

Long non-coding RNA BRE-AS1 inhibits proliferation, migration and invasion of clear cell renal cell carcinoma by downregulating miR-106b-5p

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Summary. Objective. The objective of this study was to investigate the involvement of the long non-coding RNA (lncRNA) BRE-AS1 in clear cell renal cell carcinoma (ccRCC) and to explore its potential therapeutic role.

Methods. The expression of BRE-AS1 and miR-106b-5p was determined by qRT-PCR. Overexpression of BRE-AS1 and miR-106b-5p were performed to study their relationship. Transwell assays were used to evaluate cell movement. Methylation-specific PCR (MSP) was performed to explore the role of BRE-AS1 in the methylation of the miR-106b-5p gene.

Results. The results showed that the expression levels of BRE-AS1 were decreased, while those of miR-106b-5p were increased in ccRCC tissues. BRE-AS1 was found to be closely associated with the prognosis of patients with ccRCC. The expression of BRE-AS1 was inversely correlated with that of miR-106b-5p in tumor tissues. Overexpression of BRE-AS1 led to decreased expression levels of miR-106b-5p and increased methylation of the miR-106b-5p gene, whereas miR-106b-5p did not affect the expression of BRE-AS1. BRE-AS1 inhibited the movement and proliferation of ccRCC cell lines, while miR-106b-5p suppressed the role of BRE-AS1.

Conclusion. BRE-AS1 may suppress ccRCC by downregulating the expression of miR-106b-5p.

Key words: ccRCC, BRE-AS1, miR-106b-5p, Survival

Introduction

Studies on human genome sequences have shown that only 1.2% of the human genome is responsible for encoding protein products (Carninci et al., 2005; Birney et al., 2007), and there are over 7,000 genes that encode for long non-coding RNAs (lncRNAs) which lack the ability to encode proteins (Johnson et al., 2005; Furuno et al., 2006). lncRNAs play pivotal regulatory roles in the form of RNAs (Fatica and Bozzoni, 2014). lncRNAs have been found to play a role in regulating cancers, and some specific lncRNAs may potentially serve as targets for cancer treatment (Spizzo et al., 2012). However, the clinical application of lncRNAs in cancer therapy is limited due to the unknown functionality of most of them.

Renal cell carcinoma (RCC) accounts for 90% of renal cancers (Garcia and Gottschalk, 2018). RCC is commonly considered as a major cause of death in clinical practices (Hsieh et al., 2017). With the improvement of surgical management and increased understanding of the molecular mechanisms of this disease, treatment outcomes of RCC have been significantly improved during the past 15 years (Jonasch et al., 2014). However, the survival rates of some RCC patients remain poor, particularly for those who have developed tumor metastasis (Jonasch et al., 2014). The occurrence and development of RCC involve in multiple signaling pathways, which can be targeted to treat cancers. lncRNAs have also been shown to play critical roles in RCC (Blondeau et al., 2015). However, the functions of most lncRNAs in RCC remain unclear. It is reported that lncRNA BRE-AS1 could inhibit the development of several cancers, such as lung cancer and breast cancer (Zhang et al., 2018). In the field of cancer biology, BRE-AS1 has been found to have a dual function of upregulating tumor suppressors, such as NR4A3, and also acting as a sponge for oncogenic microRNAs (miRNAs), such as miR-21, thereby inhibiting cancer progression. However, the role of BRE-AS1 in RCC is unknown. In

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our preliminary study, we observed increased expressions levels of BRE-AS1 in RCC and its significant correlation with miR-106b-5p (data not shown), which is a characterized oncogenic miRNA in ccRCC, where miR-106b-5p targets SETD2 to promote cell proliferation and inhibits cell apoptosis (Xiang et al., 2015). Therefore, we hypothesized that BRE-AS1 may inhibit clear cell RCC (ccRCC, a major subtype of RCC) by downregulating miR-106b-5p, potentially through methylation.

Materials and methods

Subjects

A total of 78 patients (30 females and 48 males, mean age 50.2±6.4 years old) with ccRCC were enrolled at the Second Affiliated Hospital of Nanchang University from May 2010 to May 2013. Ethical approval was obtained from the Ethics Committee of this hospital. Diagnosis of ccRCC is performed by combined techniques, such as imaging and biopsy. All patients signed the informed consent. Based on AJCC staging method, the 78 patients were grouped into stage I (n=12), II (n=20), III (n=26) and IV (n=20), respectively.

Tissues and cell lines

Biopsies of tumor tissue and adjacent healthy tissue were obtained from each participant, and all specimens were confirmed by three experienced pathologists. Additionally, two ccRCC cell lines (Caki-2 and 786-O) and a normal renal cell line (HK-2) obtained from ATCC were used. Cells were cultivated and collected following the protocols from ATCC.

Treatment and follow-up study

Treatment strategies, such as surgical resection, chemotherapy, radiotherapy and their combinations were applied. Patients were followed up every month for 5 years. Overall survival conditions were recorded.

Cell transfection

BRE-AS1-expressing vector (pcDNA3.1) and Hsa-miR-106b-5p miRNA and NC miRNA were prepared by Sangon. Cell transfections were performed using Lipofectamine 2000 with 10 nM vectors or 50 nM miRNAs. Briefly, vector and/or miRNA were first mixed with Lipofectamine 2000 to prepare the transfection mixture, which was then mixed with cells and incubated for 6 h to achieve transfection. After that, cells were washed with fresh medium immediately to reduce cytotoxicity.

RT-qPCR

RNA samples were prepared using RNazol (Sigma-Aldrich). After the synthesis of cDNA using SSRT-IV kit (ThermoFisher Scientific), RT-qPCR was performed

using SYBR qPCR kit (Toyobo) to detect the expression of BRE-AS1 and miR-106b-5p with GAPDH and U6 as the internal control, respectively. The expression levels of BRE-AS1 and miR-106b-5p were calculated using the $2^{-\Delta\Delta CT}$ method.

Cell proliferation assay

A 96-well plate was used to cultivate cells (3×10^3 cells in 0.1 ml per well). CCK-8 (10 μ l) was added every 24h until 96h. OD values were measured at 450 nm at 4h after the addition of CCK-8. OD values of each group at each time point were normalized to C group at 24h to calculate the relative cell proliferation.

Transwell assay

To study cell movement, Transwell assays were conducted. Cells were seeded in the upper chambers of the Transwell inserts and cultivated in non-serum medium. To induce cell movement, FBS was added to the lower chamber at a concentration of 10%. Cells were analyzed after staining the membrane with 0.5% crystal violet. Invasion and migration assays were performed using the same method. The only difference is that Matrigel (0.4 mg/ml) was used to coat the inserts at room temperature for 24h prior to invasion assay mimic *in vivo* invasion conditions, while uncoated inserts were used in migration assay.

Methylation-specific PCR

Following transfection, genomic DNA was isolated from the cells. The isolated DNA was then subjected to conversion using the EpiScope MSP Kit (Takara Bio) followed by MSP and routine PCR using Taq polymerase (NEB) to amplify the methylated (M) and unmethylated (U) promoter region of miR-106-5p.

Western blot analysis

Cells were collected and total proteins were isolated using RIPA solution (Sangon). BCA assay was performed to determine protein concentration, followed by protein separation using 10% SDS-PAGE gels. After gel transfer (PVDF membranes) and blocking, the membranes were incubated with SETD2 (ab184190, Abcam) and GAPDH (ab8245, Abcam) in a cold room for 16h. After that, incubation with anti-rabbit IgG-HRP secondary antibody (1:1,000, MBS435036, MyBioSource) was performed at room temperature for 4h. Finally, signal was produced using ECL solution (Invitrogen). MyECL imager (Thermo Fisher) was used to capture signals.

Statistical analysis

Data analysis and image preparations were performed using GraphPad Prism 6 software. Two groups were compared by Student's t test. Based on

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Youden's index, patients were divided into high (n=35) and low (n=43) BRE-AS1 level groups. Survival curves were plotted and compared by log rank t test. Chi-squared test was used to analyze the association between the expression levels of BRE-AS1 and patients' clinical data. Differences with $p < 0.05$ were considered as statistically significant.

Results

The expression of BRE-AS1 and miR-106b-5p in ccRCC tissues and cell lines

Gene function is determined by gene expression levels. Therefore, the expression levels of BRE-AS1 and miR-106b-5p were determined in tissue samples from 78 ccRCC patients using RT-qPCR prior to any treatment. Compared to healthy tissues, BRE-AS1 was significantly downregulated in ccRCC tissues (2.11-fold, Fig. 1A, $p < 0.05$), while miR-106b-5p was highly upregulated in ccRCC tissues (1.72-fold, Fig. 1B, $p < 0.05$). Moreover, the expression of BRE-AS1 and miR-106b-5p in ccRCC cell lines Caki-2 and 786-O, as well as the normal renal cell line HK-2 were measured. Consistently, BRE-AS1 was downregulated in ccRCC cell lines (Fig. 1C, $p < 0.05$), while miR-106b-5p was highly upregulated in ccRCC cell lines (Fig. 1D, $p < 0.05$). It is worth noting that the expression levels of BRE-AS1 and miR-106b-5p were

not significantly different among tumor grades and stages (data not shown). Therefore, altered expression of BRE-AS1 and miR-106b-5p may participate in ccRCC.

Downregulation of BRE-AS1 in tumor tissues is closely correlated with poor survival

A 5-year follow-up was performed on all 78 ccRCC patients to explore the prognostic value of BRE-AS1 for ccRCC. It showed that no significant difference was found in treatment strategies between high and low BRE-AS1 level groups. Survival analysis showed that patients in the low BRE-AS1 group experienced more deaths (Fig. 2). Therefore, downregulation of BRE-AS1 may serve as a biomarker for the poor prognosis of ccRCC patients. Chi-squared test showed that BRE-AS1 was not associated with patients' clinical stages (stage I, II, III and IV), gender (males or females), age ($>$ or ≤ 50 years old), BMI ($>$ or ≤ 24), smoking habit (yes or no) or drinking habit (yes or no) (all p values < 0.05).

Correlations between BRE-AS1 and miR-106b-5p

Correlations may suggest possible interactions. To explore the potential interaction between BRE-AS1 and miR-106b-5p, associations between BRE-AS1 and miR-106b-5p were evaluated using Pearson's correlation coefficient. It was observed that BRE-AS1 and miR-

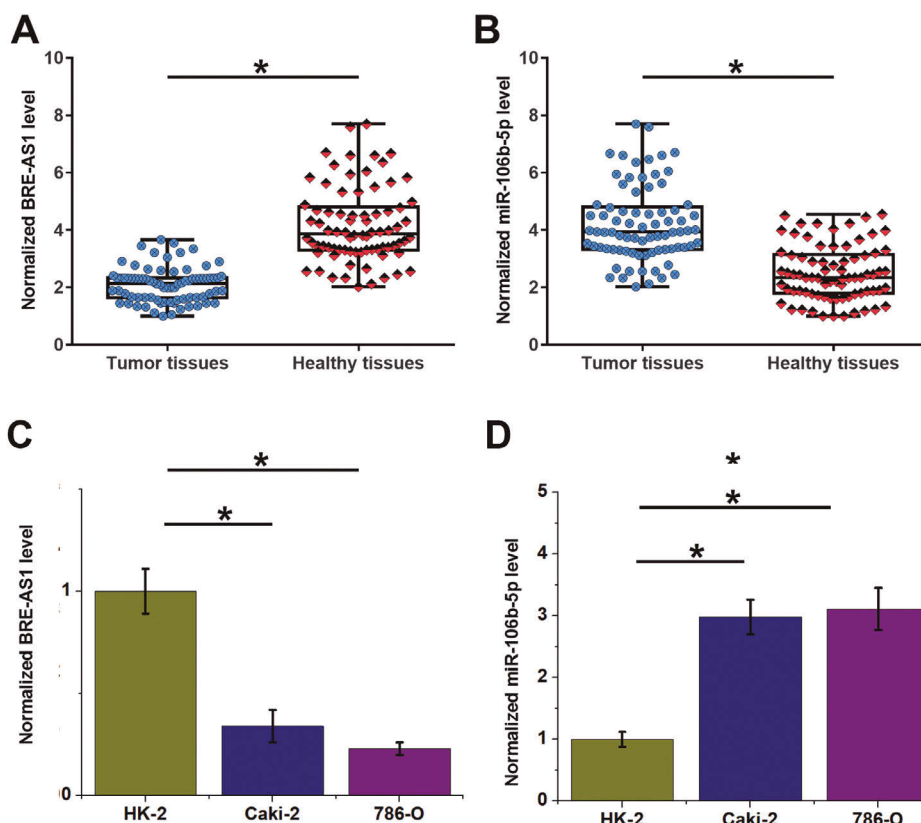


Fig. 1. The expression of BRE-AS1 and miR-106b-5p in ccRCC. RT-qPCR and unpaired t test analysis showed that BRE-AS1 was expressed in low amounts (A), while miR-106b-5p was accumulated in high amounts (B) in ccRCC. Moreover, BRE-AS1 was lowly expressed in ccRCC cell lines (C), while miR-106b-5p was highly expressed in ccRCC cell lines (D). (*, $p < 0.05$).

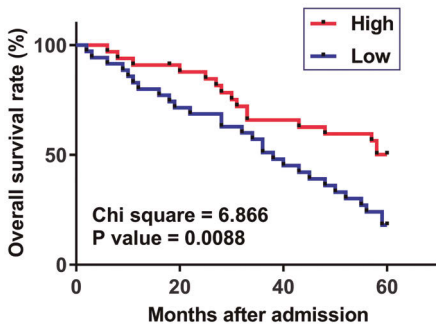


Fig. 2. The predictive role of BRE-AS1 in patients' survival. Based on Youden's index, patients were divided into high (n=35) and low (n=43) BRE-AS1 level groups. Survival curves were plotted and compared by log

rank t test. Low level of BRE-AS1 in tumor tissues were closely correlated with patients' death and short-term survival.

106b-5p were inversely correlated across ccRCC tissues (Fig. 3A). In contrast, this correlation was not observed across healthy tissues (Fig. 3B). Therefore, BRE-AS1 and miR-106b-5p may interact with each other in ccRCC.

Overexpression of BRE-AS1 mediated the down-regulation of miR-106b-5p

The close correlation between BRE-AS1 and miR-106b-5p indicates a potential interaction between BRE-AS1 and miR-106b-5p. To this end, BRE-AS1 and miR-106b-5p were overexpressed in Caki-2 and 786-O cells to study the crosstalk between them. Overexpression rates of BRE-AS1 and miR-106b-5p higher than 250% were achieved at 24h after transfection (Fig. 4A). In

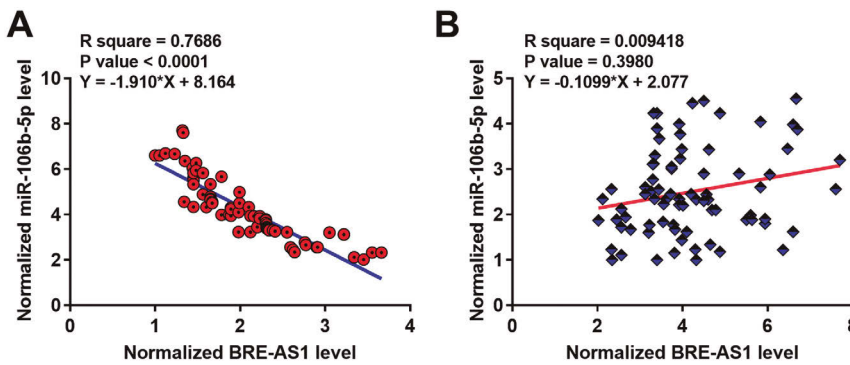


Fig. 3. Correlations between BRE-AS1 and miR-106b-5p. The correlation of BRE-AS1 to miR-106b-5p across tumor tissues (A) and control tissues (B) was analyzed by Pearson's correlation coefficient.

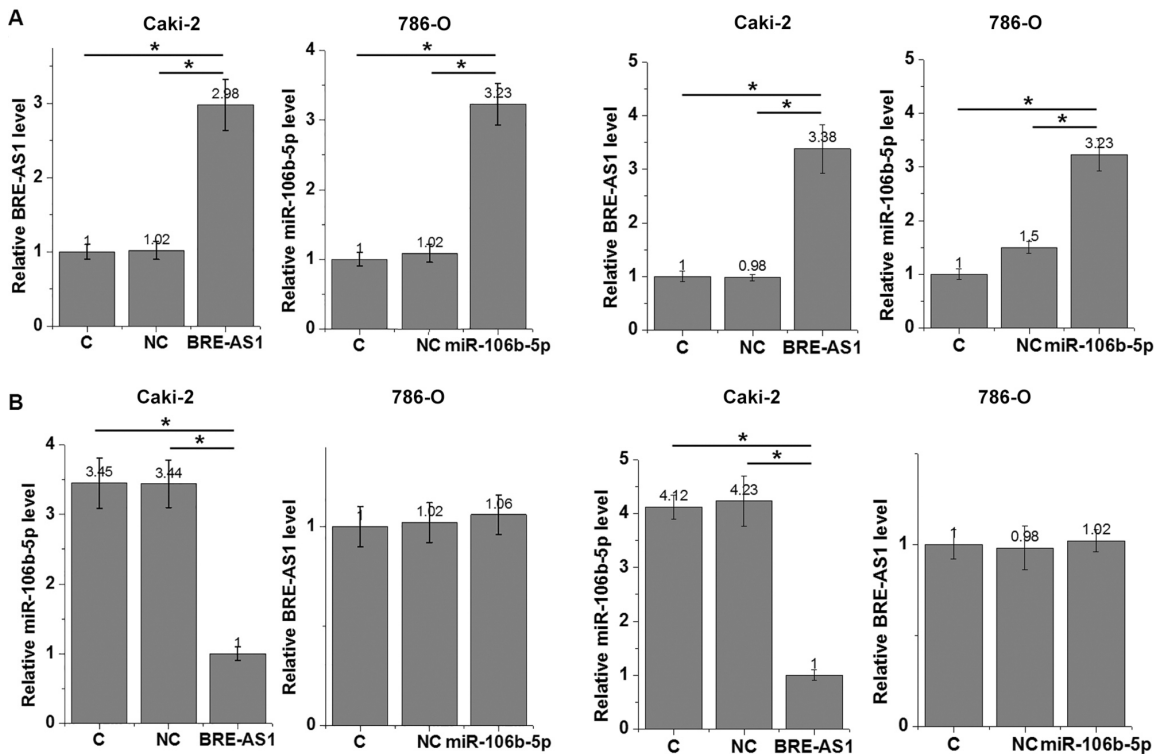


Fig. 4. The role of BRE-AS1 in the expression of miR-106b-5p. Overexpression of BRE-AS1 and miR-106b-5p was reached to explore the interaction between BRE-AS1 and miR-106b-5p (A). The role of BRE-AS1 in regulating the accumulation of miR-106b-5p and the role of miR-106b-5p in regulating the expression of BRE-AS1 (B) was analyzed by RT-qPCR. (*, p<0.05).

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addition, it was observed that overexpression of BRE-AS1 mediated the downregulation of miR-106b-5p (Fig. 4B, $p < 0.05$), while overexpression of miR-106b-5p did not affect the expression of BRE-AS1 (Fig. 4B). Therefore, BRE-AS1 may downregulate miR-106b-5p in ccRCC.

BRE-AS1 regulated ccRCC cell behaviors by regulating miR-106b-5p

CCK-8 assay and Transwell assays were conducted to explore the role of BRE-AS1 and miR-106b-5p in regulating ccRCC cell proliferation and movement. It

showed that overexpression of BRE-AS1 inhibited, while overexpression of miR-106b-5p promoted proliferation (Fig. 5A), migration (Fig. 5B) and invasion (Fig. 5C) of Caki-2 and 786-O cells ($p < 0.05$). In addition, overexpression of miR-106b-5p attenuated the effects of BRE-AS1. Therefore, BRE-AS1 may suppress the proliferation and movement of ccRCC cells through miR-106b-5p.

BRE-AS1 increased the methylation of miR-106b-5p gene and upregulated SETD2 through miR-106b-5p

To explore the mechanism underlying the regulation

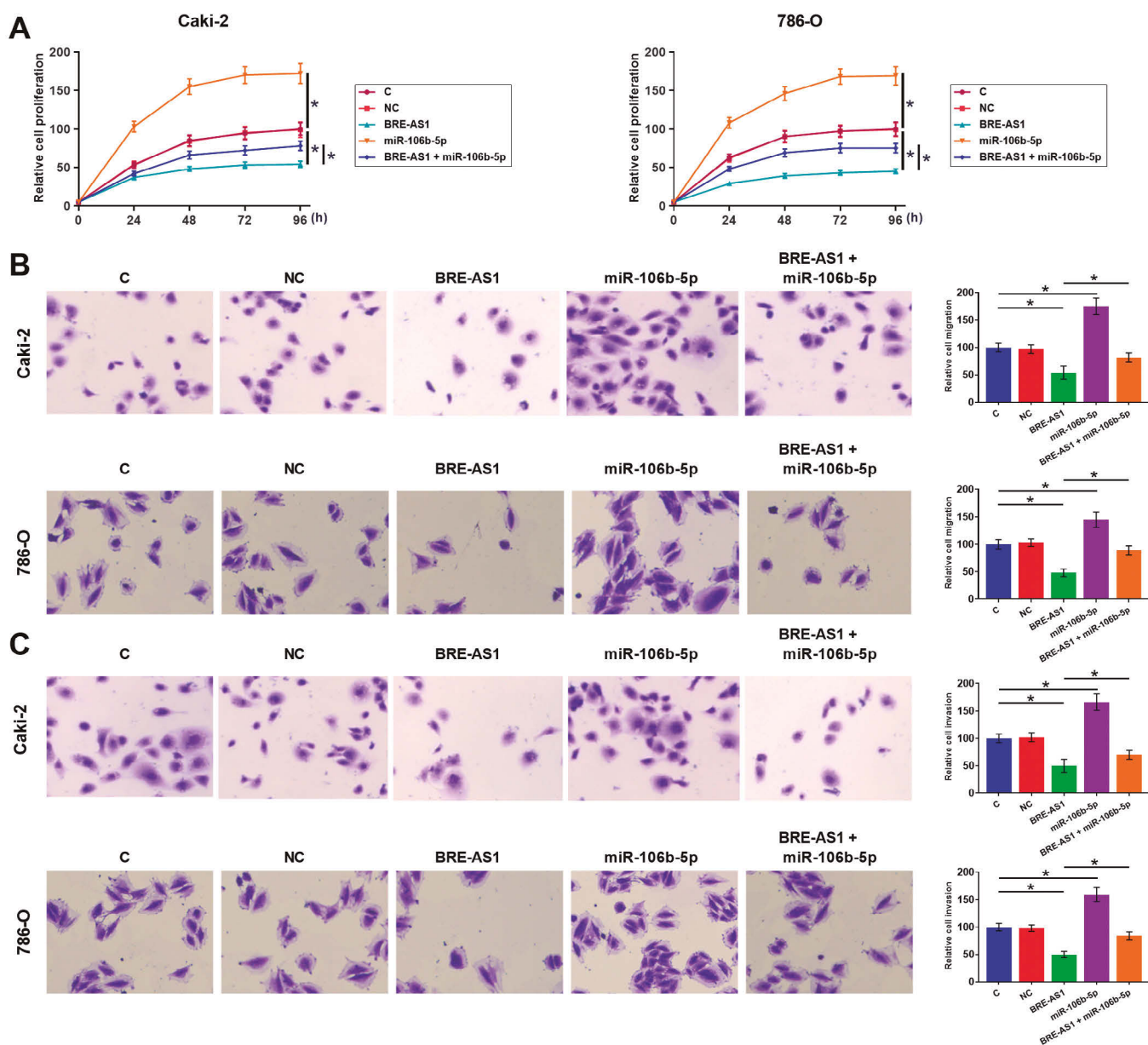


Fig. 5. BRE-AS1 regulated ccRCC cell behaviors through miR-106b-5p. BRE-AS1 mediated the inhibited, while miR-106b-5p mediated the promoted proliferation (A), migration (B) and invasion (C) of cells of ccRCC cell lines Caki-2 and 786-O. In addition, miR-106b-5p suppressed the role of BRE-AS1. (*, $p < 0.05$).

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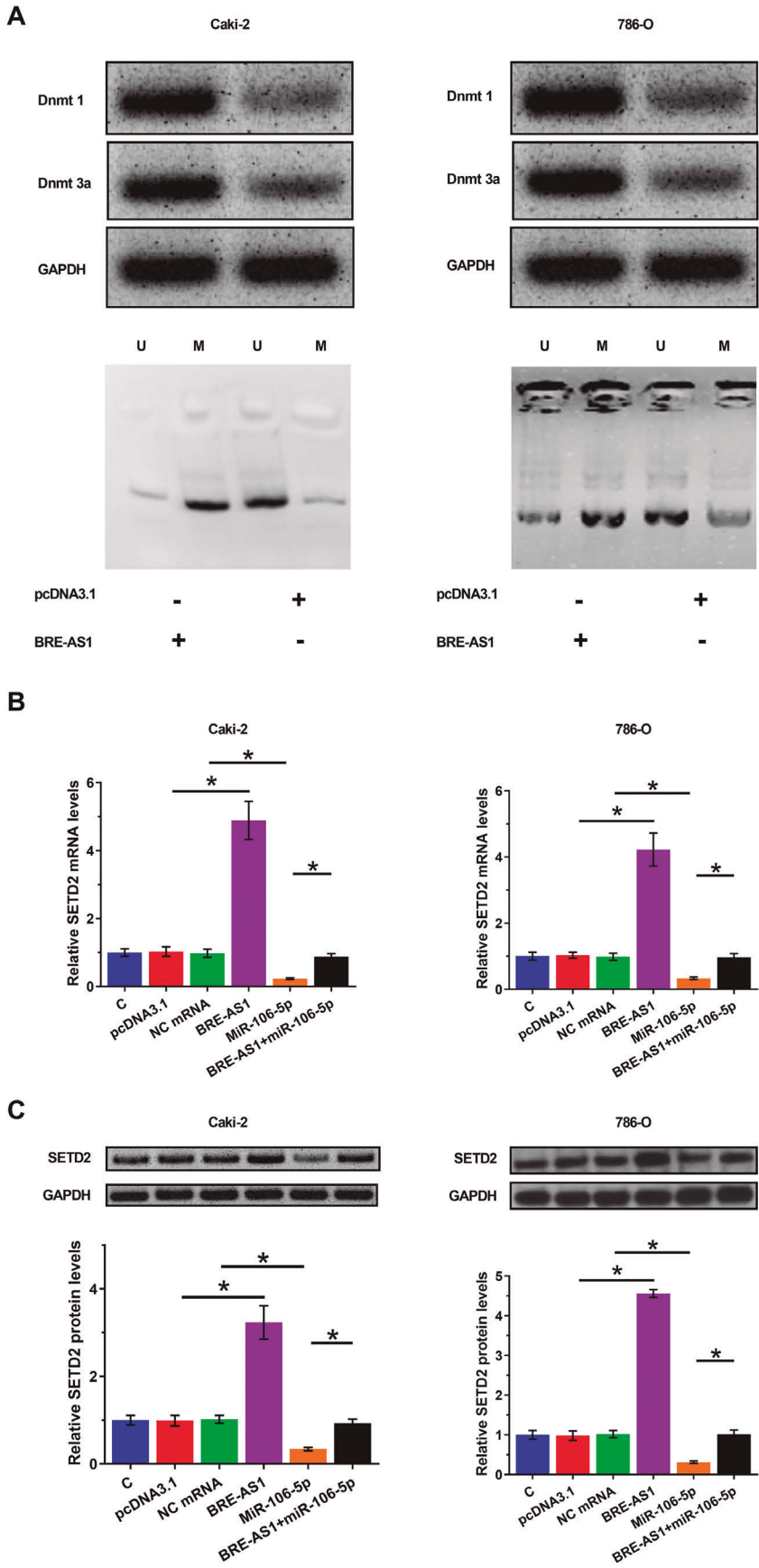


Fig. 6. BRE-AS1 increased the methylation of miR-106b-5p gene and upregulated SETD2 through miR-106b-5p. The role of BRE-AS1 in the methylation of miR-106b-5p was analyzed by MSP (A). The role of BRE-AS1 and miR-106b-5p in the expression of SETD2 was analyzed by performing RT-qPCR (B) and Western blot analysis (C) at mRNA and protein levels, respectively. U, unmethylation; M, methylation (M); (*, $p < 0.05$).

of miR-106b-5p by BRE-AS1, the role of BRE-AS1 in the methylation of miR-106b-5p was evaluated by MSP. The results showed that BRE-AS1 increased methylation of miR-106b-5p promoter (Fig. 6A). SETD2 is a downstream target of miR-106b-5p. The role of BRE-AS1 and miR-106b-5p in regulating the expression of SETD2 was explored by performing RT-qPCR (Fig. 6B) and Western blot analysis (Fig. 6C). It was observed that BRE-AS1 increased the expression levels of SETD2, while miR-106b-5p decreased the expression levels of SETD2. Moreover, BRE-AS1 suppressed the role of miR-106b-5p in downregulating SETD2. Therefore, BRE-AS1 may downregulate miR-106b-5p through methylation, thereby upregulating SETD2.

Discussion

The present study focused on examining the functions of BRE-AS1 in ccRCC, a common malignancy worldwide. Our findings indicate that BRE-AS1 plays a tumor-suppressive role in ccRCC by modulating cancer cell behavior. These actions are likely mediated through interactions with miR-106b-5p. Our study investigated the role of miR-106b-5p in ccRCC. MiR-106b-5p can promote cancer development by targeting multiple downstream tumor suppression pathways, such as SETD2 (Xiang et al., 2015) and PTEN (Chen et al., 2018). Besides the direct functions in cancer development, overexpression of miR-106b-5p also reduces the sensitivity of cancer cells to chemotherapy (Yu et al., 2017a). Thus, the inhibition of miR-106b-5p may be a potential therapeutic strategy for the treatment of cancers (Xiang et al., 2015; Yu et al., 2017a; Chen et al., 2018). It is known that miR 106b-5p promotes RCC (Li et al., 2016). In the present study we also observed the role of miR 106b-5p in increasing ccRCC cell proliferation and movement, further confirming the role of miR 106b-5p as an oncogene in ccRCC.

The functions of BRE-AS1 have been investigated in only a few cancers, including lung cancer. In lung cancer, BRE-AS1 has been shown to negatively regulate cancer cell survival and growth by promoting the expression of the tumor suppressor NR4A3 (Zhang et al., 2018). We observed the upregulated expression of BRE-AS1 in ccRCC tissues and its inhibitory effects on cancer cell proliferation and movement, suggesting the tumor suppressive role of BRE-AS1 in ccRCC. To date, the prognosis of ccRCC is still critical due to the high recurrence rate, especially in patients at advanced stages (Ko et al., 2015). In the present study we also observed the predictive value of decreased expression levels of BRE-AS1 in patients' survival, indicating the potential application of BRE-AS1 as a prognostic biomarker for ccRCC, while expression levels of BRE-AS1 were not significantly different among tumor grades and stages. Moreover, BRE-AS1 was inversely correlated with miR-106b-5p, and expression levels of miR-106b-5p were not significantly affected by tumor grades and stages. This discrepancy is likely caused by the sample size. Our

conclusions should be further verified by studies with a bigger sample size.

In cancer development, there is known to be interaction between lncRNAs and miRNAs (Paraskevopoulou and Hatzigeorgiou, 2016; Yu et al., 2017b). At present, studies of the interactions between lncRNAs and miRNAs mainly focus on the role of lncRNAs as the endogenous competing RNAs for miRNAs, while the role of lncRNAs in regulating the expression of miRNAs is not fully understood. Our findings demonstrated that BRE-AS1 could decrease the expression levels of miR 106b-5p through methylation to regulate ccRCC cell behaviors. Moreover, SETD2, which is a tumor suppressor that can be targeted by miR 106b-5p, was upregulated in ccRCC by BRE-AS1, suggesting the role of SETD2 as the downstream effector of the BRE-AS1/miR 106b-5p pathway. In view of the oncogenic effects of miR 106b-5p on ccRCC, BRE-AS1 may be overexpressed to treat ccRCC. In this study, BRE-AS1 and miR 106b-5p showed similar effects on the proliferation of both VCaP and 22Rv1 cells. Expression levels of BRE-AS1 and miR 106b-5p were not significantly different between these two cell lines. VCaP is the only PC cell model which expresses the AR-V7, an Androgen receptor splice variant, AR-V7, and the TMPRSS2-ERG gene fusion. Therefore, the functions of BRE-AS1 and miR 106b-5p in PC are unlikely to be affected by AR-V7 and TMPRSS2-ERG.

Conclusion

In collusion, BRE-AS1 plays a tumor suppressive role in ccRCC by affecting cancer cell behaviors through the downregulation of miR-106b-5p.

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Conflict of interest. The authors declare that they have no conflict of interest.

Authors contribution. Gaode Zou, Yi Yu concept, manuscript editing Lian Guo, Shaochen Shen, Zhen Song, Zheyang Ouyang, data collection and analysis, manuscript preparation

All authors have read and approve the submission of the manuscript.

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Availability of data and materials. The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate. The present study was approved by the Ethics Committee of the Second Affiliated Hospital of Nanchang University. The research has been carried out in accordance with the World Medical Association Declaration of Helsinki. All patients and healthy volunteers provided written informed consent prior to their inclusion within the study.

Consent for publication. Not applicable.

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