Original Article
Differential expression of miR-200c in breast cancer stem cells

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Abstract: Breast cancer has become the most popular malignant tumors in females. Breast cancer stem cells (BCSCs) play an important role in metastasis and recurrence. Previous study showed close correlation between miR-200c and behavioral regulation of breast cancer stem cells. This study aimed to investigate differential expression of miR-200c in breast cancer stem cells, and its role in regulation tumor formation property. DNA microchip assay and qRT-PCR analyzed differential expression of microRNA between BCSCs and non-tumor formation NTG cells. Bioinformatics and luciferase reporter gene assay determined targeted site of miR-200c. MiR mimic and inhibitor were transfected to change miR-200c expression level, followed by Western Blot for detecting BMI1 gene expression. Cell clonal assay determined cell proliferation, whilst tumor xenograft was performed to analyze tumor formation potency. MiR-200c was down-regulated in BCSCs compared to NTG cells (P<0.05). Bioinformatics and luciferase reporter gene assay attributed BMI1 gene as targeting site of miR-200c. Transfection of miR-200c and inhibitor altered miR-200c expression. Results showed that miR-200c could suppress BMI1 expression, and suppressed proliferation and tumor formation rate (P<0.05). Compared to NTG cells, BCSCs has significantly down-regulated expression of miR-200c. BCSCs facilitate BMI1 gene expression via inhibiting miR-200c expression, further elevating BCSCs proliferation and tumor formation potency.

Keywords: Breast cancer stem cells, MicroRNA-200c, cell proliferation, tumor formation

Introduction

Statistics showed breast cancer as the most popular female specific malignant tumors, and occupies about 29% of all female cancers [1]. Surgery is still the major treatment strategy, in addition to chemotherapy and hormone replacement, with chemotherapy as the major approach [2]. Common chemotherapy reagents including docetaxel and adriamycin frequently lead to drug resistance, which severely compromises treatment efficacy and increases patient's burden [3, 4]. Therefore, the study of mechanism underlying drug resistance of breast cancer is of critical importance for improving treatment efficacy and decreasing patient burden.

Tumor stem cells are a certain sub-type of cells in solid tumors, with self-renewal potency causing heterogeneity of tumors [5]. Tumor stem cells have multiple features similar as those of normal stem cells. Studies shown the important role of tumor stem cells in multiple pathology and cellular behaviors of malignant tumors [6]. The self-renewal and rapid proliferation potency of tumor stem cells are critical for tumor maintenance and recurrence. Meanwhile, multiple mechanisms inside tumor stem cells are closely correlated with drug resistance after routine tumor treatment [7, 8]. Therefore, the study of behavioral regulation of malignant tumor stem cells and related mechanism are of critical importance for identifying effective treatment against malignant tumors including breast cancer.

MiR-200c belongs to microRNA family. Previous study showed that microRNA could affect multiple cell behaviors via RNA interference mechanism [9]. By analyzing microRNA expressional profile of malignant tumor cells, it has been found to play an important role in regulating behaviors of malignant tumor cells [10]. Recently, study has shown important roles of

Table 1. qRT-PCR primer sequences

Name	Sequence
miR-200-F	5'-GGTTCTTCCCTGGGCTTC-3'
miR-200-R	5'-GAGTAGGAGCTCCGGATGTG-3'
U6-F	5'CTCGCTTCGGCAGCACA3'
U6-R	5'AACGCTTCACGAATTTGCGT3'

Figure 1. MiRNA expression in BCSCs by gene chip assay.

PCR miRNA test kits were purchased from Ambion (US). Real-time PCR was purchased from Bio-Rad (US).

Cell culture and miRNA expression profile analysis

BCSCs were cultured in improved DMEM culture medium containing 15% FBS. NTG and HEK293 cells were cultured in dual-resistant culture medium containing 10% FBS. Cells were cultured at 37°C with 5% CO_2.

BCSCs and HEK283 cells were collected and cultured. RNA extraction kit was used to collect total RNA. FlashTagBiotinHSR test kit (Affymetrix) was used for adding tail tag and biotin label on miRNA of sample RNA. MiRNA4.0 microarray was used for reaction after washing and rinsing. Collected data were analyzed by ExpressionConsole software for obtaining mean values and standard deviation (SD).

qRT-PCR

qRT-PCR primers were firstly designed based on miR-200c sequence (GeneBank access number, NR_029779) as shown in **Table 1**. Using total RNA extracted from HEK293 cells as the control, qRT-PCR was used to test miR-200c expression level in HTB-106 cells. MirVanat qRT-PCR miRNA test kit was used for qRT-PCR assay. Built-in software of instrument (version 2.02) was used for analyzing expression level by $2^{-\Delta\Delta Ct}$ method using U6 sequence as the internal control [13].

Functional prediction of miR-200c

TargetScan Release 5.1 (www.Targetscan.org) was used to predict functional targeted gene of miR-200c. Luciferase reporter gene assay was used to demonstrate possible targets of miR-200c. Based on 3'UTR sequences of BMI1 (Genebank access number, NM_005180), primers were synthesized as 5'-TATATC TAGAT TCTTG TTATT ACGCTG TTTTG-3' and 5'-AGATT CTAGA ATGTCA TATACC AATATG GC-3'. 3-UTR sequence of BMI1 mRNA was obtained by PCR amplification, followed by the insertion into downstream site of luciferase gene coding region of pmiRGLO plasmid to construct pmirGLO-BMI1 and pmirGLO plasmid. Those cells with successful transfection were transfected with miR-200c mimic to elevate miR-200c level
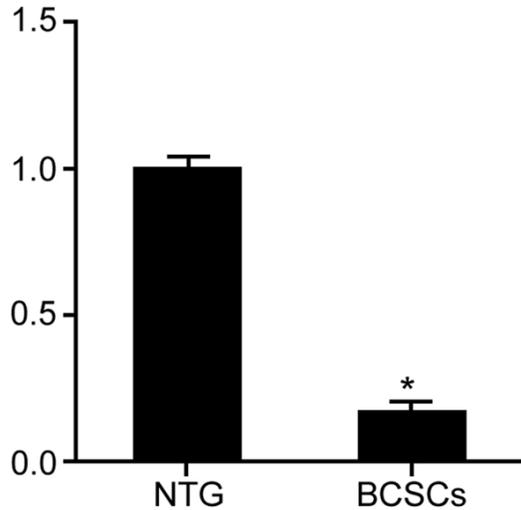
microRNA in regulating malignant tumor cells, as miR-200c and miR-222 had abnormal expression level in breast cancer cell lines with drug resistance [11, 12]. This study aimed to investigate differential expression of miR-200c in breast cancer stem cells (BCSCs) and related mechanism.

Materials and methods

Materials

BCSCs and non-tumor formation breast cancer cell NTG, plus HEK293 cells were purchased from Chinese Medical Academy. DMEM culture medium, penicillin and streptomycin were purchased from Gibco (US). RNA extraction kit was purchased from Qiagen (US). Improved DMEM medium was purchased from Invitrogen (US). Model GCS3000 microchip scanner and miRNA4.0 test array were purchased from Affymetrix (US). MirVanat qRT-

Figure 2. RT-PCR assay for BCSCs cell relative expression. *, P<0.05 compared to NTG cells.
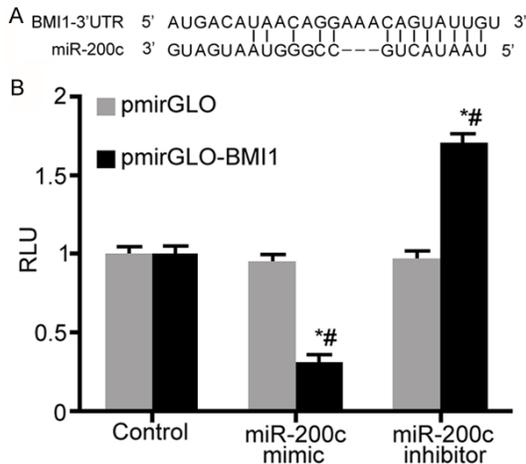

Figure 3. Correlation between miR-200c and BMI1 gene. A: Sequence homology between miR-200c and 3'UTR of BMI1 gene; B: Effects of miR-200c on BMI1 gene expression. *, P<0.05 compared to control group; #, P<0.05 compared to pmir-GLO vector.

as described below. 48 h after transfection, cells were analyzed for fluorescent intensity. Dual luciferase reporter gene assay system (Promega) and MicroLumatPlus LB96V spectrometry (Berthold) were used for analyzing fluorescent intensity.

Transfection of miR-200c

MiR-200c mimic or inhibitor sequence was transfected into BCSCs to elevate or suppress miR-200c expression level. MiR-200c mimic and inhibitor sequence were purchased from

Gimma (China). Liposome INTERFERin™ transfection kit (Polyplus transfection) was used for cell transfection. After culture, digestion, cells were counted and inoculated into 96-well plate for 24 h incubation. Transfection was performed according to manual instruction of test kit.

Western blot analysis

BCSCs with successful transfection were collected and lysed for extracting total proteins, which were tested for BMI1 expression in cells by Western Blot. Primary antibody (mouse anti-human BMI1 antibody at 1:1000 or anti-human β-actin at 1:1000) and secondary antibody (goat anti-mouse IgG at 1:200) were used. Western Blot results were analyzed by computer assisted gel imaging system to calculate SMI expression across different cell lines.

Cell clone formation assay

BCSCs cell line with successful transfection was cultured until log-growth phase. Cells were digested in trypsin and re-suspended into fresh culture medium. Cells were then inoculated into 10 mL fresh medium at 100 cells concentration in triplicates. Cells were incubated for 10 days. After that, culture medium was removed and cells were fixed in paraformaldehyde for staining in crystal violet. Staining buffer was removed, and clones were counted on the inverted culture dish with meshed film to calculate clonal formation rate = (clone number/100) ×100%.

Xenograft for human breast cancer cells

Breast cancer cell xenograft assay was performed to test the effect of miR-200c on tumor formation potency of BCSCs. Those cells with successful transfection were cultured at 37°C for 2 h, and were re-suspended for counting. Using NOD/SCID mice as research objects, 12 adult mice (6 weeks age, body weight at 32.6±2.5 g, 6 males and 6 females) were randomly assigned into three groups. Each mouse received 10^5 cells and was fed with normal diet. Animals were cultured at 25°C with 60 ±10%. Animal protocols followed guidelines of animal experimental stipulated by NIH.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA). Results were presented as mean ±
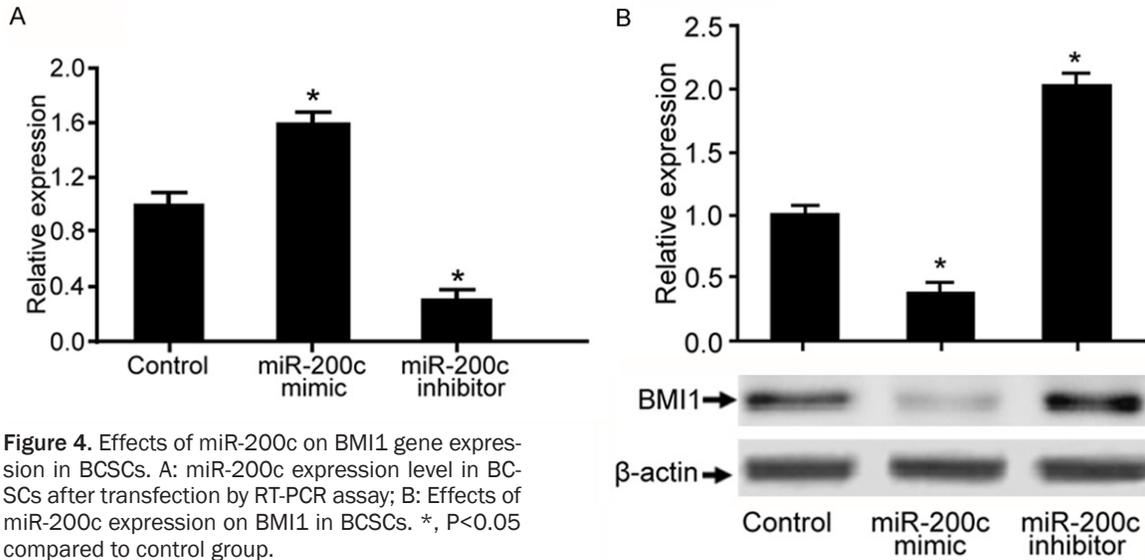
Figure 4. Effects of miR-200c on BMI1 gene expression in BCSCs. A: miR-200c expression level in BCSCs after transfection by RT-PCR assay; B: Effects of miR-200c expression on BMI1 in BCSCs. *, P<0.05 compared to control group.
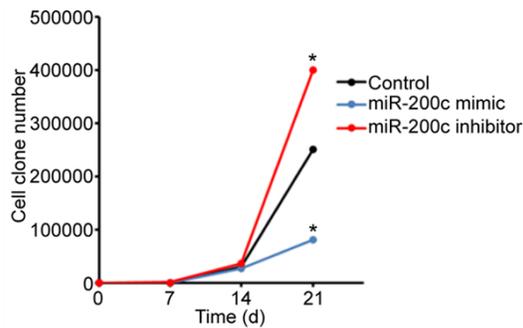

Figure 5. Cell clonal formation ability. *, P<0.05 compared to control group.

standard deviation (SD). SPSS20.0 was used for student t-test. A statistical significance was defined when P<0.05, and an extreme significance was defined when P<0.01.

Results

Differential expression of miRNA in BCSCs

Total RNA was extracted from BCSCs and NTG cells. MiRNA expression was examined by gene chip assay in triplicates. Expression Console software was used for analysis using miRNA expression in NTG cells as baseline level, to analyze miRNA expression in BCSCs. As shown in **Figure 1**, in BCSCs, expression levels of miR-214, miR-127, miR-1555 and miR-34b were significantly elevated (P<0.05), whilst miR-429, miR-183, miR-200a and miR-200c were all down-regulated (P<0.05).

qRT-PCR for miR-200c expression level

qRT-PCR was used to confirm results of gene chip assays. As shown in **Figure 2**, miR-200c expression level in BCSCs was significantly lowered (P<0.05 compared to NTG cells), as consistent with gene chip assay.

Relationship between miR-200c and BMI1 gene

TargetScan Release 5.1 was used to predict target genes of miR-200c. We found certain homology between miR-200c sequence and 3'UTR region of BMI1 (**Figure 3A**), thus predicting that BMI1 gene might be the target of miR-200c. We then constructed luciferase reporter gene expression system for confirmation. As shown in **Figure 3B**, we found significantly decreased fluorescent intensity after miR-200c mimic transfection (P<0.05), and elevated cellular fluorescent intensity after miR-200c inhibitor transfection (P<0.05), indicating that 3'UTR of BMI1 gene was functioning target of miR-200c.

Effects of miR-200c on BMI1 gene expression in BCSCs

By transfecting mimic or inhibitor, miR-200c expression was enhanced or suppressed in BCSCs. After transfection, cells were cultured until reaching log-growth phase. Total RNA was extracted from cell lines for RT-PCR assay measuring miR-200c relative expression level
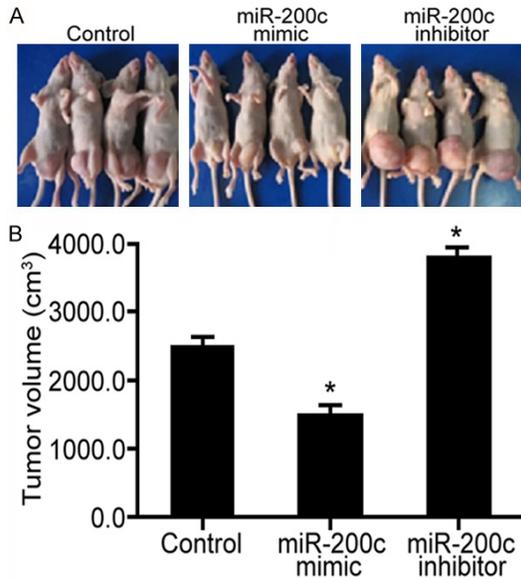
Figure 6. Tumor formation ability of BCSCs. A: Results of breast cancer cell xenograft; B: Tumor size of mouse breast cancer cell xenograft. *, P<0.05 compared to negative control group.

(**Figure 4A**), BMI protein expression level was shown in **Figure 4B**. Our results showed that miR-200c mimic and inhibitor could facilitate and inhibit miR-200c expression in BCSCs. Meanwhile, we also found significantly decreased BMI1 gene expression in BCSCs transfected with miR-200c mimic (P<0.05), whilst miR-200c inhibitor transfection remarkably facilitated BMI1 expression in BCSCs (P<0.05).

Proliferation potency of BCSCs

Cell clonal formation assay was performed to test effects of miR-200c mimic and inhibitor transfection on proliferation potency of BCSCs. As shown in **Figure 5**, after 21 days of cell culture, BCSCs with miR-200c mimic transfection had significantly decreased clonal formation potency (P<0.05 compared to control group). Whilst transfection of miR-200c inhibitor elevated clonal formation of BCSCs (P<0.05), indicating that miR-200c could inhibit proliferation activity of BCSCs.

Tumor formation potency of BCSCs

Tumor cell xenograft assay was performed to test tumor formation ability of BCSCs after transfecting with miR-200c mimic or inhibitor. As shown in **Figure 6**, we found significantly

lowered tumor formation potency of BCSCs in those transfected with miR-200c mimic (P<0.05 compared to control group). Whilst those transfected with miR-200c inhibitor had elevated tumor formation ability (P<0.05).

Discussion

Various studies have suggested major difference of microRNA expressions including miR-21, miR-10b and miR-200c between breast cancer cells and normal tissues [14-16]. Cancer cells are proposed to regulate pathology and cell behaviors of malignant tumors via changing normal expression of microRNA [15]. MiR-200c also plays an important role in proliferation of breast cancer cells [14]. Some studies also suggested the close correlation between microRNA expression and drug resistance of breast cancer cells [10]. It is commonly believed that tumor stem cells are critical factor for proliferation, metastasis and drug resistance of malignant tumors. Therefore, it is speculated that miR-200c plays an important role in behavioral regulation of BCSCs [6]. This study firstly analyzed miRNA expressional spectrum in NTG cells and BCSCs, and found down-regulation of multiple miRNA including miR-200c. We further employed bioinformatics prediction and luciferase reporter gene assay to determine functioning site of miR-200c as BMI1. Cell clonal assay and breast cancer xenograft assay found that miR-200c could inhibit proliferation or tumor formation potency of BCSCs.

Currently lots of studies showed down-regulation of miRNA in BCSCs, but leaving the mechanism of how malignant tumor cells achieve differential expression of miRNA is still debatable [16, 17]. Some studies treated tumor cells with demethylation reagent and found up-regulation of microRNA, thus proposing that methylation or other irreversible modification of transcription regulatory sequence caused different expression levels of microRNA in malignant tumor cells [17]. Moreover, some studies also showed that expression products of certain targeted genes of miRNA can also regulate miRNA expression, although more evidences are required as support [18].

As one important regulatory gene in animal cells, BMI1 gene participates in embryonic development of mammalian bones and nervous system [19]. BMI1 has close correlation with

proliferation of normal stem cells. It is also one oncogene as it can facilitate self-renewal and proliferation of tumor stem cells [20]. BMI1 gene is found to be up-regulated in various human malignant tumor cells including breast cancer [21]. Previous findings showed that the binding between BMI1 and c-Myc can inhibit Ink4a/Arf gene loci to regulate $p16^{Ink4a}$ and $p19^{Arf}$ transcription, eventually inhibiting cell aging, facilitating cell proliferation and self-renewal of stem cells [22]. This study showed that miR-200c could suppress BMI1 gene expression. Meanwhile, miR-200c was found to be down-regulated in BCSCs. Therefore up-regulation of BMI1 gene expression eventually facilitates proliferation and tumor formation ability of BCSCs.

As the most common female specific malignant tumor, breast cancer severely threatens women health [1]. The recurrence, metastasis and drug resistance of breast cancer mainly depends on BCSCs. Therefore, the inhibition of BCSCs is of critical importance for treating breast cancer. This study found significantly suppressed miR-200c expression in BCSCs, which can up-regulate BMI1 gene expression via suppressing miR-200c expression, thus facilitating proliferation and tumor formation ability of BCSCs. This conclusion may provide novel insights for future treatment of breast cancer. By molecular biology approach, one can elevate miR-200c expression in BCSCs, thus inhibiting recurrence and metastasis of breast cancer.

Conclusion

MiR-200c is down-regulated in BCSCs, thus elevating target gene BMI1 expression level and enhancing proliferation and tumor formation potency of BCSCs.

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Disclosure of conflict of interest

None.

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