer's instructions. The concentration of total RNA was measured by ultraviolet spectrophotometry; an optical density (OD) 260/280 ratio between 1.8 and 2.0 was deemed to be acceptably pure. Reverse transcription was performed according to the manufacturer's instructions. Briefly, 1 μg of total RNA and the Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Fermentas, Burlington, Canada) in a 20 μl reaction volume consisting of 0.5 μg/μl of oligo d(T) primer, 1 μl of random primers (0.2 μg/μl) and 10 μl of DEPC-H_2O. The reaction mix was first pre-denatured at 65˚C for 10 min. Following the addition of 200 U M-MLV reverse transcriptase (Fermentas), the samples were incubated at 42˚C for 1 h and annealed at 70˚C for 10 min. The synthesized cDNA was used as a template for real-time fluorescent quantitative PCR using the fluorescent dye SYBR Green and the Eppendorf Realplex 4 real-time PCR system (Eppendorf, Hamburg, Germany). The 20 μl reaction mixture consisted of 10 μl of 2X SYBR Green, 1 μl of template, 1 μl of upstream and downstream specific primers and 8 μl of deionized water. The reaction mixture was pre-denatured at 95˚C for 2 min, followed by 40 cycles at 95˚C for 15 sec, 59˚C for 20 sec and 72˚C for 20 sec. Each primer sample was run in triplicate. The primers used were as follows: CD133-forward (F): CACTTACGGCACTCTTCACCTG; CD133-reverse (R): CCAGTCTGAGCCAAGTAGCTGTC. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal standard for data calibration (GAPDH-F: GGGTGTGAACCATGAGAAGTATG; GAPDH-R: GATGGCATGGACTGTGGTCAT). The lengths of the PCR products were 213 (CD133) and 145 bp (GAPDH).

To distinguish between specific and non-specific products and primer dimers, dissociation curve analysis was conducted immediately following amplification by continuous monitoring of the SYBR Green I fluorescence signal at temperatures between 60 and 95˚C. For calculation of differential gene expression, the $2^{-\Delta\Delta Ct}$ formula was used.

Flow cytometry (FCM) and fluorescence-activated cell sorting (FACS). Cultured cells were trypsinized using 0.25% trypsin and rinsed in phosphate-buffered saline (PBS). The cells were centrifuged at 800 x g for 5 min and resuspended in up to 500 μl PBS. Cell suspensions were incubated with phycoerythrin (PE)-conjugated CD133 antibody in the dark for 30 min at room temperature. During the reaction, vortexing was performed for 5 min. Following the reaction, the cells were rinsed with PBS and resuspended in up to 400 μl PBS. Flow analysis was performed using a FACS instrument (Becton-Dickinson, Mountain View, CA, USA). CD133+ and CD133- cells were sorted. CD133-sorted cell populations were again suspended in serum-free medium (SFM; Sigma-Aldrich, St. Louis, MO, USA). The purities of sorted CD133+ and CD133- cells were evaluated by FCM.

Proliferation assays of CD133+ Hep-2 cells using the cell counting kit-8 (CCK-8) system. Cultured CD133+ and CD133- Hep-2 cells were trypsinized using 0.25% trypsin. Cell proliferation was measured using the CCK-8 system (Beyotime, Nanjing, Jiangsu, China), according to the manufacturer's instructions. Briefly, $5x10^3$ CD133+ or CD133- Hep-2 cells were seeded into 96-well culture plates. Cells were cultured in SFM at 37˚C. Following 1-6 days, 10 μl of CCK-8 reagent was added to each well and following 2 h of incubation at 37˚C, the absorbance was measured at 450 nm, using the following formula: $OD = OD_{cell}-OD_{blank}$.

Expression of Glut-1 mRNA in CD133+ and CD133- Hep-2 cells by real-time RT-PCR. Real-time RT-PCR was performed as described previously. Briefly, CD133+ and CD133- Hep-2 cells were homogenized in TRIzol reagent (Invitrogen). Total RNA was extracted from cells according to the manufacturer's instructions. Using 1 μg of total RNA and MMLV in a 20 μl reaction volume, the reaction mix was first pre-denatured at 65˚C for 10 min. Following the addition of 200 U MMLV, the samples were incubated at 42˚C for 1 h and annealed at 70˚C for 10 min. The synthesized cDNA was used as a template for real-time fluorescent quantitative PCR. The 20 μl reaction mixture consisted of 10 μl of 2X SYBR Green, 1 μl of template, 1 μl of upstream and downstream specific primers and 8 μl of deionized water. The reaction mixture was pre-denatured at 95˚C for 2 min, followed by 40 cycles at 95˚C for 15 sec, 59˚C for 20 sec and 72˚C for 20 sec. Experiments were performed in triplicate and were repeated at least twice independently. The primers used were as follows: Glut-1-forward (F): CCGCAACGAGGAGAACCG; Glut-1-reverse: GTGACCTTCTTCTCCCGCATC. GAPDH was used as an internal standard for data calibration (GAPDH-F: GGGTGTGAACCATGAGAAGTATG; GAPDH-R: GATGGCATGGACTGTGGTCAT). Dissociation curve analysis was conducted. For calculation of differential gene expression, the $2^{-\Delta\Delta Ct}$ formula was used.

Table I. Ultraviolet absorption of $CD133^+$ cells and $CD133^-$ cells on days 1, 2, 3, 4, 5 and 6 (mean ± SD).

Day	$CD133^+$ cells	$CD133^-$ cells	P-value
1	0.38±0.06	0.45±0.01	P=0.18
2	0.81±0.05	0.87±0.03	P=0.12
3	1.64±0.07	1.56±0,01	P=0.11
4	2.43±0.06	2.24±0.05	P=0.013
5	2.53±0.01	2.22±0.04	P=0.000
6	2.57±0.03	2.17±0.06	P=0.000

Glut-1 protein levels in $CD133^+$ and $CD133^-$ Hep-2 cells by western blotting. Western blotting was performed as described previously (26). The Glut-1 and β-tubulin (as a control) protein in each group of Hep-2 cells were assayed using a BAC protein quantitative kit (Wuhan Boster Biological Technology Co. Ltd., Wuhan, Hubei, China). Briefly, 80 μg of protein was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane (Millipore, Billerica, MA, USA). Skimmed milk (2%) was used as a blocking solution (room temperature, 1 h). The membrane was incubated with the primary antibody (Glut-1, 1:1,000; β-tubulin, 1:5,000) at room temperature for 3 h and with the secondary antibody (1:5,000, donkey anti-rabbit; 1:2,000, donkey anti-mouse) at room temperature for 1 h. The proteins were detected using an enhanced chemiluminescence system (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and were exposed to X-ray film. Protein expression was analyzed semi-quantitatively using the Kodak Gel Logic Analysis System (Carestream Health Inc., Rochester, NY, USA).

Statistical analysis. Statistical analyses were performed using SPSS for Windows, version 19.0. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of CD133 in the Hep-2 cell line. Real-time RT-PCR demonstrated that the sizes of the CD133 and GAPDH PCR product were 213 and 145 bp, respectively (Fig. 1). Dissociation curve analysis performed between 60-95˚C demonstrated only the expected peaks at 82.1 and 85.1˚C for CD133 and GAPDH, respectively (Fig. 2). The analysis demonstrated that each primer pair had sufficient specificity for use in the present study of CD133 expression. The mean ΔCt of CD133 expression was 10.98.

Detection of $CD133^+$ Hep-2 cells by FCM. CD133 cells were isolated from the Hep-2 cell line using FCM. To evaluate the efficiency of FCM, harvested cells were subjected to FACS analysis. Prior to isolation, the $CD133^+$ fraction was 1.2%, which increased to 76.1% following isolation (Fig. 3). Successive tests also proved that cells grew well following isolation.

Proliferation of $CD133^+$ Hep-2 cells. Following isolation, $CD133^+$ and $CD133^-$ cells were cultured separately in SFM.

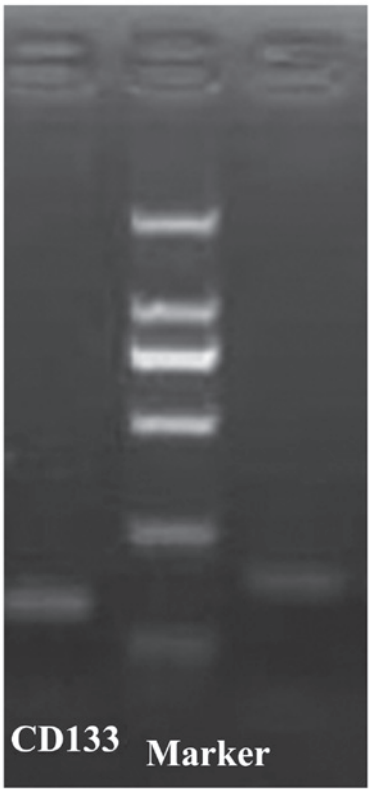

Figure 1. RT-PCR demonstrated that the CD133 and GAPDH PCR products were 213 and 145 bp, respectively. RT-PCR, real-time reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Proliferation is displayed in Table I and Fig. 4. The proliferation of $CD133^+$ and $CD133^-$ cells was not different during the first 3 days ($P>0.05$). From day 4, however, the proliferation capacity of $CD133^+$ cells *in vitro* was higher than that of $CD133^-$ cells ($P<0.05$).

Glut-1 mRNA levels in $CD133^+$ and $CD133^-$ Hep-2 cells. Real-time RT-PCR demonstrated that the sizes of the Glut-1 and GAPDH PCR products were 123 and 145 bp, respectively (Fig. 5). Dissociation curve analysis performed between 60-95˚C demonstrated only the expected peaks at 86.2 and 85.1˚C for Glut-1 and GAPDH mRNA, respectively (Fig. 6). Thus, the analysis demonstrated that each primer pair had sufficient specificity for use in the present study of Glut-1 mRNA expression. The mean ΔCt of Glut-1 mRNA expression in $CD133^+$ cells was 1.78. The mean ΔCt of Glut-1 mRNA

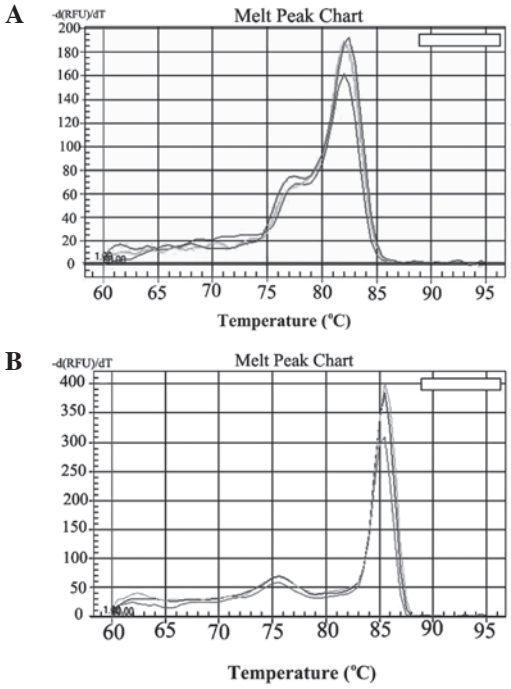

Figure 2. Real-time RT-PCR dissociation curve analysis. (A) CD133 PCR product exhibiting a dissociation temperature of 82.1˚C. (B) GAPDH PCR product exhibiting a dissociation temperature of 85.1˚C. RT-PCR, real-time reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

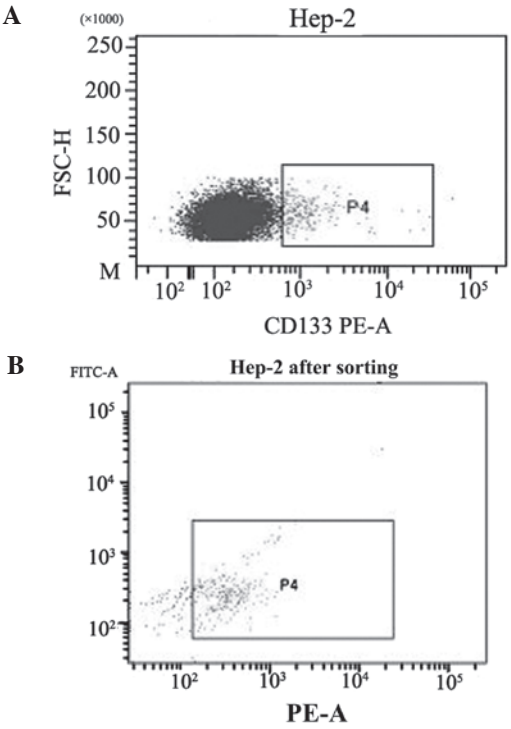

Figure 3. CD133 cells were isolated from the Hep-2 cell line using FCM. (A) Prior to isolation, the CD133+ fraction was 1.2% by FACS analysis, (B) which increased to 76.1% following isolation. FCM, flow cytometry; FACS, fluorescence-activated cell sorting; PE-A, area of phycoerythrin.

expression in CD133- cells was 1.00. The expression of Glut-1 mRNA differed significantly between CD133+ and CD133- cells (P<0.05).

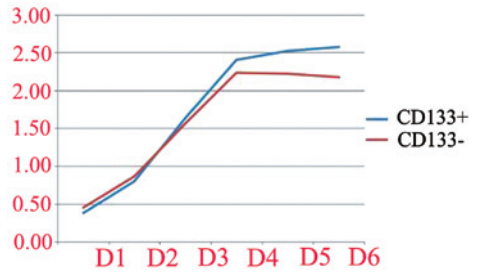

Figure 4. Proliferation of CD133+ and CD133- cells was not different during the first 3 days (P>0.05). From day 4, the proliferation capacity of CD133+ cells *in vitro* was higher than that of CD133- cells (P<0.05).

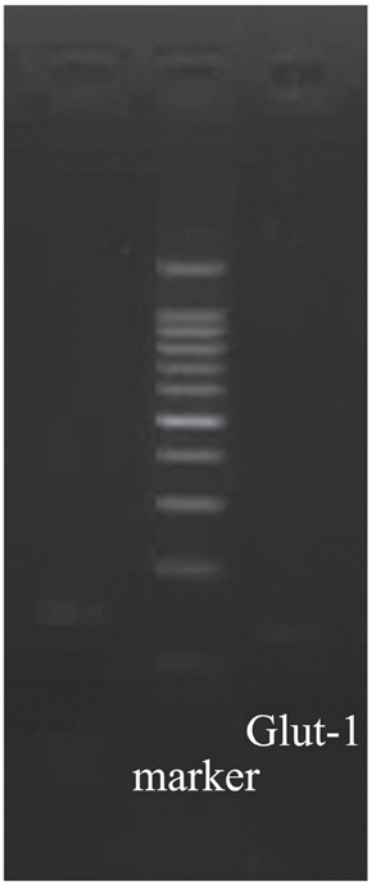

Figure 5. Real-time RT-PCR demonstrated that the Glut-1 PCR product and GAPDH PCR product were 123 and 145 bp, respectively. RT-PCR, real-time reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Glut-1, glucose transporter-1.

Glut-1 protein levels in CD133+ and CD133- Hep-2 cells. Mean Glut-1 protein levels in CD133+ Hep-2 cells and CD133- Hep-2 cells relative to β-tubulin were 0.48 ± 0.02 and 0.21 ± 0.03 $\mu g/\mu l$, respectively (P<0.05; Fig. 7).

Discussion

Glut-1 is highly expressed in numerous types of cancer, including laryngeal carcinoma (21,25) and is associated with resistance to chemoradiotherapy and poor prognosis (28-30). High expression of Glut-1 has been demonstrated to be associated with FDG uptake (17-20,22). Thus, Glut-1 is an intrinsic

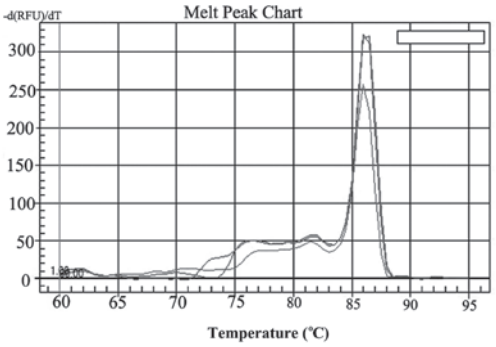

Figure 6. Dissociation curve analysis of real-time RT-PCR: Glut-1 PCR product, showing a dissociation temperature of 86.2˚C. RT-PCR, real-time reverse transcription-polymerase chain reaction; Glut-1, glucose transporter-1.

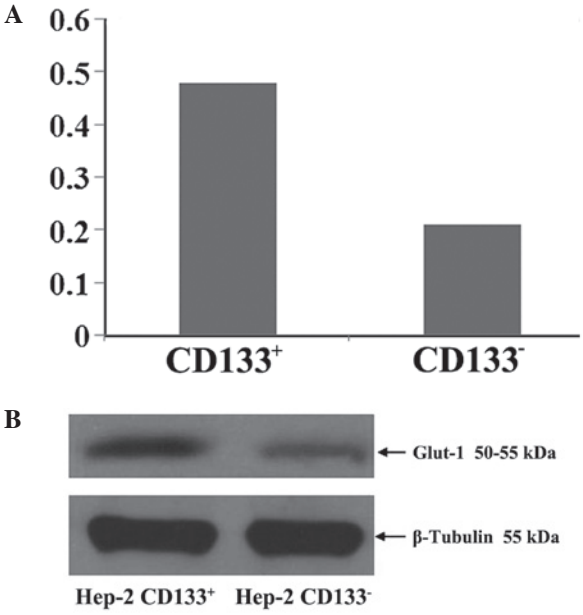

Figure 7. (A) Mean of Glut-1 protein values in $CD133^+$ and $CD133^-$ Hep-2 cells relative to β-tubulin were 0.48 ± 0.02 and 0.21 ± 0.03 $\mu g/\mu l$, respectively ($P<0.05$). (B) Western blot analysis. Glut-1, glucose transporter-1.

marker of hypoxia in cancer (31,32). However, studies of Glut-1 expression in CSCs are limited (6,23,24). To the best of our knowledge, no study of Glut-1 expression in laryngeal CSCs exists.

CD133 has been demonstrated to be a marker of CSCs in laryngeal carcinoma (1-4). In the present study, we successively isolated $CD133^+$ Hep-2 laryngeal carcinoma cells by FCM. Prior to isolation, we confirmed the expression of CD133 in the Hep-2 cells by real-time RT-PCR. Subsequently, FCM demonstrated that the proportion of $CD133^+$ Hep-2 cells was only 1.2%. Following isolation, the approximate proportion of $CD133^+$ Hep-2 cells was 76.1%. In 2007, Zhou *et al* first demonstrated that CD133 was a marker of CSCs in a Hep-2 laryngeal cell line (4). They demonstrated that the $CD133^+$ fraction was 2.45% by FACS analysis prior to isolation and this was increased to 91.26% following isolation. Wei *et al* demonstrated that the $CD133^+$ fraction was 3.15% by FACS analysis prior to isolation, which increased to 90.26% following isolation (3). They demonstrated that $CD133^+$ cells have a stronger ability to form tumors *in vivo* than unsorted cells. In the present study, we also demonstrated that the proliferation capacity of $CD133^+$ cells *in vitro* was higher than that of $CD133^-$ cells at days 4, 5 and 6 ($P<0.05$). Thus, our study demonstrated that CD133 may be a marker of laryngeal carcinoma CSCs. However, further *in vivo* experiments are required to determine whether the $CD133^+$ cells are comparable with the *in vitro* phenotype.

Whether $CD133^+$ CSCs have metabolic programs distinct from those of the bulk of tumor cells is not well established. Certain regulatory pathways, including the Wnt (9), Notch (10), Hedgehog (11) and PI3K/Akt pathways (12), have been demonstrated to be important in the regulation of CSC metabolism and energy sensing. However, in laryngeal carcinoma, Chen *et al* demonstrated no significant difference between the expression of Notch2 and PTEN in $CD133^+$ and $CD133^-$ cells, although they were expressed at high levels in $CD133^+$ cells (2). Thus, other metabolic pathways may play a regulatory role in CSCs of laryngeal carcinoma, including the Warburg effect. Ke *et al* demonstrated that Glut-1 expression was higher in $CD133^+$ than $CD133^-$ cells in thyroid cancer following ^{131}I radiotherapy (6). Mai *et al* demonstrated that stem cells from proliferating hemangiomas may produce Glut-1 (23). The present study is to the best of our knowledge the first to demonstrate that Glut-1 expression in laryngeal CSCs and Glut-1 mRNA and protein expression were higher in $CD133^+$ than in $CD133^-$ cells ($P<0.05$). These results suggest that Glut-1 expression may play a role in the CSCs of laryngeal carcinoma. Glut-1 is a transmembrane protein and a main glucose transporter. CSCs rely primarily on glycolysis to meet their energy demands (16), which is dependent on glucose uptake. Thus, we also suggest that Glut-1 is important in the energy supply of laryngeal $CD133^+$ Hep-2 cells. Several studies have demonstrated that the inhibition of Glut-1 expression may repress glycolysis and glucose uptake in cancer cells (27,33). Phloretin is a glucose transporter inhibitor stated to induce apoptosis and overcome drug resistance under hypoxic conditions (33). In laryngeal carcinoma, we demonstrated that antisense Glut-1 may decrease glucose uptake and inhibit the proliferation of Hep-2 cells (27). We suggested that Glut-1 may be a therapeutic target in laryngeal carcinoma. However, this requires further study.

In conclusion, our data do not permit a definitive conclusion regarding the expression of Glut-1 in laryngeal CSCs. However, our findings demonstrate that Glut-1 mRNA and protein expression is higher in $CD133^+$ than in $CD133^-$ cells. Thus, Glut-1 may be important in the energy supply of laryngeal $CD133^+$ Hep-2 cells and may represent a potential therapeutic target for the inhibition of the proliferation of laryngeal CSCs.

Acknowledgements

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