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Expression of Stem Cell Markers and Dopamine D2 Receptors in Human and Rat Prolactinomas

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Background:

Dopamine agonists (DAs) are the first-line treatment for prolactinomas. DAs primarily target the dopamine D2 receptor (D2R). Tumor stem-like cells (TSLCs) are associated with the tolerance to radiotherapy and chemotherapy. TSLCs have also been identified in pituitary adenomas. We aimed to characterize the expression pattern of stem cell markers and D2R in human and rat prolactinomas.

Material/Methods:

Human prolactinoma specimens (n=14) were obtained from patients with surgical resection. The xenograft model of rat prolactinomas was generated by endermically injecting MMQ cells, HE and PRL were confirmed by immunohistochemical staining of tumor sections, and the expression of serum PRL was measured by ELISA. The expression of stem cell markers (CD133, Nestin, Oct4, and Sox2) and D2R in prolactinomas was detected by immunofluorescence. The proportion of CD133-expressing cells after DA treatment was evaluated by flow cytometry *in vitro*.

Results:

We found that a small subpopulation of cells expressing stem cell markers existed both in human and rat prolactinomas. Furthermore, the CD133-expressing cells showed negative D2R expression. Conversely, the D2R-expressing cells showed negative CD133 expression. The proportion of CD133-expressing cells in surviving tumor cells was significantly increased after DA treatment.

Conclusions:


Our results confirmed the existence of cells expressing stem cell markers in human and rat prolactinomas. Additionally, the CD133-expressing cells might resist DA therapy due to the lack of D2R expression.

MeSH Keywords:

Dopamine Agonists • Prolactinoma • Receptors, Dopamine D2 • Stem Cells • Drug Resistance

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Background

Pituitary adenomas are generally considered as benign intracranial tumors. They account for approximately 10% to 15% of all diagnosed intracranial tumors. Autopsy and radiology show a high prevalence rate of 14.4% and 22.5% in the overall populations, respectively [1]. Prolactinomas are the most predominant type of functional pituitary adenomas and account for about 40% of all pituitary tumors [2]. Dopamine agonists (DAs) are the first-line treatment for prolactinomas currently, and have been proven effective in treating 70% to 90% of prolactinomas [3–5]. DAs are effective in suppressing the hypersecretion of prolactin (PRL), reducing the size of tumors, and restoring gonadal function [5,6]. The clinical response of prolactinomas to DA treatment correlates well with dopamine D2 receptor (D2R) expression levels of tumor cells, and tumor cells with low D2R expression are resistant to DAs [7–10], which indicates that DAs primarily target D2R.

Currently, tumor stem-like cells (TSLCs) are a popular topic in tumor research. The TSLCs theory defines these cells as a unique subpopulation of tumor cells, which possess the ability to initiate tumor growth and sustain self-renewal, as well as multilineage differentiation [11,12]. TSLCs have been identified in various malignant tumors such as acute myelogenous leukemia (AML) [13], brain tumor [14], breast cancer [15], and gastric cancer [16]. Previous studies have shown that TSLCs are in quiescence or slow-cycling state, which is associated with relative drug-resistance and radioresistance [14,15,17–19]. Breast cancer stem cells retain lipophilic fluorescent dye PKH26 as a consequence of their quiescent nature, and PKH26+ breast cancer cells are highly clonogenic, spherogenic, drug-resistant, and radio-resistant [15]. The expression of aldehyde dehydrogenase 1 (ALDH1) is high in drug-resistant and radio-resistant cells isolated from head and neck squamous cell carcinoma (HNSCC) tumors. Gene expression microarray analysis demonstrated that the epithelial-mesenchymal transition (EMT) pathway and EMT-related genes were significantly up-regulated in ALDH1+ HNSCC cells. Moreover, the increased incidence of ALDH1 expression positively correlates with the clinical stage of HNSCC patients [18]. These data demonstrate that TSLCs possess resistance to chemo- and radio-therapy [17–19].

Although most studies concerning TSLCs have focused on malignant tumors, a few studies relate to benign tumors. Pituitary adenoma stem-like cells were first isolated from a case of human nonfunctional adenoma and another case of human somatotrophic adenoma, respectively, by Xu [20]. Their existence was established in several recent studies [21–23]. Stem cell markers are widely used for the identification of TSLCs. The presence of stem cell markers in pituitary adenomas has been demonstrated [20–24]. However, the expression pattern of stem cell markers and D2R in prolactinomas is still unknown.

In the present study, we characterized the expression pattern of stem cell markers CD133, Nestin, Oct4, and Sox2, and D2R in human and rat prolactinomas.

Material and Methods

Tissue specimens

Tumor specimens were obtained from pituitary prolactinomas resected via transsphenoidal approaches in the Department of Neurosurgery, First Affiliated Hospital of Wenzhou Medical University from March 2012 to December 2015. This research was approved by the Clinical Medicine Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University (permission: 2012-13). Tumors were classified according to the World Health Organization classification of pituitary tumors (2004 edition) [25]. Patients with prolactinomas (n=14; 8 men and 6 women) ranged in age from 22 to 54 years. All had increased prolactin serum levels from 76.7 to >2000 ng/ml and the tumor volumes varied from 365 to 9450 mm³.

Cell lines and culture

The MMQ rat prolactinoma cells were purchased from the Cell Resource Center of Shanghai Institute of Life Sciences, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in F12 medium (Gibco, USA) supplemented with 15% horse serum (Gibco, USA), 2.5% fetal bovine serum (Gibco, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco, USA). MMQ cells were maintained at 37°C and 5% CO₂, and medium was refreshed every 2 days.

Xenograft model of prolactinomas

The experimental procedure was conducted according to the institutional animal care guidelines and approved by the Laboratory Animal Ethics Committee of Wenzhou Medical University with the reference number wyd2014-0026. Four to five-week-old female BALB/c nude (nu/nu) mice were purchased from the Animal Experimental Center of Chinese Academy of Sciences (Shanghai, China). MMQ cells (1×10⁶) were resuspended in 100 μL phosphate-buffered saline (PBS) and subcutaneously injected into flanks of each nude mouse, and controlled nude mice (n=9) underwent the same type of injection with PBS simultaneously. After MMQ cells injection, tumor size was measured by a vernier caliper weekly and calculated as (length×width²)/2. When the tumors increased to about 10 mm in diameter, the tumor-bearing nude mice (n=9) and controlled nude mice (n=9) were euthanized, xenograft tumors were harvested, and blood was collected from the angular vein. The serum PRL levels of tumor-bearing and controlled nude mice were determined using a rat PRL ELISA kit

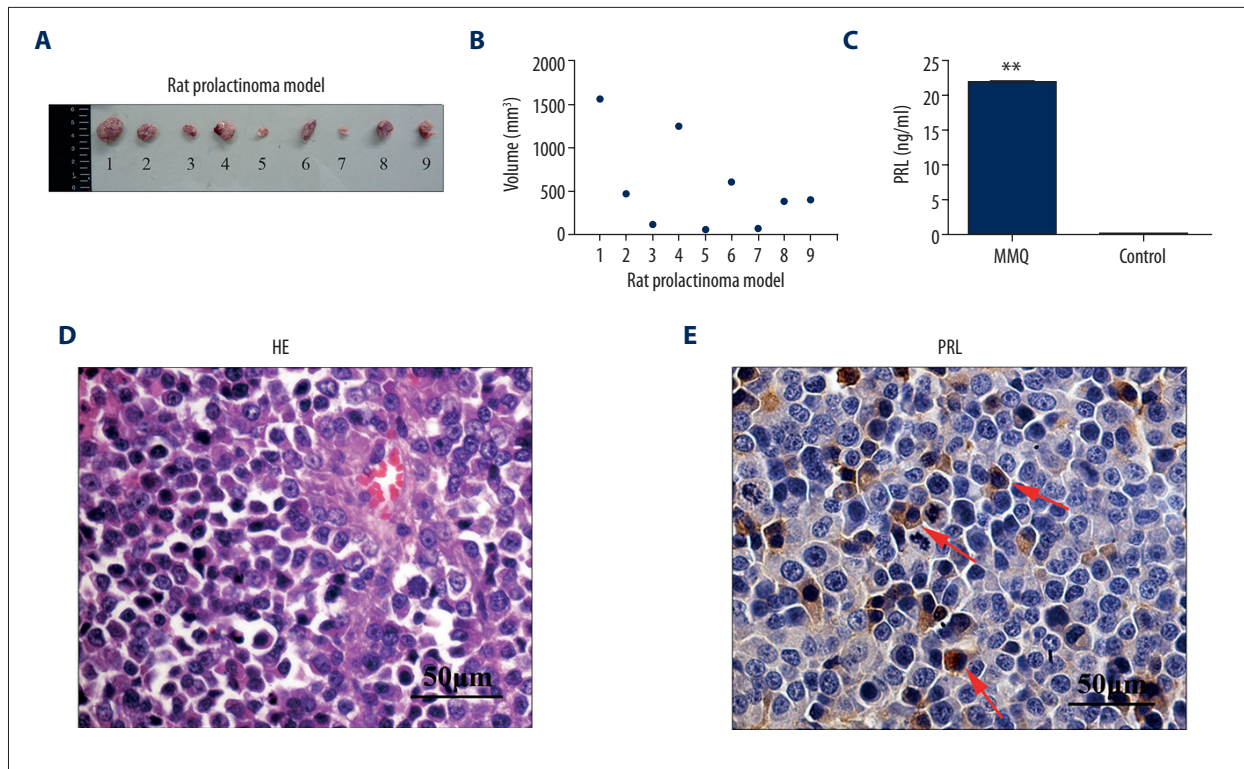


Figure 1. The construction of rat prolactinoma model. MMQ cells (1×10^6) were subcutaneously injected into flanks of each nude mouse ($n=9$), and controlled nude mice ($n=9$) underwent the same type of injection with PBS simultaneously. After injection, tumor size was measured by a vernier caliper weekly and calculated as $(\text{length} \times \text{width}^2)/2$. When the tumors increased to about 10 mm in diameter, the tumor-bearing nude mice ($n=9$) and controlled nude mice ($n=9$) were euthanized, xenograft tumors were harvested, and blood was collected from the angular vein. The representative images for xenograft tumor on the nude mouse are shown in (A), and the tumor volumes are shown in (B). (C) The serum PRL levels of tumor-bearing and controlled nude mice were determined using a rat PRL ELISA kit, showing that the expression of PRL in tumor-bearing nude mice increased significantly. (D) An HE Staining Kit was used to characterize the histological features of xenograft tumors. (E) The PRL expression of tumor tissues was detected according to the standard protocol of immunohistochemistry. ** $P < 0.001$.

(Abnova, Taiwan). The HE Staining Kit (Solarbio, China) was used to characterize the histological features of xenograft tumors. The PRL expression of tumor tissues was detected according to the standard protocol of immunohistochemistry, using Rabbit anti-PRL (1: 200, Abcam, USA) and HRP-conjugated Goat anti-Rabbit IgG (ZSGB-Bio, China).

Immunofluorescence analysis

The human prolactinoma specimens ($n=14$) and rat xenograft tumors ($n=9$) were fixed, paraffin-embedded, and cut into 4- μm -thick sections. The sections were deparaffinized, rehydrated, and rinsed with PBS, followed by antigen retrieval in citrate buffer (pH 6.0) for 15 min at 95-100°C. Subsequently, non-specific antigens were blocked in 5% donkey serum for 60 min at 25°C. Then sections were incubated with primary antibodies (Rabbit anti-CD133, 1: 100, MyBioSource, USA; Goat anti-Nestin, 1: 50, Santa Cruz, USA; Rabbit anti-Oct4, 1: 100, Proteintech, USA; Rabbit anti-Sox2, 1: 100, Proteintech, USA; Mouse anti-D2DR, 1: 50, Santa Cruz, USA) overnight at 4°C.

After being rinsed with PBS, sections were incubated with secondary antibodies (FITC or PE conjugated Donkey anti-Rabbit, anti-Mouse or anti-Goat IgG, 1: 200, Santa Cruz, USA) for 60 min at 25°C. Finally, sections were counterstained with DAPI (Sigma, USA) and mounted with Antifade Mounting Medium (Beyotime, China) before being examined by fluorescence microscopy (Olympus, Tokyo, Japan).

Pharmacological studies *in vitro*

The dopamine agonist cabergoline was purchased from Tocris Bioscience (Bristol, UK). Cabergoline was dissolved in dimethylsulfoxide (DMSO) according to the manufacturer's instructions. MMQ cells were assigned into 3 treatment groups (1×10^6 cells per group): (1) Group A was treated with 0 μM cabergoline (DMSO) and regarded as a control; (2) Group B was treated with 50 μM cabergoline; (3) Group C was treated with 100 μM cabergoline. All groups were treated at 37°C and 5% CO_2 for 24 h.

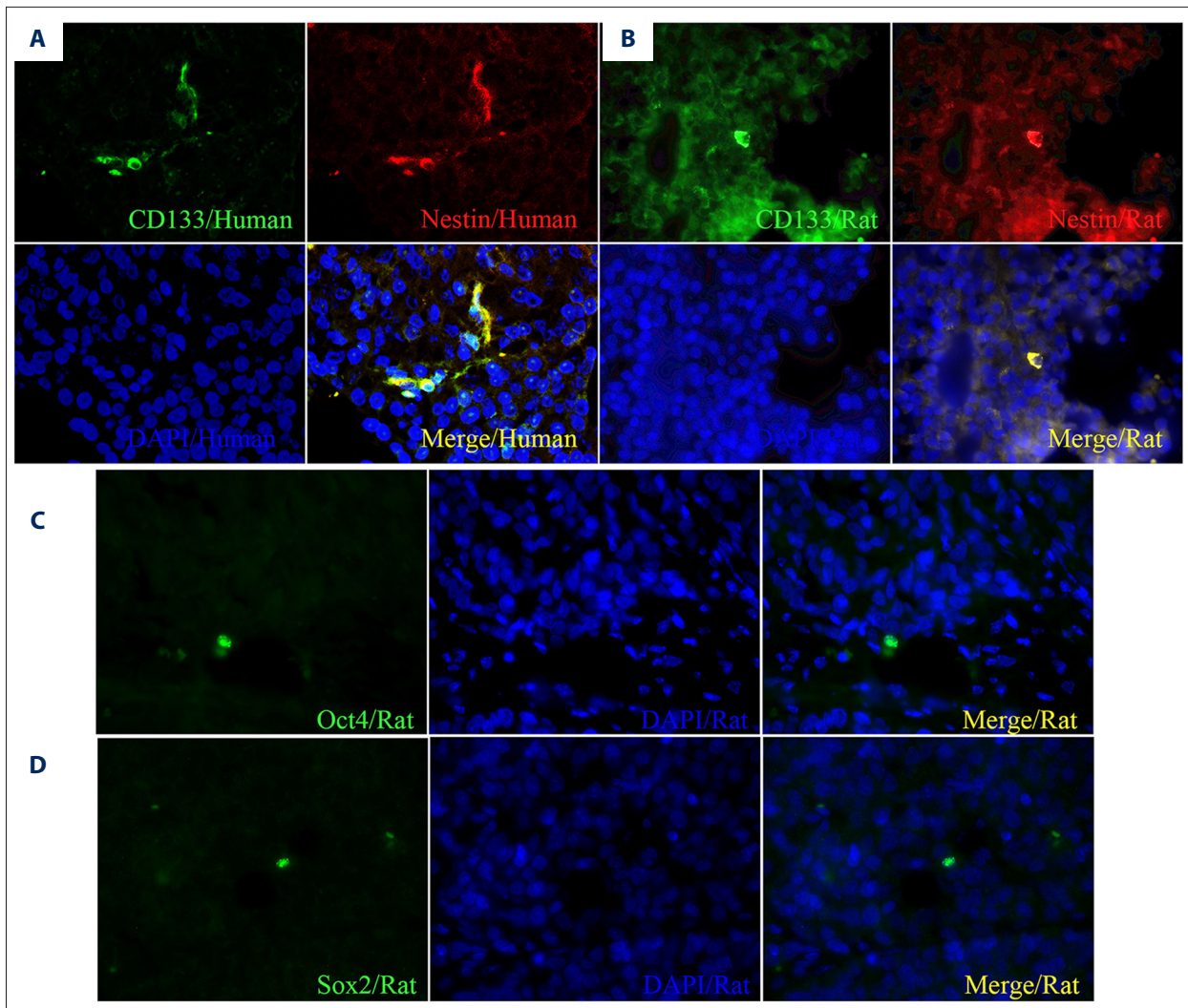


Figure 2. Tumor cells expressed stem cell markers in prolactinomas. The expression of stem cell markers in human and rat prolactinomas was detected by immunofluorescence. As shown in (A), tumor cells co-expressing CD133 (green) and Nestin (red) were presented in human prolactinoma specimens. Similarly, mouse xenografts also contained cells co-expressing CD133 (green) and Nestin (red) (B). In addition, a small fraction of mouse xenografts cells had positive expression of Oct4 (C) and Sox2 (D), which were also considered as markers of neural stem cells.

Flow cytometry analysis

After cabergoline treatment, MMQ cells were collected and washed with PBS. Then, MMQ cells were resuspended in 100 μ L staining buffer (LiankeBio, China) and incubated with Rabbit anti-CD133 (1: 50, MyBioSource, USA) for 30 min at 4°C. Negative control was incubated with equivalent PBS instead. After washing with PBS, cells were incubated with FITC-conjugated Donkey anti-Rabbit IgG (1: 50, Santa Cruz, USA) for 30 min at 4°C. Subsequently, cells were washed and resuspended in PBS. Then, the proportion of CD133-expressing cells in all groups was analyzed by a FACS Calibur Flow Cytometer (BD Biosciences). All experiments were repeated 3 times.

Statistical analysis

The data were analyzed in IBM SPSS Statistics Version 21.0. Values are represented as means \pm SD. The differences between groups were analyzed by one-way ANOVA, and differences were considered statistically significant at $P < 0.05$.

Results

Rat prolactinoma model

The xenograft tumors collected from rat prolactinoma model are displayed in Figure 1A, and their volumes varied from

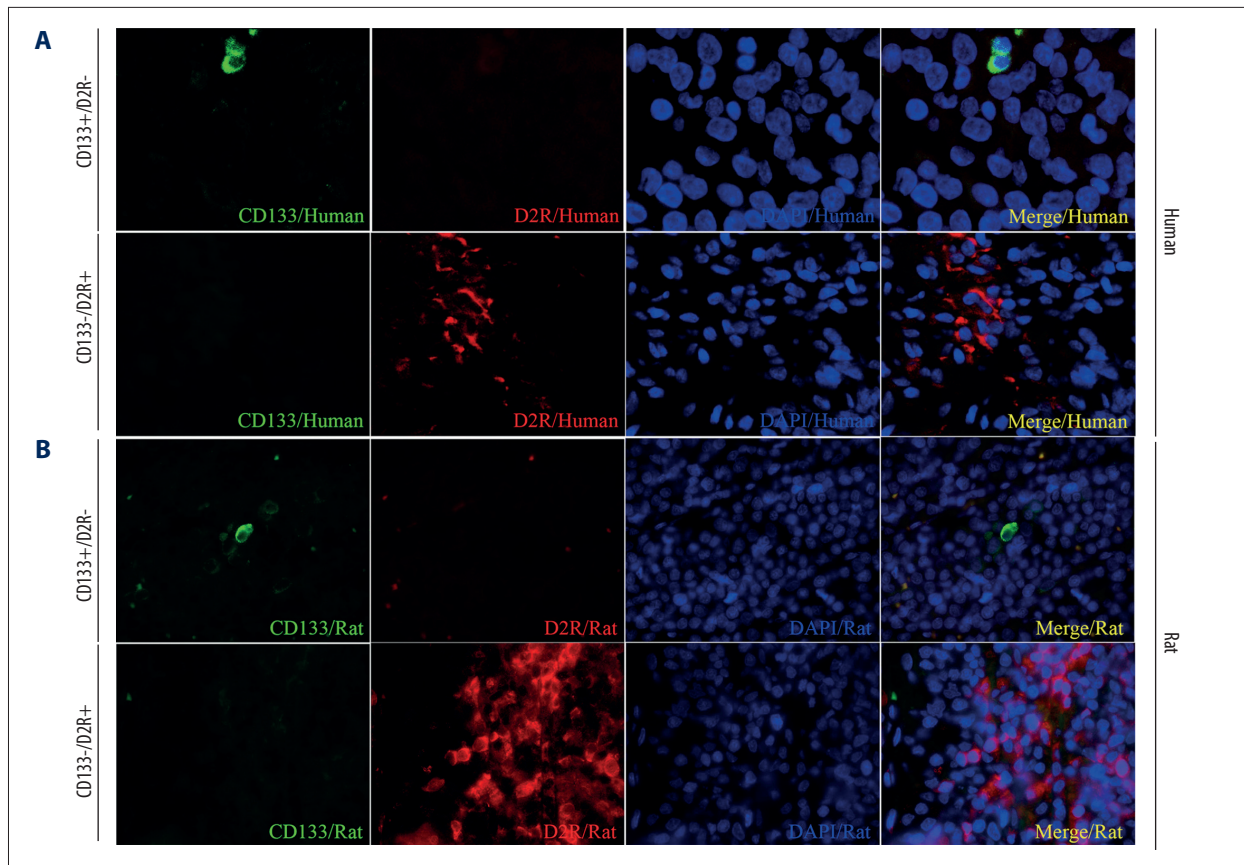


Figure 3. The expression of CD133 and D2R in human and rat prolactinomas. Double immunofluorescence was used to characterize the expression of CD133 and D2R. As shown in (A), in human prolactinomas cells, CD133-positive staining (green) was found only in D2R-negative (red) cells, but not in D2R-expressing cells. In contrast, D2R-positive staining (red) was found only in CD133-negative (green) cells, but not in CD133-expressing cells. No tumor cells co-expressing CD133 and D2R were found. Similar results were also found in rat prolactinomas cells (B).

62.78 to 1555.34 mm³ (Figure 1B). The serum PRL levels of tumor-bearing nude mice increased obviously compared to that of controlled nude mice (Figure 1C, $P < 0.01$), which was similar to clinical findings. In addition, the histological features of xenograft tumors were analogous to those of rat pituitary adenomas (Figure 1D), and immunostaining was positive for PRL in tumor tissues (Figure 1E). These results indicated the successful development of a rat prolactinoma model.

Cells expressing stem cell markers in prolactinomas

The expression of stem cell markers in human and rat prolactinomas was detected by immunofluorescence. As shown in Figure 2A, tumor cells co-expressing CD133 (green), and Nestin (red) were presented in human prolactinoma specimens. Similarly, mouse xenografts also contained cells expressing CD133 (green) and Nestin (red) (Figure 2B). Further, we investigated the expression of Oct4 and Sox2 in mouse xenografts, and identified cells with positive expression (Figure 2C, 2D). Of the 14 human prolactinomas and 9 rat prolactinomas tested

by immunofluorescence assay, there were different degrees of positive expression of CD133 in 12 human prolactinoma specimens and in 9 rat prolactinomas specimens. Nestin was positively expressed in 11 human prolactinoma specimens and in 9 rat prolactinomas specimens. Oct4 was expressed positively in 7 rat prolactinomas specimens. Sox2 was positively expressed in 8 rat prolactinomas specimens. However, these cells expressing stem cell markers in prolactinomas represented only a small subpopulation of all tumor cells.

Expression of CD133 and D2R

Double immunofluorescence was used to characterize the expression pattern of CD133 and D2R. In human prolactinomas, as shown in Figure 3A, CD133-positive staining (green) was found only in D2R-negative (red) cells, but not in D2R-expressing cells. In contrast, D2R positive staining (red) was found only in CD133-negative (green) cells, but not in CD133-expressing cells. No tumor cells co-expressing CD133 and D2R were found. An identical expression pattern of CD133 and D2R

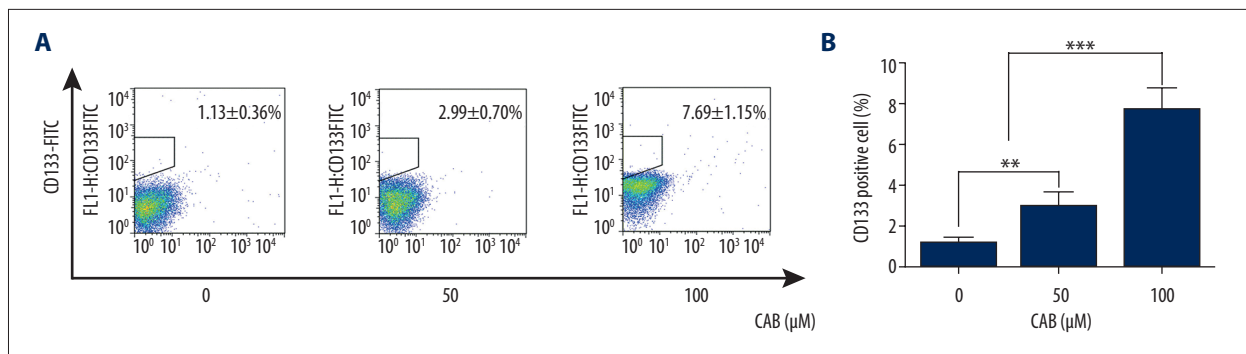


Figure 4. Increased proportion of CD133-expressing cells after DA treatment. The proportion of CD133-positive cells after DA treatment was evaluated *in vitro*. After being treated with different concentrations of cabergoline for 24 h, the proportion of CD133-positive cells was calculated under FACS. The results in Figure 4 show that the proportion of CD133-positive cells in different groups increased as the concentration of cabergoline increased. ** $P < 0.01$; *** $P < 0.001$.

was also found in rat prolactinomas (Figure 3B). These results showed that D2R, the primary target of DAs, was absent in the CD133-positive cells of human and rat prolactinomas.

Increased proportion of CD133-expressing cells after DA treatment

Since the CD133-expressing cells showed negative D2R expression, we evaluated their response to DA treatment. The proportion of CD133-expressing cells in MMQ after DA treatment was evaluated *in vitro*. As shown in Figure 4, the CD133-expressing cells accounted for $1.13 \pm 0.36\%$ of all surviving cells in the group treated with $0 \mu\text{M}$ cabergoline (vehicle). In contrast, this proportion increased to $2.99 \pm 0.70\%$ and $7.69 \pm 1.15\%$ in the groups treated with $50 \mu\text{M}$ and $100 \mu\text{M}$ cabergoline, respectively. Significant differences existed among the 3 groups in terms of CD133-positive cells (Figure 4B, $P < 0.05$).

Discussion

The TSLCs hypothesis was proposed decades ago. TSLCs have been identified in various malignant tumors [13–16], and possess resistance to chemo- and radio-therapy [17–19,26]. Despite the limited number of studies involving benign tumors, the presence of TSLCs in benign pituitary adenomas has been demonstrated [20–23]. DAs are the first-line treatment for prolactinomas [3–5], and dopamine D2 receptor (D2R) is the main target for DA treatment. D2R expression in TSLCs of prolactinomas is unknown. We believe the present study is the first of its kind to characterize the expression pattern of stem cell markers and D2R in human and rat prolactinomas.

One of the methods widely used for TSLC identification involves expression of stem cell markers in tumor cells. CD133 and Nestin are 2 widely used neural stem/progenitor cell markers [27]. Recent studies also showed that cells expressing

CD133 and Nestin in pituitary adenomas partially exhibit stem cell properties [20,22]. Oct4 and Sox2 are 2 typical embryonic stem cell markers and are established markers of pituitary stem cells [28]. Their existence in pituitary adenomas has also been demonstrated [24,29]. In our study, we found tumor cells co-expressing CD133 and Nestin in human and rat prolactinomas. Additionally, rat prolactinomas cells also contained Oct4- and Sox2-positive cells. Therefore, our results suggest that these cells, expressing stem cell markers, exist in both human and rat prolactinomas.

D2R is the primary target of DAs in prolactinomas [30]. Previous studies showed that the clinical response of prolactinomas to DA treatment correlated well with D2R expression in tumor cells, and tumor cells with low D2R expression were resistant to DAs [8–10]. In the present study, we found that D2R expression was absent in CD133-expressing cells, both in human and rat prolactinomas, which suggests that CD133-expressing cells might be resistant to DA treatment. Our *in vitro* pharmacological studies further support this finding. We found that the proportion of CD133-expressing cells in surviving tumor cells increased after DA treatment. This finding demonstrated that CD133-positive cells survived in DA therapy, which also indirectly reflects their resistance to DAs. This finding is consistent with the characteristics of chemotherapy resistance reported in TSLCs [19].

Tumor recurrence after withdrawal of DAs is a key clinical concern in treatment of prolactinomas [4,31]. Treatment cessation leads to recurrence of hyperprolactinemia in over 60% of the patients, even though serum prolactin (PRL) is reduced to normal for more than 2 years after continuous drug therapy [9,10]. In our study, we found that D2R expression was absent in CD133-expressing cells in human and rat prolactinomas, and CD133-expressing cells were resistant to DA treatment. Previous studies have shown that TSLCs possess resistance to radio- and chemo-therapy, and are the root of tumor

recurrence [17,19,32,33]; therefore, our findings indicate these CD133-expressing cells may play a role in tumor recurrence after drug withdrawal in prolactinomas, but further studies are needed to confirm our findings.

The present study has certain limitations that must be mentioned. The direct response of CD133-expressing cells isolated from prolactinomas to DA treatment was not monitored, and the increased proportion of CD133-expressing cells after DA treatment was not confirmed *in vivo*. Further, it is not sufficient to define TSLCs based solely on stem cell markers. Therefore, additional evidence is needed to support our speculation in future.

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Conclusions

Our results confirmed the presence of cells expressing stem cell markers in human and rat prolactinomas. Additionally, the CD133-expressing cells might resist DA therapy due to the lack of D2R and their increased proportion after DA treatment.

Conflicts of interest

The authors have no conflicts of interest.