

Original Article

CD151 up-regulation contributes to the invasion of pituitary adenomas

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Abstract: CD151 is up-regulated in several types of tumors and is involved in tumor invasion and metastasis. However, the potential role of CD151 in pituitary adenomas remains unclear. Here, we performed quantitative real-time PCR and immunochemistry to measure the expression levels of CD151 in samples of pituitary adenomas and investigated the effects of CD151 knock-down on tumor phenotypes in the HP75 pituitary adenoma cell line. We found that, compared to normal pituitary tissues, CD151 expression is increased in non-invasive pituitary adenomas and further increased in invasive pituitary adenomas. CD151 knock-down inhibited cell proliferation, invasion, clone formation, *in vivo* tumor development, and promoted apoptosis in the pituitary adenoma cell line. Our results fully support that CD151 plays an important role in promoting invasion and aggression of pituitary adenomas and thus might be a therapeutic target.

Keywords: CD151, pituitary adenomas, invasion, targeting therapy

Introduction

Pituitary adenomas are primary tumors of the pituitary gland and account for 10% to 25% of all intracranial neoplasms. The estimated prevalence rate of pituitary adenomas in the general population is around 17% [1]. Although most cases of pituitary adenomas are benign, about 35% of cases show invasive and aggressive behavior [2]. Current treatment of pituitary adenomas relies on surgery, radiation, and chemotherapy. But the prognosis of patients with invasive pituitary adenomas remains poor [3]. Unfortunately, the molecular mechanism underlying the invasion of pituitary adenomas is far from unclear. Comprehensive understanding of invasive pituitary adenomas might provide new therapeutic targets for its treatment.

CD151 is a member of the transmembrane protein superfamily (transmembrane 4 super family, TM4SF) which binds specifically to integrins to regulate cell adhesion, migration, and proliferation [4]. Previous studies have shown that CD151 is up-regulated in several types of

tumors and is involved in tumor invasion and metastasis [5]. Moreover, CD151 expression level correlates with tumor histological grading, clinical staging, and prognosis [6-8]. More importantly, CD151 is a promising drug target as several anti-CD151 monoclonal antibodies have shown anti-cancer effects in *in vivo* mouse models [9]. However, the potential role of CD151 in pituitary adenomas is unknown. Here, we measured the expression level of CD151 in pituitary adenoma samples and investigated the effects of CD151 knock-down on cellular behavior of the HP75 pituitary adenomas cell line. Our results suggest that CD151 may play an important role in the invasion of pituitary adenomas. The therapeutic efficacy of anti-CD151 monoclonal antibodies in pituitary adenomas thus deserves further investigation.

Materials and methods

Clinical samples

The study was approved by the Institutional Review Board of Wannan Medical College and

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written informed consent was obtained from each patient. A total of 60 cases of pituitary adenomas from patients without radiation or chemotherapy and 7 cases of normal pituitary tissues from autopsy were collected in the Department of Neurosurgery, Affiliated Hospital of Wannan Medical College from May 2011 to May 2014 by surgical resection. Samples were stored in liquid nitrogen. All cases of pituitary adenomas were confirmed by histopathological examination. Twenty nine cases were male and thirty one cases were female. The average age of 60 cases was 48.5 ± 9.8 , ranging from 23-69. Invasive pituitary adenomas were defined as meeting the following criteria: Hardy-Wilson grading ≥ 3 or C to D stage or Knosp grading ≥ 3 . Thus, 24 cases were invasive adenomas and 36 cases were non-invasive adenomas.

Cell cultures

Human pituitary adenomas cell line HP75 from ATCC was maintained in Dulbecco's Modified Essential Medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were cultured in a humidified atmosphere with 5% CO₂ at 37°C.

CD151 knock-down

The short hairpin RNA (shRNA) targeting human CD151 mRNA sequence (forward: TGGGAGATCATCGCTGGTATCTTCAAGAGAGATACCAGCGATGATCTCC CTTTTTTC and reverse: TCGAGAAAAAGCCTCTAGTAGCGACCATAG TCTCTGAACTATGGTCGCTACTAGAGGCA) and its scrambled shRNA were constructed into the pLentiLox3.7 (pLL3.7) lentiviral vector. The lentivirus was packaged and amplified in HEK293T cells. The HP75 cells were infected at an MOI of 5.

Invasion assay

Invasion assay was performed in a Transwell (Corning). The well was filled with Matrigel (BD Biosciences) at 1 mg/ml in DMEM medium without FBS. The lower chamber was filled with 600 ml medium with 10% FBS. The cells were collected and 100 ml of suspension was plated at the concentration of 8×10^5 cells/ml into the well. The cells were cultured at 37°C with 5% CO₂ for 36 h. The upper surface of the membrane was wiped with a cotton tip and the

migrant cells attached to the lower surface were stained with crystal violet. Cell numbers in 5 random visual fields were counted and the data are presented as fold change relative to the control group.

Apoptosis assay

For Annexin V-FITC apoptosis assay, cells were trypsinized, washed, and stained with Annexin V-FITC Apoptosis kit (abcam, ab14085) in the dark for 15 min at room temperature. Then, the stained cells were analyzed by MoFlo XDP (Beckman Coulter, Inc). For TUNEL assay, cells were fixed with 4% PFA, permeated with 0.25% Triton[®]X-100, and stained with Click-iT[®] TUNEL Alexa Fluor[®] Imaging Assay kit (C10245). The stained cells were then analyzed by fluorescence microscopy.

MTT assay

Cells were seeded into a 96-well plate in triplicate at a concentration of 4×10^3 cells per well. Cell growth was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) bromide assay every 24 hours for 5 days. Cells were incubated with 5 mg/ml MTT for 4 h, and subsequently solubilized in DMSO (100 ul/well). The absorbance at 570 nm was measured using an ELISA reader.

Clone formation assay

Cells were plated in duplicate in a 6-well plate. After incubation at 37°C for 14 days, the colonies were stained with Crystal Violet solution in methanol for 15 min. Colonies >50 μ m in diameter were counted. The data are presented as fold change relative to the control group.

In vivo xenograft study

The animal experiments were conducted in accord with the animal welfare guidelines of Wannan Medical College. Female athymic nude mice (6 weeks old, BALB-c/nu/nu strain) were kept in specific pathogen-free condition and were randomly assigned to two groups: CD151 RNAi and scrambled group, 7 animals per group. Pituitary adenomas cell line HP75 were infected with CD151 RNAi or scramble lentivirus at MOI of 10. A total of 1×10^6 cells were implanted into subcutaneous tissue of bila-

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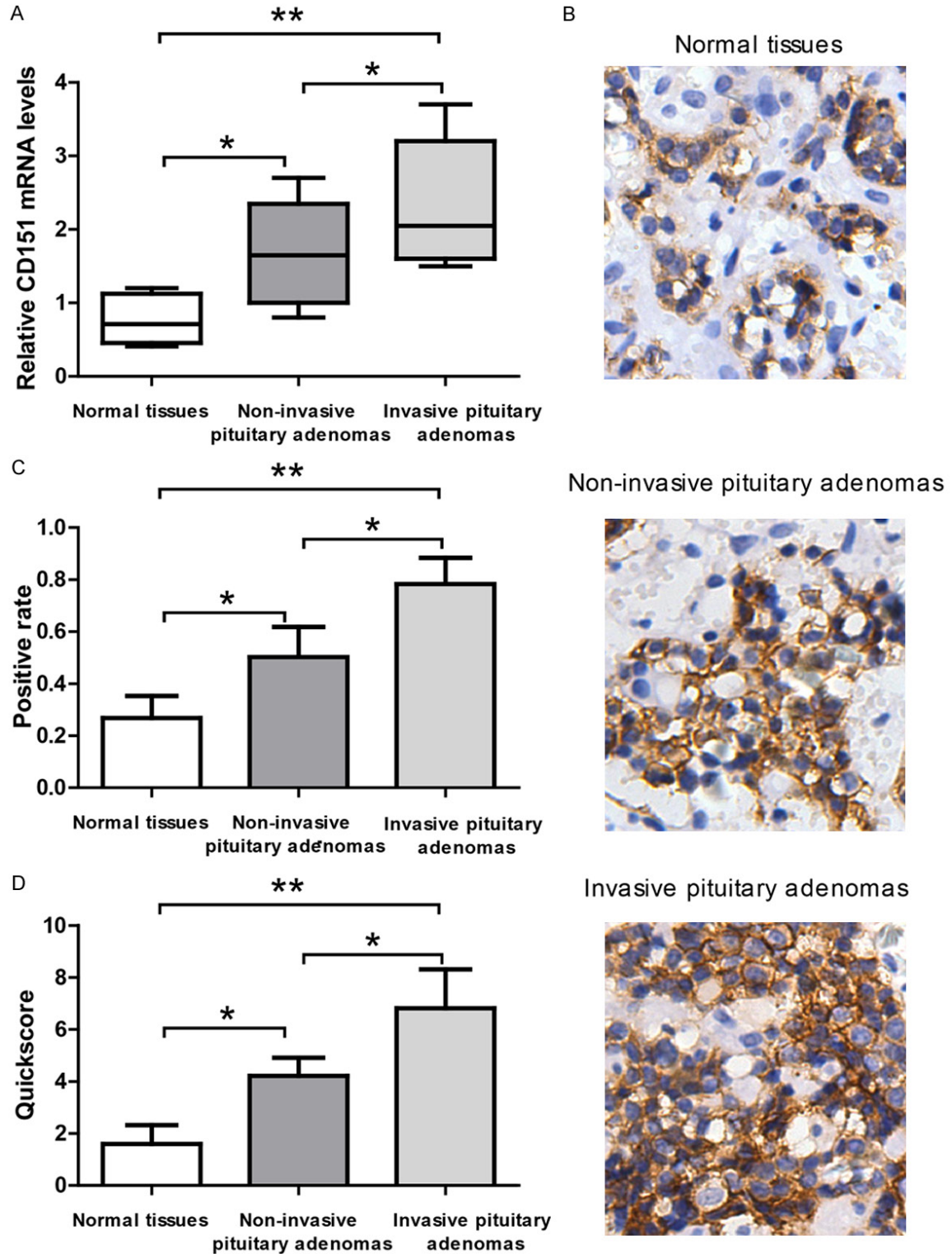


Figure 1. CD151 expression in pituitary adenomas. A. The quantification of CD151 mRNA levels in indicated samples using real-time PCR. B. The representative images of CD151 staining in indicated samples using IHC. C. The positive rate of CD151 staining in indicated samples. D. The quickscore of CD151 staining in indicated samples. For all, * $P < 0.05$; ** $P < 0.01$.

teral forelimb armpit with a 26-gauge needle/1 ml syringe. Tumor size was measured after 4

weeks by measuring the length (L) and width (W) of xenografted tumors with a vernier cali-

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per. Tumor size was calculated as follows: $L \times (W)^2 / 2$.

Real time-PCR

Total RNA was extracted using Trizol from tissues or cultured cell line. The reverse transcription was performed with 1 μ g RNA under the following condition: 25°C 10 min, 50°C 30 min, 85°C 5 min. The cDNA was used as template for SYBR Green real-time PCR. The relative expression levels of target genes were calculated by comparative Delta-delta CT method. The primers for CD151 were as follows: F, 5-GCACCGTTTGCTCAAGT-3; R, 5-ACCACCAGATGTAGGCTGT-3. The primers for b-actin were as follows: F, 5-GAAGGTGAAGTCGGAGTC-3; R, 5-GAAGATGGTATGGGATTTC-3.

Western blot

Total protein was extracted with sodium dodecyl sulfate lysis buffer (2% sodium dodecyl sulfate, 10% glycerol, 0.1 mM dithiothreitol, and 0.2 M Tris-HCl, pH 6.8) and boiled at 95°C for 10 min. Protein samples were resolved by SDS-PAGE electrophoresis and blotted with anti-human CD151 antibody (1:400, ab217357, Abcam).

Immunohistochemistry

Serial sections of paraffin-embedded tissues at the thickness of 5 μ m were prepared. Immunostaining of the sections with rabbit polyclonal anti-human CD151 antibody (ab217357, Abcam) was performed according to the instruction of VectaStain Universal ABC kit. The slides were then counter-stained with hematoxylin. CD151 staining was scored by the Quickscore method [10]. Briefly, each case was represented by five random visual fields. The positive cells were counted and the positive rate (P) was calculated by the total number of positive cells divided by the total number of all cells in five fields. The positive rate was further scored as: 1, <25%; 2, <50%; 3, >50%. The staining intensity (I) was scored as 1 for weak, 2 for moderate, and 3 for strong. The quickscore equals to $P \times I$. The quickscore of each case was calculated as the average quickscore of five fields. Analysis of the immunohistochemistry results was performed in a double-blind manner.

Statistical analysis

Statistical analysis was performed by two-tailed Student t test for two groups and one way ANOVA with Newman-Keuls post hoc test for more than two groups using SPSS 16.0 software. The data are expressed as mean \pm SD. Statistically significant differences are defined as $P < 0.05$.

Results

CD151 expression in pituitary adenomas

The mRNA levels of CD151 in tissue samples were measured by real time-PCR and the results show that, compared to normal pituitary tissue (n=7), CD151 mRNA levels are increased in non-invasive pituitary adenomas (n=36) and further increased in invasive pituitary adenomas (n=24) (**Figure 1A**). We also measured CD151 protein levels using immunohistochemistry in the same tumor and normal tissues. The staining results show that CD151 expression is mainly found in the cell membranes (**Figure 1B**). Semi-quantitative analysis shows that, compared to normal pituitary tissue, both the positive rate (**Figure 1C**) and the quickscore (**Figure 1D**) which integrates positive rate and intensity were increased in non-invasive pituitary adenomas and further increased in invasive pituitary adenomas. Taken together, these results suggest that CD151 up-regulation is associated with the invasive behavior of pituitary adenomas.

CD151 knock-down inhibits cell proliferation and induces apoptosis in pituitary adenomas

We sought to determine if CD151 affects tumor phenotypes in the human pituitary adenoma cell line HP75. CD151 knock-down was performed using lentiviral vector-based shRNA targeting human CD151. When HP75 cells were infected at an MOI of 5 for 48 h, CD151 protein level was greatly reduced (**Figure 2A**). The potential effect of CD151 RNAi on cell proliferation was evaluated by MTT assay. The results showed that CD151 RNAi inhibited cell proliferation (**Figure 2B**). The effect of CD151 RNAi on apoptosis was evaluated using TUNEL assay (**Figure 2C**) and Annexin V-FITC assay (**Figure 2D**). Both results show that CD151 RNAi induced apoptosis. All these results from *in vitro* cultured HP75 cells support that CD151

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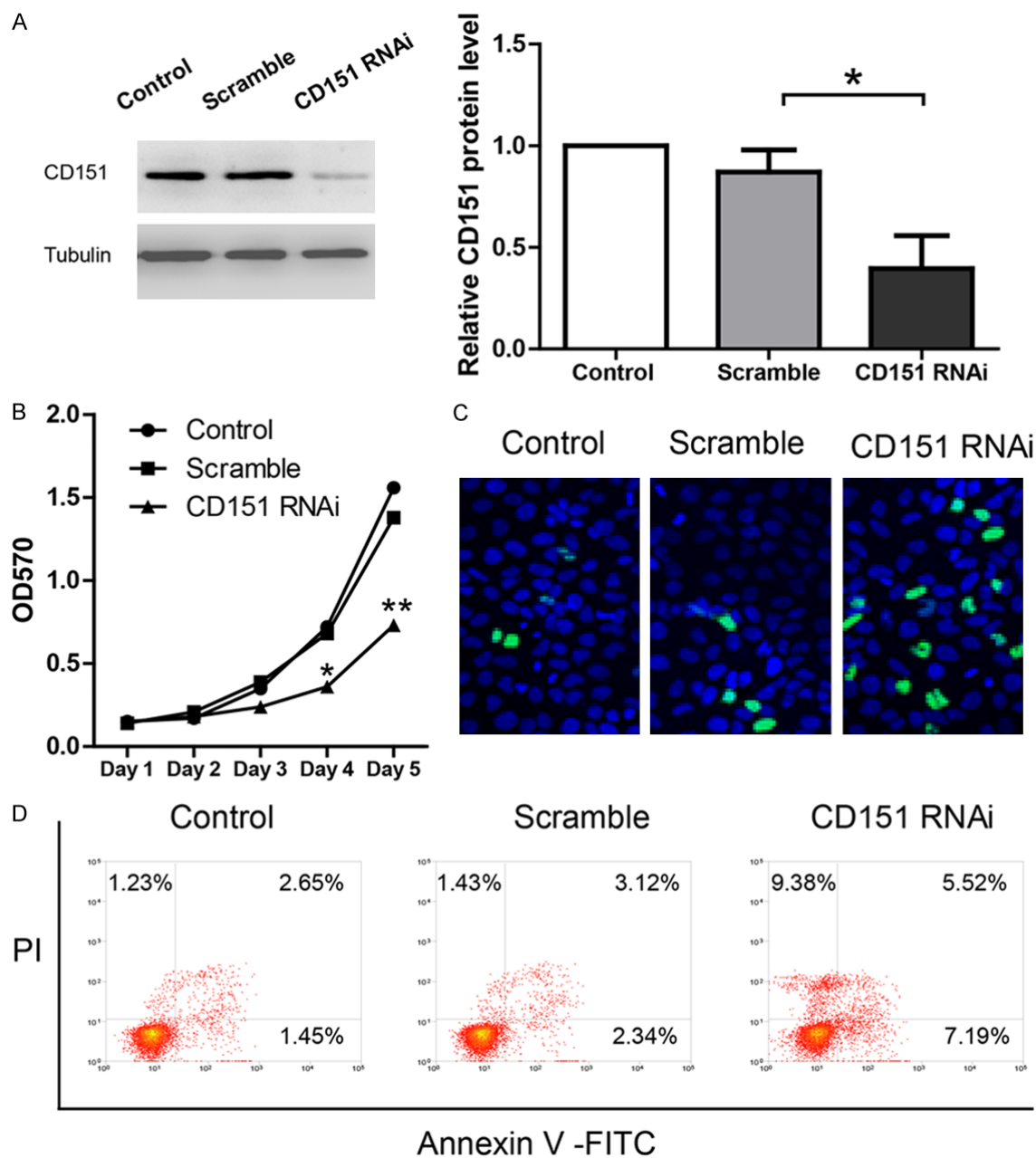


Figure 2. CD151 knock-down inhibits cell proliferation and induces apoptosis. A. The representative Western blot of CD151 protein levels after CD151 RNAi in HP75 cell line and its quantification. B. MTT assay of HP75 cells after CD151 RNAi. C. TUNEL assay of HP75 cells after CD151 RNAi. D. Annexin V-FITC apoptosis assay of HP75 cells after CD151 RNAi. For all, * $P < 0.05$; ** $P < 0.01$.

plays an important role in promoting aggressive phenotypes of pituitary adenomas.

CD151 knock-down inhibits invasion and tumor formation in pituitary adenomas

The effect of CD151 RNAi on invasion and tumor formation was further evaluated by Matrigel invasion assay (**Figure 3A**) and clone

formation assay (**Figure 3B**). The results show that CD151 RNAi greatly reduced the number of migrated cells and clone numbers. To provide *in vivo* evidence that CD151 is indeed important for the development of pituitary adenomas, we established a subcutaneous xenograft model in nude mice. A total of 14 nude mice were randomly assigned to two groups: CD151 RNAi ($n=7$) and scrambled group ($n=7$).

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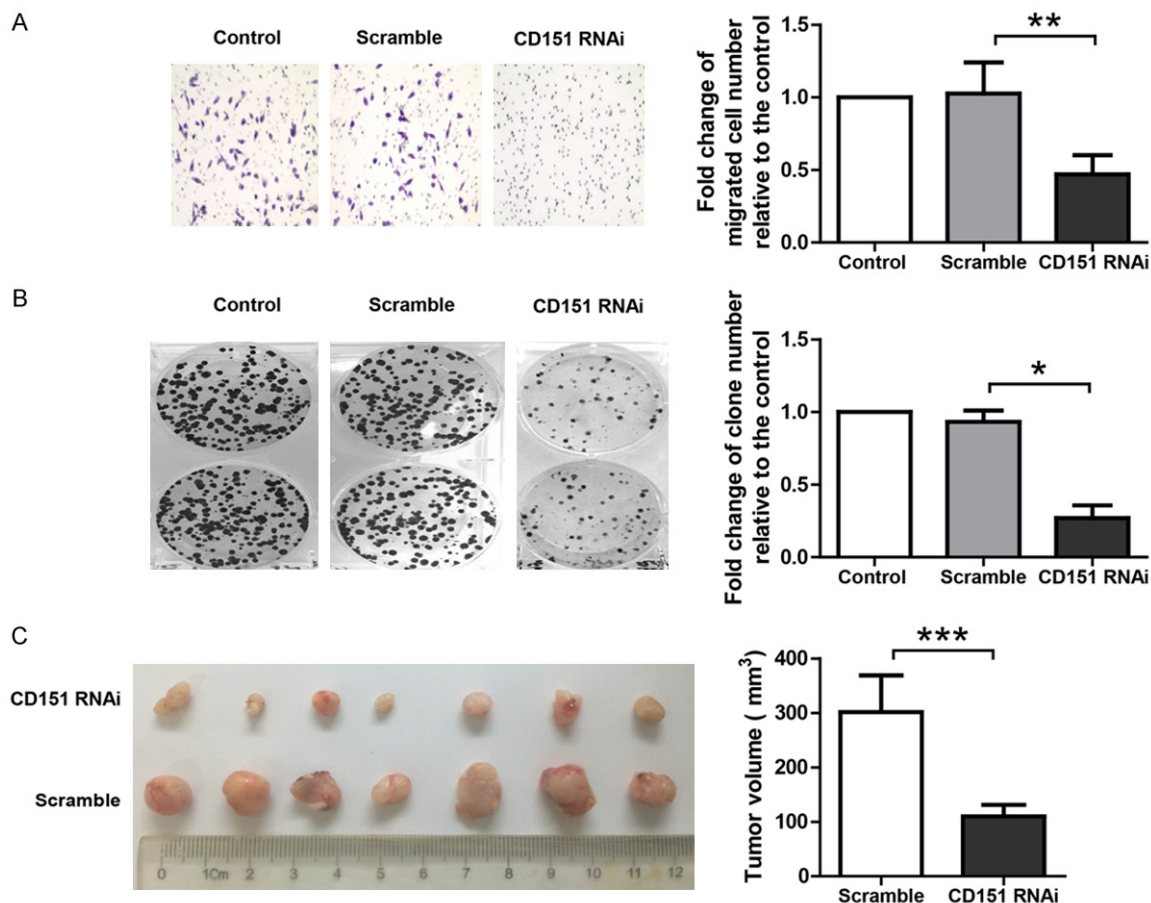


Figure 3. CD151 knock-down inhibits invasion and tumor formation. The representative images and quantification of invasion assay (A), clone formation assay (B) and in-vivo xenografts (C). For all, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Four weeks after implantation, mice were sacrificed and the tumor tissues were collected. The results show that tumor size of CD151-RNAi group was significantly smaller than that of scrambled group (**Figure 3C**). The mean tumor volume of CD151-RNAi and scrambled groups was 110.0 mm³ ($P < 0.001$ vs scramble) and 301.5 mm³, respectively.

Discussion

As a member of the TM4SF family, human CD151 is located on chromosome 11P15.5 [11]. Although CD151 is widely expressed among different tissues, CD151 expression is up-regulated in a variety of tumor types such as ovarian cancer [12], breast cancer [13], and pancreatic cancer [14]. Ang et al. measured CD151 expression in prostate cancer using immunochemistry [16]. They showed that CD151 expression in prostate cancer tissue was significantly higher than benign prostatic hyperplasia, and CD151 expression in poorly

differentiated prostate cancer is higher than well-differentiated prostate cancer. Similarly, Hashida et al. detected CD151 expression using immunochemistry and RT-PCR in 146 cases of colon cancer [17]. They found up-regulation of CD151 in 81 cases and the rate of 3-year survival of high-CD151 patients was significantly lower than the low-CD151 patients. Thus, CD151 up-regulation plays important roles in promoting aggressive behaviors of tumors.

In this report, we measured the expression level of CD151 in 60 cases of pituitary adenomas and 7 cases of normal pituitary tissues. We found that CD151 expression was up-regulated in pituitary adenomas compared with normal pituitary tissues. Moreover, CD151 up-regulation was associated with invasion of pituitary adenomas. Further results from HP75 cells indicate that CD151 promoted cell proliferation, invasion, clone formation, *in vivo* tumor development, and inhibited apoptosis. Our

results are fully consistent with previous studies showing that CD151 promotes tumor cell proliferation, migration, and invasion via the formation of CD151- α 3 β 1/ α 6 β 1 complexes [18-20]. However, the molecular mechanism underlying the effects of CD151 in pituitary adenomas remains unclear. As the function of CD151 relies on molecular interactions with integrins, it's possible that CD151- integrin complexes underlies the invasion of pituitary adenomas. Indeed, there is one study investigating integrin β 1 expression in pituitary adenomas from 49 Chinese patients and they found that integrin β 1 expression was higher in high-grade pituitary adenomas [21]. However, a French study showed that expression of integrins is reduced in 25 cases of pituitary adenomas compared to 6 cases of normal tissues [22]. This discrepancy may result from different ethnic groups or bias from small sample size. Thus, further experiments are required to elucidate the potential involvement of integrins in pituitary adenomas.

In addition, anti-CD151 monoclonal antibodies have shown anti-cancer effects in gallbladder carcinoma [23], rectal carcinoma [24], and colon cancer [25]. Antibody-based cancer therapy has superior specificity and efficacy than the conventional chemotherapy in several cancers [26]. Although the efficacy of CD151 antibody treatment remains to be validated in pituitary adenomas, our results support that CD151 is a plausible target for pituitary adenomas. Thus, the combination of anti-CD151 monoclonal antibodies, surgery, radiation, and chemotherapy might significantly improve the prognosis of pituitary adenomas.

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Disclosure of conflict of interest

None.

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