ORIGINAL ARTICLE

hsa-miR- 574-5p Down Regulates the Expression of CBR1 in A549 Cells and Enhances their Invasive Ability

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ABSTRACT

Objective To explore the impact of CBR1 expression on A549 cell proliferation and invasive ability, and investigate the regulatory function of hsa-miR-574-5p on these processes.

Methods A549 cells with high and low CBR1 expression were established by transfection with a CBR1-pENTER plasmid and an hsa-miR-574-5p analog, respectively, MTT assays were performed to measure cell proliferation ability, RT-PCR and western blot were used to detect the expression of CBR1, and E-cadherin, and transwell migration assays were used to detect the invasive ability of the cells.

Results Cells transfected with CBR1-pENTER showed increased CBR1 and E-cadherin expression and reduced invasive ability; those transfected with hsa-miR-574-5p showed reduced CBR1 and E-cadherin expression and increased invasive ability. There was no significant difference in proliferation ability among the groups.

Conclusions In A549 cells, the expression of CBR1 is irrelevant to cell proliferation ability but affects invasive ability; hsa-miR-574-5p can enhance the invasive ability of cells by down regulating CBR1 expression; hsa-miR-574-5p may not only be biological marker for diagnosis, but also may be an important prognostic index for patients with non-small cell lung cancer.

KEYWORDS A549 cells, carbonyl reductase 1, hsa-miR-574-5p, invasive ability, non-small cell lung cancer

INTRODUCTION

Carbonyl reductases (CBRs) are widely distributed in the cytoplasm of cells from various tissues including the liver, heart, kidney, brain, ovary, adrenal gland, testis and blood, in mammals and non-mammals[1]. They exist in both dissociated and membrane-bound states[2–4]. Recently, it was discovered that the human carbonyl reductase gene family includes four members: carbonyl reductase 1 (CBRl), DCXR(known as HCR2 and CBR2), CBR3 and CBR4, among these, CBR1 shows the highest expression levels in various tissues.

Changes in human CBR1 activity occur during tumorigenesis in different tissues, activity was shown to increase in lung cancer, colon cancer, and breast cancer tissues[5,6]; but decreased in primary liver cancer and prostate cancer[7,8]. In some cancers, the expression of CBR1 is related to prognosis. For example, low expression of CRB1 increases the invasive ability of endometrial carcinoma cells and correlates with reduced patient survival[9], in non-small cell lung cancer, patients with reduced CBR1 expression also had shorter survival periods[10]. It has not been reported whether low expression of CBR1 in A549 tumor cells can increase their invasive ability. Patients with non-small lung cancer also showed increased, serum levels of hsa-miR-574-5p[11], a microRNA that can reduce CBR1 expression in Chinese hamster ovary cells and human lymphocytes[12]. Elucidating whether hsa-miR-574-5p can impact the invasive ability of non-small cell lung cancer cells through the regulation of CBR1 is worthy of investigation, with a view to using it as a prognostic marker.

In this study, we established a high-CBR1 expression model, by transfecting a CBR1-pENTER vector into A549 cells, and investigated cell proliferation and invasive ability. Cells were also transfected with hsa-miR-574-5p to investigate its regulatory effects on CBR1 expression in A549 cells, and the subsequent impact on the invasive ability of A549 cells.

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MATERIALS AND METHODS

Cell lines

A549 cells from human non-small cell lung cancer cell lines were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cell lines were maintained at 37°C and 5% CO_2 in RMPI 1640 (Thermo), supplemented with 10% heat inactivated fetal bovine serum (Gibco).

Transfection

The A549 cell is divided into three groups, CBR1-pENTER group, hsa-miR-574-5p group and control group. The CBR1-pENTER group: Inoculated cells 1.5×10^5/hole in 24-well plates with each hole adding 500 µl antibiotic-free medium, discard nutrient solution before transfection. Refer to the instruction manual of Lipofectamine TM 2000 transfection kit (Invitrogen), transfect A549 cell with CBR1-pENTER plasmid (4 µg) (Vigenebio, Shandong, China), after transfection for 48 hours, Collect cells for spare; The hsa-miR-574-5p group: inoculated cells 1.5×10^5/hole in 24-well plates, with each hole adding 500 µl antibiotic-free medium, discard culture medium before transfection. Refer to the instruction manual of LipofectamineTM2000 transfection kit (Invitrogen), use hsa-miR-574-5p (15 nM) analogue (mimics) (Ribobio, Guangzhou, China) to transfect A549 cells, after transfection for 48 hours, collect cells for spare. The control group: normal A549 cells.

RT-PCR

The total RNA from A549 cells was extracted and reverse transcribed in complementary (c)DNA using the PrimeScript® RT Master Mix (Perfect Real Time) kit (Takara, Dalian, China), RT products were then used as templates to examine the expression of CBR1 using real-time PCR with the SYBR® Premix Ex TaqTM II (Perfect Real Time) kit (Takara, Dalian, China). The oligonucleotide primer sequences were as follows: CBR1, 5'-CTGATCCCACACCCTTTCAT-3' (forward); 5'-TTAAGGGCTCTGACGCTCAT -3'(reverse); β-actin, 5'-CGTCTTCCCCTCCATCGT-3'(forward); 5'-GAAGGTGTGGTGCCAGATTT-3'(reverse). Methods CBR1-mRNA was tested with the real-time fluorescent quantitative PCR (Bio-Rad, Hercules, CA), and use instrument matching software to record Ct value of each sample. β-actin, an endogenous housekeeping gene was used to normalise Ct (cross-over threshold) values for A549 experiments, $-2^{\Delta\Delta Ct}$ method to compare the relative expression volume of CBR1 mRNA for different samples. CBR1mRNA values were expressed relative to the normalized CBR1mRNA content of negative control-transfected cells.

Western blotting

Cells were lysed using Cell lysis buffer for Western and IP (Beyotime Institute of Biotechnology, Jiangsu, China). BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Jiangsu, China) was used to detect protein concentration. These samples were boiled for 10 min and insoluble materials were removed by centrifugation at 15,000 rpm for 10 min at 4°C. About 200 µg of total protein was electrophoresed on a 10% of SDS–polyacrylamide gel and electro-blotted to polyvinylidene fluoride (PVDF) membranes. Membranes were incubated with antibodies included anti-E-cadherin (Sangon Biotech, Shanghai, China), Vimentin (Sangon Biotech, Shanghai, China), and β-actin (Sangon Biotech, Shanghai, China), overnight, Blots were visualized with an enhanced chemiluminescent method and analyzed using a gel image analysis system.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The A549 were mechanically disrupted. The mechanically disrupted cells were seeded into 96-well plates at a cell density of 5×10^3 cells/ml, followed by cultivation in RMPI 1640 (Thermo) with 15% FBS at 37°C under 5% carbon dioxide for 24 h, 48 h, and 72 h, respectively. Subsequently, the culture medium was replaced with 5 mg/ml of MTT solution (GBCBIO Technologies, Guangzhou, China) diluted in PBS. The plates were incubated again for 4 h at 37°C and a volume of 150 µl DMSO was added to each well. The plates were then agitated for 10 min to ensure the dissolution of any remaining crystals. Optical density (OD) was measured at 490 nm (A490).

In vitro *invasion assay*

Invasion assay was used to determine cell invasion. Cells were cultured in 24-well culture plates. An insert was placed into each well of the plate, effectively creating a lower chamber and upper chamber. The bottom of the insert was made of 8 µm pore size polycarbonate membranes which were either coated or not coated with Matrigel (6 µg/ml). 40 µl (BD Biosciences, NY, USA). The cells (the control group, CBR1-pENTER group, and hsa-miR-574-5p group) were then cultured in the culture medium without FBS at a density of 2×10^5 cells/well in the upper chamber, and the lower chamber was filled with the culture medium supplemented with 10% FBS. After incubation for 24 h, the upper surface of the membranes was wiped with cotton swabs to completely remove the cells. The filters were fixed and stained with xylene for 10 minutes. Dye it with crystal violet (GBCBIO Technologies, Guangzhou, China) for 5 minutes. After that, rinse with distilled water for three times; The cells on the lower surface were counted at

400 × magnification in five randomized field views. Each experiment was done in triplicate and calculated the mean ± standard deviation.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD) of three independent experiments, each performed in duplicate triplicate. Significant differences between groups were statistically analyzed using Student's *t* test. Differences in *P* values below 0.05 were considered significant. All tests were performed with SPSS 16.0 software (SPSS Inc., Chicago, IL) and Microsoft Excel (Microsoft Corporation, Redmond, WA, USA).

RESULTS

Establishment of CBR1 high-expression and low-expression cell model in A549 cells

After transfecting CBR1-pENTER plasmid in A549 cell for 48 h, quantitative RT-PCR gene expression detection of CBR1mRNA showed that the expression CBR1mRNA increases (1.04 ± 0.10 versus 1.51 ± 0.08, $P = 0.004$) (Fig. 1A), CBR1 protein expression increases (1.13 ± 0.17 versus 1.47 ± 0.08, $P = 0.035$) (Fig. 1B), the difference has statistical significance. This result suggests that CBR1 high-expression cell model was successfully established.

After transfecting hsa-miR-574-5p analog in A549 cell for 48 h, quantitative RT-PCR gene expression detection of CBR1mRNA showed that the expression CBR1mRNA declines(1.04 ± 0.10 versus 0.74 ± 0.06, $P = 0.013$) (Fig. 1A), CBR1 protein expression declines (1.13 ± 0.17 versus 0.74 ± 0.11, $P = 0.029$) (Fig. 1B), the difference has statistical significance. This result suggests that CBR1 low-expression cell model was successfully establish.

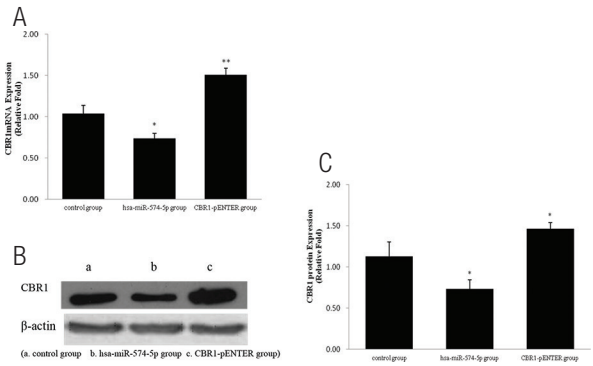

Fig. 1 Carbonyl reductase 1(CBR1) expression. (A) CBR1 mRNA expression of A549 cells transfected with CBR1-pENTER or hsa-miR-574-5p and untreated control cells (B) Representative and quantitative western blot results of CBR1 protein expression in the different cell groups, (C) Shown first are representative western blots of CBR1and β-actin protein, three independent experiments. The results are presented as the means ± SD, Statistical significance was determined using student's *t*-test. $^{*}P < 0.05$. $^{**}P < 0.01$.

The expression of CBR1 is irrelevant to cell proliferation ability in A549 cells

The MTT OD value can be found in Table 1. After transfecting CBR1-pENTER plasmid or hsa-miR-574-5p analogue (mimics) in A549 cell for 48 h, no significant difference in the absorbance was observed at 24, 48 h or 72 h among the three groups (Fig. 2). But for each group, the OD value increases with time. There are significant changes in cell proliferation ability within 72 hours. These results suggest that in A549 cells, whether transfected CBR1-pENTER plasmid or hsa-miR-574-5p analogue (mimics), the proliferation of cells does not change, the expression of CBR1 is irrelevant to cell proliferation ability.

Expression of CBR1 is relevant to cell invasive ability in A549 cells

After transfecting hsa-miR-574-5p analog in A549 cell for 48 h. The migration capacity of A549 increases and the number of cells that permeated through the artificial membrane was less than that of the control cells (123.67 ± 13.20 versus 82.66 ± 9.71, respectively; $P < 0.05$) (Fig. 3A). On the other hand, after transfecting

Table 1 MTT value.

	24 h	48 h	72 h
control group	0.63 ± 0.03	1.04 ± 0.05	1.65 ± 0.05
hsa-miR-574-5p group	0.70 ± 0.03	0.93 ± 0.06	1.73 ± 0.02
CBR1-pENTER group	0.64 ± 0.04	0.87 ± 0.08	1.68 ± 0.05

Values are shown as the mean ± standard deviation, MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

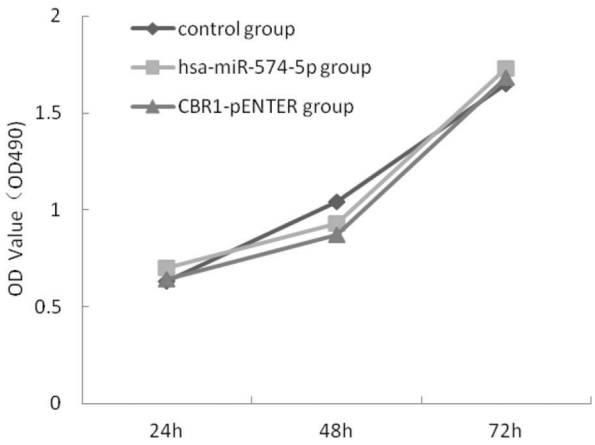

Fig. 2 Determination of cell proliferative potential by MTT assay. For each group, the OD value increases with time. The three groups of cells showed roughly the same proliferative potential in 72 h. $P = 0.947$, Control group versus hsa-miR-574-5p group; $P = 0.848$, Control group versus CBR1-pENTER group; $P = 0.803$, hsa-miR-574-5p versus CBR1-pENTER group. All $P > 0.05$. Values are shown as the mean, MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, OD optical density.

CBR1-pENTER plasmid in A549 cell for 48 h. The migration capacity of A549 declined and the number of cells that permeated through the artificial membrane was less than that of the control cells (55.67 ± 11.59 versus 82.66 ± 9.71, respectively; $P < 0.05$) (Fig. 3A).

For this result, we then examined epithelial-to-mesenchymal transition (EMT)-associated proteins, factor. Representative western blot results indicate the expression levels of the epithelial to mesenchymal transition (EMT) markers E-cadherin and vimentin in the different groups. In hsa-miR-574-5p group, E-cad expression declined, Vimentin expression increased (Fig. 3B). In CBR1-pENTER group, E-cad expression increased, Vimentin expression declined (Fig. 3B), These results suggest that CBR1 affects the ability of cell invasion to be altered by the EMT pathway.

DISCUSSION

CBR1 activity increases after lung cells become cancerous. Lopez de Cerain et al. showed that the activity in lung cancer is 2–40 times higher than that in normal tissues[6]. Schlager and Powis also found that CBR1 activity significantly increases after lung cells become cancerous[13]. However, low expression of CBR1 may not be an advantageous clinical sign. Research by Takenaka et al. on 59 cases of non-small lung cancer found that the survival rate of patients with high CBR mRNA expression after surgery was higher than that of the patients with lower expression in tumor tissues[10]. CBR1, as prostaglandin (PG) 9-ketoreductase, can metabolize PGE2 to PGF2a[14]. PGE2 can increase the expression of vascular endothelial growth factor, facilitating neovascularization and aggressive tumor invasion and metastasis. After metabolism by CBR1, PGE2' activity is reduced, which impacts tumor invasion and metastasis. This could explain patients with high CBR1 expression demonstrating a higher survival rate.

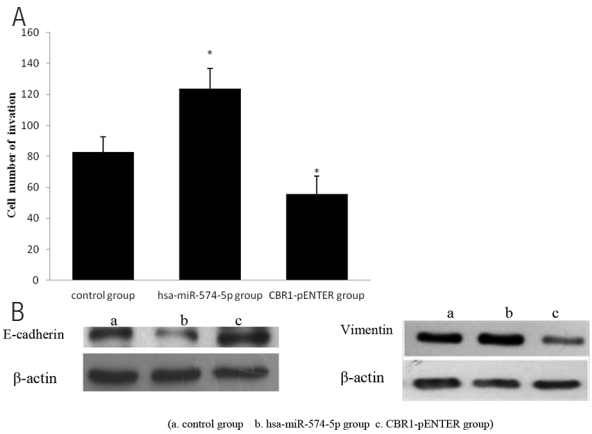

Fig. 3 Different A549 groups tumor invasion *in vitro*. (A) The number of cells that crossed the Transwell invasion chamber in different groups. (B) Representative western blot results indicating the expression levels of the epithelial to mesenchymal transition (EMT) markers E-cadherin and vimentin in different groups. The results are presented as the means ± SD, three independent experiments. Statistical significance was determined using student's *t*-test. *$P < 0.05$.

Our high CBR1 expressing A549 cell model showed that the level of CBR1 expression is irrelevant for cell proliferation. This suggests that the higher postoperative survival rate shown by patients with non-small cell lung cancer who have higher CBR1 expression is not due to the impact of CBR1 expression differences on proliferative ability. Instead, changes in CBR1 levels are more likely to cause changes in invasive ability. Our model showed that with increasing CBR1 expression, cell invasive ability reduces and E-cad expression increases. In the low CBR1 expression cell model, established using hsa-miR-574-5p transfection. With reduced CBR1 expression, cell invasive ability is enhanced and E-cad expression decreases. This result corroborates with endometrial carcinoma cells that was reported by Murakami et al. After establishing a high CBR1 expression model, cell invasive ability decreased and E-cad expression increased with their low CBR1 expression model. Cell invasive ability increased and E-cad expression decreased[9].

Research using Chinese hamster ovary cells and human lymphocytes showed that hsa-miR-574-5p can interact with CBR1 at the 3'-untranslated region and reduce its expression[12]. In the current study, A549 cells transfected with hsa-miR-574-5p showed reduced CBR1 expression, which indicates that the hsa-miR-574-5p mechanism for regulating CBR1 expression mechanism exists in A549 cells. In addition, research by Foss et al. found significantly increased serum has-miR-574-5p levels in patients with non-small lung cell cancer, suggesting that hsa-miR-574-5p could be a biological marker for non-small cell lung cancer diagnosis. Our results showing that hsa-miR-574-5p can reduce the expression of CBR1 in A549 cells and increase cell invasive ability as well as reducing E-cad expression suggesting that hsa-miR-574-5p may not only be a biological marker for non-small cell lung cancer diagnosis, but also an important index of prognosis for patients with non-small cell lung cancer. Yanaihara et al. detected multiple miRNAs in patients with non-small cell lung cancer and found that high expression of hsa-miR-155 and low expression of hsa-let-7a-2 were associated with a poor prognosis[15], however, their studies did not include hsa-miR-574-5p, and to date no study correlating hsa-miR-574-5p expression with non-small cell lung cancer prognosis has been reported. According to the results of our study, it is possible that patients with non-small cell lung cancer, who have higher serum hsa-miR-574-5 levels will have decreased expression of CBR1 and increased cancer cell invasive ability, which would cause these patients to demonstrate a lower survival rate.

In summary, in A549 cells, CBR1 expression is relevant to cell invasive ability; hsa-miR-574-5p can reduce CBR1 expression and subsequently enhance cell invasive ability. hsa-miR-574-5p may not only be a biological

marker for non-small cell lung cancer diagnosis, but also a very important prognostic index for patients with non-small cell lung cancer. These conclusions are limited by the in vitro nature of our study, and further investigation is required to obtain evidence by clinical observation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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