miR-200c inhibits the invasive activity of primary adamantinomatous craniopharyngioma cells

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Abstract

Backgrounds Craniopharyngiomas are benign epithelial tumors and difficult to complete due to the digitate brain infiltration. miR-200c has been studied in terms of development, stemness, epithelial-mesenchymal transition (EMT), and therapy resistance in many cancers. However, the role of miR-200c remains to be elucidated in adamantinomatous craniopharyngioma.

Methods Quantitative real-time polymerase chain reaction was used to evaluate the expression of miR-200c, ZEB1, ZEB2, and CTNNB1. Immunohistochemistry, Western blot, and immunofluorescence analyses were used to evaluate the expression of E-cadherin and β-catenin at the protein level. A Transwell assay was used to evaluate the invasiveness of ten primary craniopharyngioma cell.

Results miR-200c was significantly downregulated in adamantinomatous craniopharyngioma compared with papillary craniopharyngioma. Conversely, ZEB1, ZEB2, and CTNNB1 were overexpressed in adamantinomatous craniopharyngioma. Inhibition of miR-200c significantly promoted the invasion of primary adamantinomatous craniopharyngioma cells. Moreover, E-cadherin was overexpressed and β-catenin was downregulated in miR-200c mimic primary adamantinomatous craniopharyngioma cell culture.

Conclusion Our data demonstrated that miR-200c maybe reduce the invasive activity of adamantinomatous craniopharyngioma cells through E-cadherin/β-catenin. These findings suggest that the targets of miR-200c may regulate the EMT of adamantinomatous craniopharyngioma.

Background
Craniopharyngiomas are benign epithelial tumors that are histopathologically divided into adamantinomatous craniopharyngioma (ACP) and papillary craniopharyngioma (PCP) [1]. However, increased morbidity and mortality was associated with the invasion of adjacent structures, including the sella turcica, hypothalamus, optic nerves and third ventricle[2]. Long-term morbidities of craniopharyngioma include hypopituitarism, increased cardiovascular mortality, reduced quality of life and impaired cognitive function[3]. A craniopharyngioma is a benign tumor, but it shows a clinically malignant tumor-like outcome.

MicroRNAs (miRNAs), which are short single-stranded noncoding RNAs (~21 nucleotides in length), negatively regulate gene expression and induce mRNA degradation or translation inhibition[4]. miR-200c is a member of the miR-200 family, which consists of miR-200a, miR-200b, miR-200c, miR-141 and miR429. miR-200c has been demonstrated to be involved in many biological processes, including epithelial-mesenchymal transition (EMT), metastasis, cell invasion, proliferation, autophagy, apoptosis, and therapy resistance in several cancer types[5]. However, the role of miR-200c in craniopharyngioma remains to be elucidated.

In the present study, we hypothesized that miR-200c may reduce the invasive activity of adamantinomatous craniopharyngioma cells through EMT.

Methods

Patients and sample collection

This study was carried out after approval by the Ethics Committee of West China Hospital, Sichuan University (Sichuan China), and written informed consent was obtained from all participants. A total of ten tumor tissue samples were collected from patients with craniopharyngioma at West China Hospital, Sichuan University
(Sichuan China). The inclusion criteria were as follows:

(1) Primary surgical removal in the neurosurgical department of West China Hospital, Sichuan University;

(2) No preoperative or postoperative radiotherapy or chemotherapy;

(3) Histological diagnosis of craniopharyngioma.

Cell culture

Primary cell culture was performed as described previously[6]. Briefly, the tumor sample was washed with phosphate-buffered saline (PBS) containing 1% penicillin/streptomycin (Life Technologies, Gibco BRL, Grand Island, USA), dissected into pieces and digested with 0.25% trypsin (Sigma-Aldrich, St Louis, MI, USA) and 1 mg/mL collagenase II (Sigma-Aldrich, St Louis, MI, USA) for 30 minutes at 37°C in an incubated shaker. The cells were cultured with DMEM (Life Technologies, Gibco BRL, Grand Island, USA) containing 10% FCS (Life Technologies, Gibco BRL, Grand Island, USA) in an atmosphere at 37°C with 5% CO₂.

RNA isolation and quantitative reverse transcription PCR

Total RNA was extracted from samples using TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions. The complementary DNAs (cDNAs) were synthesized using the Reverse Transcription System Bestar qPCR RT Kit according to the manufacturer’s instructions with the ABI 7500 Real-Time PCR System (Applied Biosystems, Lincoln Center Drive Foster City, CA 94404, USA) and reverse-transcribed with M-MLV reverse transcriptase (Promega, Madison, Wisconsin, USA). U6 and GAPDH were used as internal controls. The primers were as follows:

miR-200b:

forward, ACACCTCCAGCTGGTGTAATACTGCCTGGTAA,

loop primer, CTCAACTGGTGTGAGTCGGCAATTTCATCATTGAG TCATCATT;
miR-200a:
forward, ACACCTCCAGTGGG TAACCTGCTCTGGTAA,
loop primer, CTCACACTGCTGGTACCGAATTCAGTTGAG ACATCGT;

miR-200c:
forward, ACACCTCCAGTGGG TAATATCTGCCGGTAA,
reverse, CTCACACTGCTGGTACCGAATTCAGTTGAGACCATCAT;

miR-429:
forward, ACACCTCCAGTGGG TAATATCTGCTTGTTA,
loop primer, CTCACACTGCTGGTACCGAATTCAGTTGAGACCTT;

miR-141:
forward, ACACCTCCAGTGGG TAACCTGCTCTGGTAA,
loop primer, CTCACACTGCTGGTACCGAATTCAGTTGAGCCATCTT;

ZEB1:
forward, ATGTGGCTAGTTTGCTCCTC,
reverse, AGCAAGATTTTCCCTCCAGT;

ZEB2:
forward, TCTGCGACATAAACGTAACGA,
reverse, GAGTGAAGCCTTGAGTGC;

CTNNB1:
forward, ACCCCATCTCTCGTCCTCCTC,
reverse, CCCCCTACAGCCTACCTTCT;

U6: forward, CTC GCT TCG GCA GCA CA,
reverse, AAC GCT TCA CGA ATT TGC GT;

GADPH:
forward, AAATTGAGCCCGCAGCTCCC,
reverse, GCGCCCAATACGACCACAAATCCGT.

**Transwell assay**

Transwell assays were performed according to the manufacturer’s instructions. Briefly, the cells (~5 x 10⁴) were harvested and resuspended in serum-free medium and then added to the Transwell upper chambers, in which the upper surface of the 8-μm pore size membrane (Corning, Steuben, New York, USA) was coated without or with Matrigel (BD Biosciences, Waltham, Massachusetts, USA). After incubation for 24 h, the cells that had migrated through the membrane were fixed, stained, and counted with an inverted microscope.

**Immunohistochemistry**

Immunohistochemical staining was performed as described previously[7]. Briefly, sections were blocked for endogenous peroxidase by incubation in 3% H₂O₂ for 20 minutes and then washed in PBS containing 0.05 M ethylenediamine tetraacetic acid (EDTA) followed by 4% paraformaldehyde. Then, the sections underwent heat-induced antigen retrieval for 20 minutes in 0.01 M citrate buffer (pH 6.0) in the microwave. Tissues were blocked with Blocking Serum from the ABC Vectastain Kit (Vector Labs, Burlingame, CA) for 20 minutes to block nonspecific binding. Then, 4 μm sections were incubated overnight at room temperature with anti-E-cadherin (Abcam) and anti-β-catenin antibodies (CST) in the presence of 10% rabbit serum. After the sections were washed, they were incubated for another 2 h with horseradish peroxidase (HRP) goat anti-rabbit IgG secondary antibody. Slides were dehydrated and examined under a light microscope.

**Western blotting**

Western blotting was performed as described previously[7]. Briefly, 50 μg of protein
lysate was loaded onto each well and resolved and then transferred to polyvinylidene fluoride (PVDF) membranes. The transferred membranes, blocked with 5% milk, were incubated overnight with primary antibodies against E-cadherin (Abcam) and β-catenin (CST) and washed with Tris-buffered saline with Tween-20 (TBST) (10 minutes, three times). The membranes were then probed with the appropriate secondary antibody (1:5000; Abcam). Immunoreactivity was determined and observed using enhanced chemiluminescence (Millipore, Billerica, MA, USA). β-Actin was used as a control. ImageJ was used to calculate the relative expression of E-cadherin and β-catenin at the protein level.

**Immunofluorescence**

Primary cells were washed with PBS and then fixed in 75% ethanol for 20 minutes at room temperature. After the cells were washed with PBS, they were blocked with 5% bovine serum albumin (BSA) for 1 h at 37°C. The cells were then incubated with a monoclonal anti-human E-cadherin (Abcam) and β-catenin antibody (CST) overnight at 4°C. The cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, 10 µg/mL, 32670; Sigma-Aldrich) to mark the nuclei. The cells were examined with a confocal laser scanning microscope.

**Statistical analysis**

All data are presented as the mean ± SEM. All experiments were performed at least three independent times. Using one-way analysis of variance followed by the Duncan’s multiple-comparison test using SPSS 19.0 (SPSS Inc., Chicago, IL), we calculated the statistical significance. P < 0.05, P < 0.01, or P < 0.001 was regarded as statistically significant.

Results
The invasive activity between adamantinomatous and papillary craniopharyngioma

Craniopharyngioma was defined as invasive if the tumor impaired the integrity of the brain parenchyma, hypothalamus, walls of the ventricles, cavernous sinus or optic chiasm or if it exhibited glio-arachnoidal adhesions[8]. To identify craniopharyngioma invasion, we examined the histological characteristics of the tumor tissues from a cohort of five ACPs and five PCPs. The results indicated that ACP had "whirl-like" or "island-like" cell clusters that penetrated into the surrounding brain tissue, showing obvious invasiveness (Fig 1a, as indicated by the black arrow); the PCP and the surrounding tissue boundary line were relatively flat, with no obvious signs of extension to the surrounding tissue (Fig 1b). Immunohistochemically, E-cadherin accumulation was stronger in PCP than ACP (Fig 1c-d).

The invasive activity between primary adamantinomatous craniopharyngioma cells and primary papillary craniopharyngioma cells.

To compare the invasive activity between the ACP and PCP, we performed Transwell assays of primary ACP tumor cells and PCP cells. We detected many more ACP cells that passed through the membrane than PCP cells (Fig 2a-c). Western blotting showed that the expression of E-cadherin was low and that β-catenin was high in primary ACP cells compared with primary PCP cells (Fig 2d-f). The expression levels of E-cadherin and β-catenin were significantly different between primary ACP and PCP cells. Immunofluorescence showed that β-catenin was distributed in the cytoplasm or nucleus of ACP but tended to be distributed in the cell membrane in PCP. The results indicated that E-cadherin was obviously downregulated in primary
ACP cell culture compared with primary PCP cell culture. In contrast, β-catenin was obviously upregulated in primary ACP cell culture compared with primary PCP cell culture (Fig 2g-h). These results suggest that ACP may be more aggressive than PCP.

**miR-200c is downregulated in adamantinomatous craniopharyngioma**

To confirm the involvement of miR-200c in craniopharyngiomas, we first tested the miR-200 family levels in five ACP and five PCP tissues. The results indicated that miR-200c expression was significantly lower in craniopharyngioma tissues than normal brain tissues, especially miR-200c expression, which was obviously lower in ACP than PCP (Fig 3a-b). Compared with the PCP tissues, the ACP tissues showed upregulation mRNA levels of ZEB1, ZEB2, and CTNNB1 (Fig 3c). These findings suggest that miR-200c may negatively regulate the expression of ZEB1, ZEB2, and CTNNB1 in ACPs.

**Increased expression of miR-200c inhibits adamantinomatous craniopharyngioma cell invasion**

To investigate the function of miR-200c in primary ACP cells, we performed Transwell assays and found that the invasion of primary ACP cells was higher than that of primary PCP cells (Fig 2c). The number of cells in miR-200c mimic group was significantly lower than that in miR-200c inhibitor group (Fig 4a-c). These findings indicated that miR-200c obviously decreased the invasion of ACP cells.

**miR-200c affects the expression of E-cadherin and β-catenin**

To explore the mechanism by which miR-200c inhibited invasion of primary ACP cells, we focused on the association between miR-200c and E-cadherin/β-catenin. Immunofluorescence was used to determine the relative expression of E-cadherin and β-catenin at the protein level. The results indicated that E-cadherin was
obviously downregulated by the miR-200c inhibitor and upregulated by the miR-200c mimic compared with the control. In contrast, β-catenin was obviously upregulated by the miR-200c inhibitor and downregulated by the miR-200c mimic compared with the control (Fig. 4 d-e). The expression levels of E-cadherin and β-catenin were significantly different in the miR-200c control, miR-200c mimic, and miR-200c inhibitor groups (Fig 5a). The expression of E-cadherin protein was the highest and β-catenin protein was the lowest in the miR-200c mimic group (Fig 5 b-c). These results suggest that miR-200c suppresses ACP invasive activity through E-cadherin/β-catenin.

Discussion

Recently, an increasing number of studies have reported the dysregulation of miRNAs in many types of cancer, indicating that the expression of proto-oncogenes or tumor suppressor genes is regulated by interactions between miRNAs and their corresponding mRNAs, which determine the malignant characteristics of tumor cells[9]. In the present study, we found that the expression levels of miR-200c were significantly downregulated in ACP tissues compared with PCP tissues. Notably, these data indicated that a low expression of miR-200c was clearly associated with the migration of ACP, which may explain the function of miR-200c in ACP. The results demonstrated that the miR-200c inhibitor markedly promoted the invasive capabilities of ACP cells. Taken together, these data demonstrated that miR-200c may be suppressed in the invasion of primary adamantinomatous cells.

In early studies, miR-200c overexpression was shown to promote the invasive phenotype of A549 cells through inducing the upregulation of E-cadherin and downregulating the expression of ZEB1[10], an important activator in EMT that
inhibits the expression of basement membrane components and cell polarity factors[11]. All of the miR-200 family members were shown to suppress EMT by activating E-cadherin expression through direct targeting of ZEB1 and its homeobox ZEB2 [11]. Interestingly, ZEB1 is not only a target of the miR-200 family but could also strongly inhibit miR-200c and another miR-200 family member, miR-141, and promote invasion of cancer cells. In our study, miR-200c was shown to inhibit the expression of ZEB1, ZEB2, and CTNNB1. Therefore, miR-200c may play an important role in the invasive activity of ACP.

E-cadherin, which is transcribed from CDH1 gene into a 135 kDa precursor polypeptide, plays crucial role in cell adhesion and cancer progression. It links to the polarized epithelial phenotype and tissue morphology[12]. Loss of the E-cadherin has been considered as the hallmark of the EMT. The previous research reported that E-cadherin and β-catenin can form a cadherin–catenin complex, which can stabilize the cell-cell contacts. Cadherin–catenin complex has been considered as the key regulator in the Wnt signaling pathway[13, 14]. CTNNB1 and β-catenin overexpression are frequently present in adamantinomatous craniopharyngiomas, whereas papillary craniopharyngiomas do not show these variations[15, 16]. Similarly, we found that miR-200c promoted the expression of E-cadherin and suppressed the expression of β-catenin at the protein level in ACPs. Therefore, these findings maybe demonstrate that miR-200c inhibit invasion through E-cadherin/β-catenin in ACP.

Conclusions

the present study provides evidence that miR-200c may play a potential role in the EMT of ACPs. The inhibition of miR-200c is closely associated with the migration of
primary ACP cells. The upregulation of miR-200c was closely linked to overexpression of E-cadherin and downregulation of β-catenin at the tumor invasive front of ACP. We conclude that miR-200c may play an important role in the EMT of craniopharyngioma; however, a precise understanding of the mechanisms of miR-200c regulating the E-cadherin and β-catenin in craniopharyngiomas requires further investigation.

Declarations

**Ethical approval:** All procedures performed in studies involving human participants were in accordance with the ethical standards of the Ethics Committee of West China Hospital, Sichuan University (Sichuan China) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from individual or guardian participants.

**Consent for publication**

Not applicable

**Availability of data and materials**

All data generated or analysed during this study are included in this published article.

**Competing interests**

The authors declare that they have no competing interests

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**Authors’ contributions:** "HL analyzed and interpreted the data and was a major contributor in writing the manuscript. DZ and ZL contribute to acquisition of the data. RCL and MT have substantively revised the manuscript. JGX have made
substantial contribution to design of research and was in charge of the research. All authors read and approved the final manuscript.

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Abbreviations

ACP: adamantinomatous craniopharyngioma
PCP: apillary craniopharyngioma

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Figures

Figure 1
Immunohistochemical demonstration of the invasive border of craniopharyngioma

Figure 2
The invasive activity between primary ACP cells and primary PCP cells. a-b, Trans
Figure 3

miR-200c is downregulated in adamantinomatous craniopharyngioma. a, qPCR analysis of miR-200 family expression in adamantinomatous craniopharyngioma and normal brain tissues. *P < 0.05. ACP, adamantinomatous craniopharyngioma; PCP, papillary craniopharyngioma.

Figure 4

Effects of miR-200c on adamantinomatous craniopharyngioma cell invasion. a, The relative expression of miR-200c in the miR-200c control, miR-200c mimic, and miR-200c inhibitor groups. 

Figure 5

Western blot analysis of the effect of miR-200c on the expression of E-cadherin and β-catenin protein in primary adamantinomatous craniopharyngioma cells. a, The protein expression of E-cadherin and β-catenin in the miR-200c control, miR-200c mimic, and miR-200c inhibitor groups. b-c, The relative protein expression of E-cadherin and β-catenin was evaluated via ImageJ in the miR-200c control, miR-200c mimic, and miR-200c inhibitor groups. *P < 0.05. ACP, adamantinomatous craniopharyngioma; PCP, papillary craniopharyngioma.