Hyper-Expressed BCYRN1 Promotes Proliferation and Brain Metastasis of Small Cell Lung Cancer by Mir-378c/AJM1 Axis

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Background: *The purpose of this study is to explore the functions and mechanism of brain cytoplasmic RNA1 (BCYRN1) in proliferation and brain metastasis of small cell lung cancer (SCLC) cells.*

Materials and Methods*: The BCYRN1 levels in SCLC cell lines and serums of patients were measured via quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR). The downstream miRNA of BCYRN1 and downstream gene of miR-378c were predicted with StarBase software, and was verified by dual-Luciferase reporter assay. Colony formation assay, MTT assay, wound healing assay and transwell assay were performed to examine the influences of BCYRN1/miR-378c/AJM1 on the proliferation, migration and invasion abilities SCLC cells. The protein levels of proliferation marker Ki-67 and key proteins of epithelial-mesenchymal transition (EMT) (E-cadherin, N-cadherin, Vimentin, Snail, Slug) were detected by western blot. Animal model of brain metastasis was conducted to verify in vitro.*

Results: *The levels of BCYRN1 were upregulated in SCLC cell lines and serum of SCLC patients. BCYRN1 knockdown inhibited the proliferation, migration and invasion, Ki-67 expression and EMT of SCLC cells. The miR-378c was identified as a direct target of BCYRN1 in SCLC cells. Knockdown of BCYRN1 suppressed Ki-67 expression and EMT by up-regulating miR-378c, thereby inhibiting the proliferation, migration and invasion of SCLC cells. AJM1 was identified as a direct target of miR-378c. Knockdown of AJM1 reversed the effects of miR-378c knockdown on SBC-2 cells. BCYRN1 knockdown inhibited EMT on brain metastasis of SCLC cells in nude mice, but knockdown of miR-378c could reverse it.*

Conclusion: *Hyper-expressed BYNRN1 could promote proliferation and brain metastasis of SCLC cells by miR-378c/AJM1 axis.*

BACKGROUND

Small cell lung cancer (SCLC) is characterized by rapid tumor growth and early metastatic spread.[1] The patients with SCLC often have abysmal prognosis. Therefore, further clarification of the molecular mechanism of SCLC metastasis could provide new targets for the clinical treatment of SCLC. A large number of non-coding RNAs in the transcriptome, once considered to be redundant genes, are recently verified to be essential to carcinogenesis and progression.[2] Long non-coding RNAs (LncRNAs) are non-coding RNAs participating in a variety of physiological and pathological factors, including tumorigenesis and metastasis.[3] Brain cytoplasmic RNA1 (BCYRN1)

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is a LncRNA with a length of 200 nucleotides.[4] BCYRN1 is highly expressed in normal neural tissues of primates and low in other normal tissues, but it is highly expressed in human cervical cancer, esophageal cancer, and lung cancer.[5,6,7] It has been documented that BCYRN1 is associated with the metastasis of cervical cancer and lung cancer.[6-7] The expression of BCYRN1 in non-small cell lung cancer (NSCLC) cells is significantly higher than that in human bronchial epithelioid cells, and it is involved in the promotion of NSCLC proliferation, migration, invasion, and energy metabolism.[7-8] So far, there is no report on the expression and function of BCYRN1 in SCLC.

Herein, the function and mechanism of BCYRN1 in proliferation and metastasis of SCLC cells were analyzed.

MATERIALS AND METHODS

Patients and samples

The serum samples of 10 patients with small cell lung cancer diagnosed with distant metastasis for the first time by histology in Tangshan People's Hospital (Tangshan, China), 20 patients with small cell lung cancer without distant metastasis (including 2 patients without lymph node metastasis and 18 patients of hilar and mediastinal lymph node metastasis proved by chest enhanced CT examination), and 10 healthy subjects were collected from November 1, 2019, to December 31, 2020. This study was approved by the Ethics Committee of Tangshan People's Hospital (Approval number RMYY-LLKS-2021-016). Experiments including human tissues were conducted following the Declaration of Helsinki.

Cell culture

Human bronchial epithelioid epithelial cells 16-HBE were purchased from Beina Chuanlian Biotechnology Co., LTD (Suzhou, China). Human small cell lung cancer cell SBC-2 was purchased from Saibaikang Biotechnology Co., LTD (Shanghai, China). Human lung adenocarcinoma cell NCI-H1299 and large cell lung cancer cell NCI-H460 were donated by Professor Sun Guogui of the Affiliated Hospital of North China University of Science and Technology (Tangshan, China). 293T cells were provided by Tianjin Saier Biotechnology Co., LTD (Tianjin, China). 16-HBE cell line was cultured in DMEM medium containing 15% fetal bovine serum (Hy Clone, USA), SBC-2 cell line, NCI-H460 cell line, and NIC-H1299 cell line were cultured in 1640 medium containing 15% fetal bovine serum (Hy Clone, USA). All cells were cultured in a humidified incubator containing 5% CO2 at 37°C.

Dual luciferase assay

The amplified wild-type (wt) or mutant (mut) 3'-UTR of BCYRN1 was then cloned into the downstream polyclonal sites of luciferase gene which linked to pmirGLO (Saierbio, Tianjin, China) vector to construct BCYRN1-wt and BCYRN1-mut recombinant vectors. 293T cell was co-transfected with BCYRN1-wt or BCYRN1-mut vector and miR-378c mimics or mimics-NC, ASO-miR-378c or ASO-NC, using Lipofectamine® 2000 kit. The luciferase activities of firefly and renilla were measured 48h after transfection with the luciferase reporter analysis system (Promega, USA). The experiment was repeated three times.

Cell transfection

Transfections were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) per the manufacturer's instructions. BCYRN1 siRNAs and control siRNAs, miR-378c mimics and mimics-NC, ASO-miR-378c and ASO-NC, AJM1 siRNAs and control siRNAs were purchased from Saier (Tianjin, China).

qRT-PCR

Total RNAs were extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA was synthesized by reverse transcription using the cDNA Reverse Transcription Kit (Invitrogen, Carlsbad, CA, USA). RT-qPCR analysis was performed using SYBR® Premix Ex TaqTM II(Tli RNaseH Plus, Takara, Japan). The thermo cycler programs were as follows: 94°C for 4mins and 40 cycles of 94°C for 30s, 58°C annealing temperature for primer pairs for 30s, and 72°C for 30s, using GAPDH or β-actin

as an internal reference. Each reaction was performed in triplicate. The 2−ΔΔCT method was used to evaluate the results. Each reaction was performed in triplicate.

Western blot assay

The SBC-2 samples were harvested and extracted using RIPA lysis buffer (50mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol). Equal amounts of cells extracts were separated on 10% SDS-PAGE gels and then transferred the separated protein samples onto a polyvinylidene fluoride (PVDF) membrane (iBlot, Shanghai, China). After blocking with 5% Blotto for 2h, the membrane was incubated at 4℃ overnight with the primary antibody, Ki-67, E-cadherin, N-cadherin, Vimentin, Snail and Slug (1:500, Saierbio, Tianjin, China), with GAPDH (1:2000, Saierbio, Tianjin, China) as the internal control. HRP rabbit IgG secondary antibodies (1:2000, Saierbio, Tianjin, China) were added and incubated for 1.5h at room temperature. The membranes were washed four times for 5mins with TBST buffer, then put the membranes into the Chemiluminescence Reagent (Bohai, Hebei, China) for 30s, which assessed protein expression. Finally, the blot signals were visualized and analyzed by a chemiluminescent gel imaging analysis system to quantify the band intensities according to the operation manual.

colony formation assay

In colony formation assay, the cells were inoculated into a 6-well plate at 24h after transfection and let grow until visible colonies formed. After 14d, the colonies were fixed using methanol and stained with hematoxylin. Finally, they were visually observed and manually counted under a microscope. The experiment was repeated three times.

MTT

Cell proliferation assays were performed using MTT (Saierbio, Tianjin, China). The 200 μL cell suspension of 1 × 104 cells/mL was inoculated in the 96-well plates. After 24h, 48h, and 72h, 20μl of MTT with a concentration of 5mg/mL was added into each well, and cells were cultured in an incubator at 37°C for 4h. After incubation, cells were shocked and blended for 10 min. The absorbance of cells at 570nm was measured by a microplate reader (Multiskan MK3, Thermo). Each experiment was carried out in three replicate wells three times.

wound healing assay

The wound healing assay was used to test collective cell migration. Briefly, the cell suspension of 4 × 105 cells/well were seeded into 6-well plates. After adherence, the indicated expression vectors were used to cells transfection. After the unattached cells being rinsed with medium, the confluent layer of cells was scratched using a sterile pipette head to create an artificial wound when the cells spread to 90% of the well. The medium without FBS was used to culture the remaining cells at 37°C with 5% CO2. Cell migration to the wounded gap was then observed by microscopy after 0h, 24h, and 48h, respectively. The wound area in a different time period was measured, and the wound healing rate was calculated using the Image J software. The experiment was repeated three times.

Transwell assay

The 24-well transwell chamber with polycarbonate filters (pore size, 8μm; Corning, Cambridge, MA) was used for the invasion assay. In brief, 3×104 per well of SBC-2 cells were resuspended in 200μL of medium without FBS and seeded in the upper section, while the bottom section was filled with 800μL of medium supplemented with 10% FBS. Then the section was placed into a 24-well plate which contained 600μL of culture media with 20% FBS. The upper chamber coated with matrigel was incubated at 37°C for 24h. After incubation, the cells were fixed with 33% acetic acid (glacial acetic acid: methyl alcohol is 1:3) and dyed with 0.1% crystal violet (Solarbio, Beijing, China) for 20mins. Cells on the upper membrane were carefully removed with a cotton stick. The number of cells per field was counted with an Olympus IX 71 (Tokyo, Japan). The experiment was repeated three times.

Animal Experiment

After generating stably transfected pSlience-NC+ASO-NC, shR-BCYRN1+ASO-NC, and shR-BCYRN1+ASO-miR-378c cell lines, each nude mouse was anesthetized by intraperitoneal injection of 100μL 0.1% pentobarbital and fixed supine. A median incision was made in the neck to separate one side of the common carotid artery. The transfected SBC-2 cells (10μL) were subcutaneously injected into the carotid artery of female BALB/c-nu nude mice. After injection, the wound was closed by ligation with silk thread to stop bleeding. The next day, 200μL antagomir (80mg/kg) was injected through the tail vein for 3 consecutive days. At about 20 days, the nude mice were observed to be emasculated, sluggish and bowed, and then some mice died. After cervical dislocation, the mice were sacrificed, the brain tissue was removed, and the number of lesions was observed, weighed and photographed. All animal studies were approved by the Ethics Committee of the North China University of Science and Technology.

Statistical analysis

We used SPSS 22.0 software (IBM, USA) to perform all the statistical analyses. Each experiment was carried out at least at 3 times, and all results were presented as the mean ± SD. Student's t-test was used to do the assessment of statistical significance between two groups. One-way analysis of variance was used for comparison within three or more groups, and the Scheffe post hoc test was used for further pairwise comparison. The significance test level was set as two-sided α=0.05. *P<0.05 was considered a statistically significant difference.

RESULTS

BCYRN1 was hyper expressed in SCLC and miR-378c was identified as a negative target of BCYRN1

The mRNA levels of BCYRN1 in lung cancer cells and the serum of SCLC patients were detected by RT-qPCR. As shown in Figure 1A, there was a statistically significant difference in the expression of BCYRN1 among the four groups (F=1096.81, P<0.001). The BCYRN1 levels in the three lung cancer cell lines were all significantly higher than that in 16-HBE cells (NCI-H1299 vs. 16-HBE: P=0.009; NCI-H460 vs. 16-HBE: P=0.001; SBC-2 vs. 16-HBE: P<0.001). Moreover, the BCYRN1 level in SCLC cell line SBC-2 was markedly higher than that in large cell lung cancer cells NCI-H460 and lung adenocarcinoma cells NCI-H1299 (SBC-2 vs. NCI-460: P<0.001; SBC-2 vs. NCI-H1299: P<0.001). There was no significant difference in BCYRN1 expression between NCI-H460 and NCI-H1299 (P=0.0554).

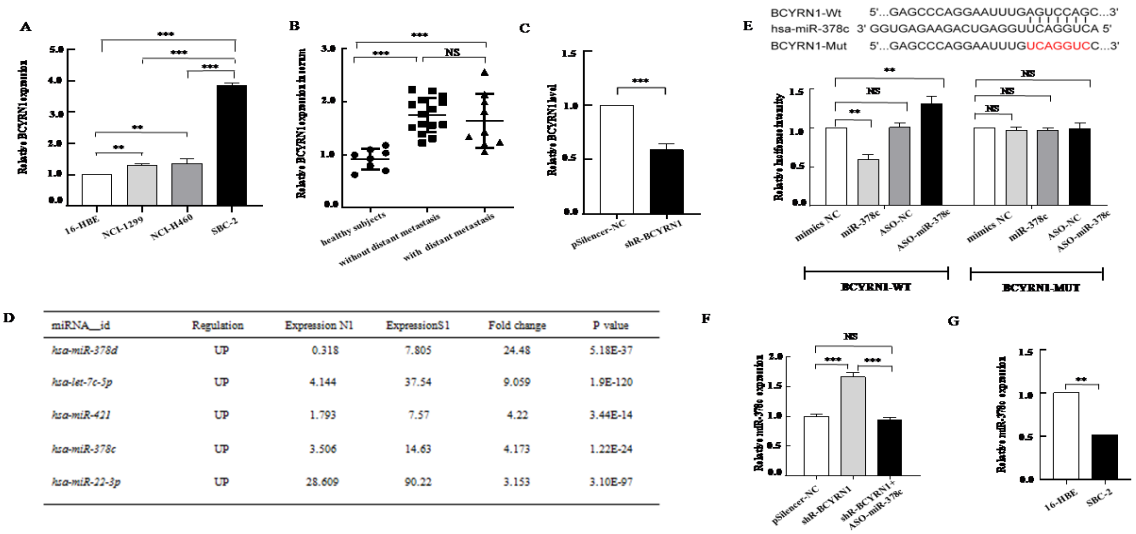
As shown in Figure 1B, there was a statistically significant difference in the BCYRN1 level in the serum of SCLC patients among the three groups (F=11.583, P<0.001). Both the BCYRN1 levels in the serum of SCLC patients without distant metastasis and with distant metastasis were all significantly higher than that of healthy subjects (SCLC without distant metastasis vs. healthy subjects: P<0.001; SCLC with distant metastasis vs. healthy subjects: P=0.003). There was no significant difference in the level of BCYRN1 in the serum of the group with distant metastasis and the group without distant metastasis (P=0.89) (Figure 1B). This indicates that BCYRN1 probably contributes to the development of SCLC. To clarify the function and mechanism of BCYRN1 in SCLC, the SBC-2 SCLC cells were transfected with pSilencer-NC and pshR-BCYRN1-3 plasmids. The BCYRN1 level in the pshR-BCYRN1-3 group was significantly lower than that in the pSilencer-NC group (t=12.87, P<0.001). Next, the total RNAs of the two group SBC-2 cells transfected with pSilencer-NC and pshR-BCYRN1 plasmids were extracted, and the miRNAs regulated by BCYRN1 were selected by microarray. The 5 upregulated miRNAs with differential expression fold greater than 3 were selected: hsa-miR-378d, hsa-let-7c-5p, hsa-miR-421, hsa-miR-378c, and hsa-miR-22-3p (Figure 1D).

Starbase website predicted that hsa-miR-378c (miR-378c) contains BCYRN1-binding sites (Figure 1E upper). Therefore, we chose miR-378c for further study. The luciferase activity (Figure 1E lower) of the pmiRGLO-BCYRN1-WT and miR-378c mimics co-transfection group in 293T cells

was significantly lower than that of pmiRGLO-BCYRN1-WT and mimics-NC co-transfection group (P<0.001). However, there was no significant difference in luciferase activity between the pmiRGLO-BCYRN1-MUT+miR-378c mimics group and pmiRGLO-BCYRN1-MUT+mimics-NC group (P=0.765). This indicated that miR-378c directly complementarily binds to BCYRN1.

As shown in Figure 1F, the level of miR-378c in the shR-BCYRN1 group was significantly higher than that in the pSilencer-NC group (P=0.0002). The rescue assay revealed that ASO-miR-378c could reverse the effect of BCYRN1 knockdown on miR-378c expression (shR-BCYRN1+ASO-miR-378c vs. shR-BCYRN1: P=0.0001). In Figure 1G, miR-378c level in SBC-2 cells was significantly lower than that in 16-HBE cells (t=5.242, P=0.006). These data suggested that BCYRN1 probably functioned as a ceRNA of miR-378c in SBC-2 cells.

Figure 1: The expressions of BCYRN1 and its downstream miRNAs in lung cancer cells



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miRNA_id	Regulation	Expression N1	ExpressionS1	Fold change	P value
hsa-miR-378d	UP	0.318	7.805	24.48	5.18E-37
hsa-let-7c-3p	UP	4.144	37.54	9.059	1.9E-120
hsa-miR-421	UP	1.793	7.57	4.22	3.44E-14
hsa-miR-378c	UP	3.506	14.63	4.173	1.22E-24
hsa-miR-22-3p	UP	28.609	90.22	3.153	3.10E-97

Note: (A): The BCYRN1 levels in human bronchial epithelioid cell 16-HBE, human small cell lung cancer (SCLC) cell SBC-2, human lung adenocarcinoma cell NCI-H1299 and human large cell lung cancer cell NCI-H460 were detected by RT-qPCR, using β-actin as the internal reference.

(B): The BCYRN1 levels in serums of healthy subjects, SCLC patients with/without distant metastasis were detected by RT-qPCR, using GAPDH as the internal reference.

(C): The BCYRN1 levels in SBC-2 cells transfected with pSilencer-NC and pshR-BCYRN1-3 plasmids were detected by RT-qPCR, using β-actin as the internal reference.

(D): The upregulated miRNAs by BCYRN1 knockdown in SBC-2 cells were selected by microarray.

(E): Upper: The miR-378c and its putative binding sequences in the 3'UTR of BCYRN1; Lower: The BCYRN1-WT or BCYRN1-MUT plasmids were co-transfected with miR-378c mimics into 293T cells, the dual-luciferase reporter system was used to analyze the luciferase activity of each group at 48h post-transfection.

(F): The miR-378c levels in SBC-2 cells transfected with pSilencer-NC, shR-BCYRN1 and shR-BCYRN1+ASO-miR-378c were detected by RT-qPCR.

(G): The miR-378c levels in 16-HBE and SBC-2 cells were detected by RT-qPCR. The above experiments are three independent repeated experiments, and the results are expressed as mean ± SD, **P＜0.01, ***P＜0.005.

Knockdown of BCYRN1 inhibited the proliferation, migration, invasion, and EMT of SBC-2 cells by upregulating miR-378c

SBC-2 cells were transfected with pSilencer-NC, shR-BCYRN1, and shR-BCYRN1+ASO-miR-378c, respectively. The colony formation assay showed that the colony number was statistically different among the three groups (F=14.611, P=0.005) Figure 2A. Among them, the colony number in the shR-BCYRN1 group was significantly lower than that in the pSilencer-NC group (P=0.0014), and the colony number in the shR-BCYRN1+ASO-miR-378c group was higher than that in the pSilencer-NC group (P=0.047), which was also significantly higher than that in the shR-BCYRN1 group (P=0.0098) Figure 2A. This indicated that knockdown of BCYRN1 could inhibit the proliferation ability of SBC-2 cells, and knockdown of miR-378c could reverse the inhibitory effect of knockdown of BCYRN1 on the proliferation of SBC-2 cells.

The transwell assay showed that the number of cells passing through the basement membrane was statistically different among the three groups (without matrigel: F=28.83, P=0.0015) Figure 2B (with matrigel: F=30.403, P=0.0013) Figure 2C. Among them, the number of cells passing through the basement membrane in the shR-BCYRN1 group was significantly lower than that in the pSilencer-NC group (without matrigel: P=0.009; with matrigel: P=0.003), and the number of cells passing through the basement membrane (without matrigel) in the shR-BCYRN1+ASO-miR-378c group was significantly higher than that in the shR-BCYRN1 group (without matrigel: P=0.0018; with matrigel: P=0.0002), but it was not statistically significant with the pSilencer-NC group (without matrigel: P=0.113; with matrigel: P=0.558). This indicated that knockdown of BCYRN1 could inhibit the migration and invasion abilities of SBC-2 cells, and knockdown of miR-378c could reverse the inhibitory effect of knockdown of BCYRN1 on the migration of SBC-2 cells.

The protein levels of epithelial marker E-cadherin were significantly different among the three groups (F=36.788, P<0.001), and the protein level of E-cadherin in the shR-BCYRN1 group was markedly higher than that in the pSilencer-NC group (P=0.0015). The protein level of E-cadherin in the shR-BCYRN1+ASO-miR-378c group was significantly lower than that in the shR-BCYRN1 group (P=0.0013) Figure 2E, and there was no significant difference between it and the pSilencer-NC group (P=0.628).

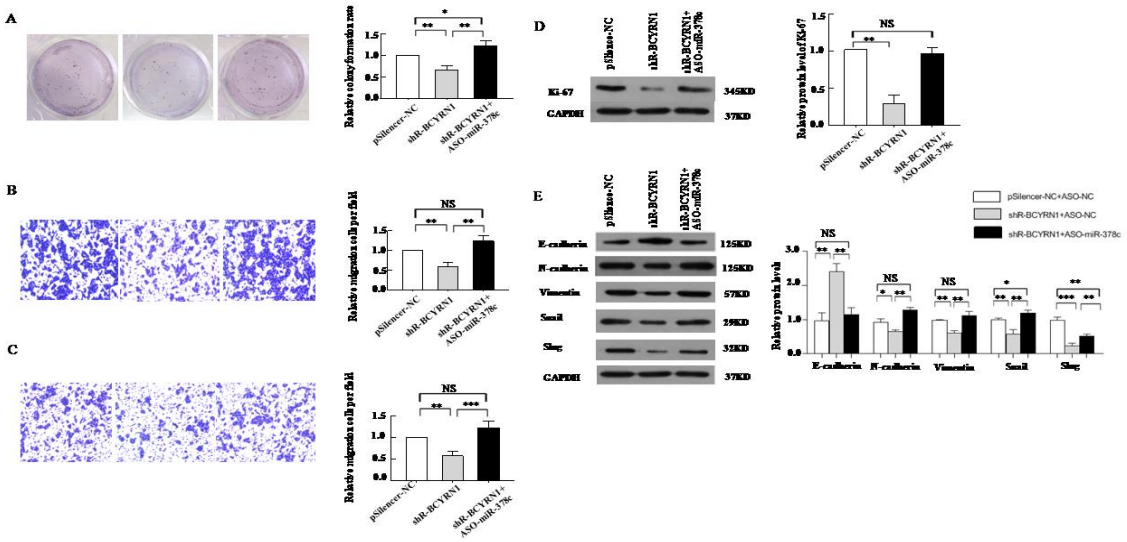
Next, transcription factors Snail and Slug were studied. The Snail and Slug protein levels were significantly different among the three groups (F=34.662, P=0.0012; F=55.181, P<0.001). The Snail and Slug protein levels in shR-BCYRN1 group were substantially lower than those in pSilencer-NC group (P=0.008; P=0.0017). The Snail and Slug protein levels in the shR-BCYRN1+ASO-miR-378c group were considerably higher than those in the shR-BCYRN1 group (P=0.0013; P=0.021). The Snail and Slug protein levels in the shR-BCYRN1+ASO-miR-378c group were substantially lower than those in the pSilencer-NC group (P=0.059; P=0.002) Figure 2E. The above results indicated that knockdown of BCYRN1 could increase the levels of transcription factors Snail and Slug by up-regulating miR-378c, inhibit the transcription of the epithelial marker E-cadherin, and inhibit the occurrence of EMT in SBC-2 cells, thereby inhibiting cell migration and invasion.

AJM1 was a target of miR-378c, and knockdown of AJM1 inhibited the effects of miR-378c on SBC-2 cells

Starbase website predicted that AJM1 contains miR-378c-binding sites Figure 3A upper. The luciferase activity of 293T cells co-transfected with pmiRGLO-AJM1-WT and miR-378c mimics was significantly lower than that of pmiRGLO-AJM1-WT and mimics-NC co-transfection group (P=0.0001) Figure 3A lower. However, there was no significant difference in luciferase activity between tpmiRGLO-AJM1-MUT+miR-378c mimics group and pmiRGLO-AJM1-MUT+mimics-NC group (P=0.1447). It was shown that miR-378c directly complementarily binds to AJM1.

The mRNA levels of AJM1 in SBC-2 cells transfected with shR-AJM1-1, shR-AJM1-2, and shR-AJM1-3 plasmids were lower than that of AJM1 in SBC-2 cells transfected with pSilencer-NC (Figure 3B) (P=0.0001). We chose shR-AJM1-1 with the best knockdown effect to continue the follow-up experiments. The SBC-2 cells which were

Figure 2: The effects of BCYRN1 on the proliferation, migration, invasion and EMT abilities of SBC-2 cells



Note: SBC-2 cell was transfected with pSlience-NC, shR-BCYRN1 and shR-BCYRN1+ASO-miR-378c, respectively.

(A): Proliferation ability of each group was detected by colony formation assay.

(B): Migration ability of each group was detected by transwell assay without matrigel.

(C): Invasion ability of each group was detected by transwell assay with matrigel. The protein levels of Ki-67

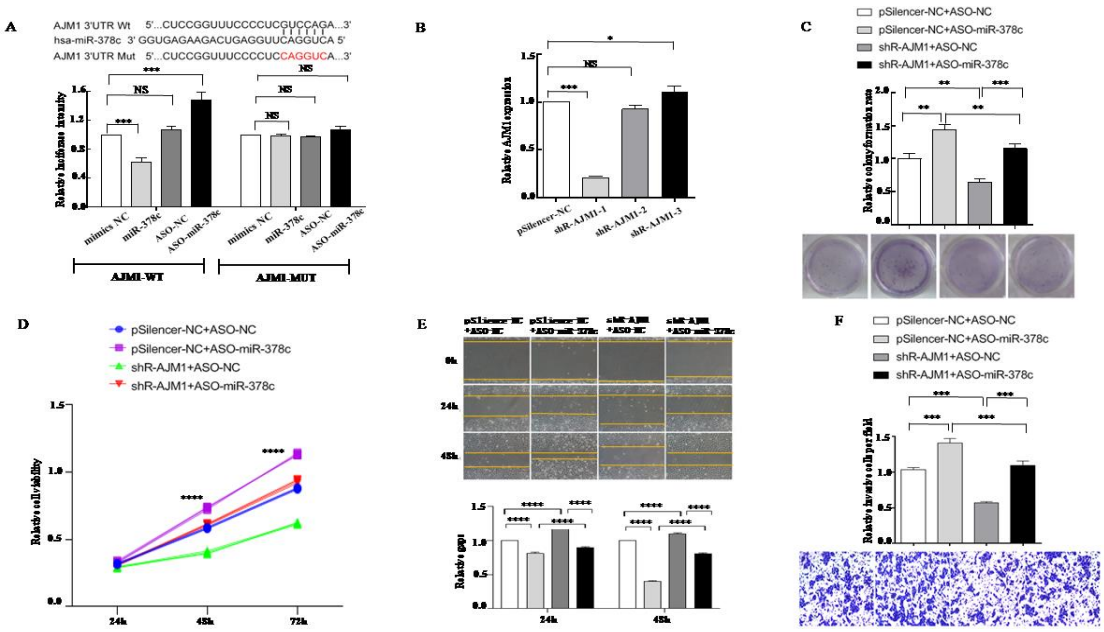
(D), EMT proteins (E: E-cadherin, N-cadherin and Vimentin) and transcription factors (E:Snail and Slug) were detected by western blot assay, using GAPDH as internal reference. The semi-quantification of the band's grayscale was analyzed by Image J software. The above experiments are three independent repeated experiments, and the results are expressed as mean ± SD, *P<0.05, **P＜0.01, ***P<0.005.

Transfected with pSilencer-NC+ASO-NC, pSilencer-NC+ASO-miR-378c, shR-AJM1+ASO-NC, and shR-AJM1+ASO-miR-378c, respectively. The colony formation assay (F=12.87, P=0.0068) showed that the colony number in the pSilencer-NC+ASO-miR-378c group was higher than that in the pSilencer-NC+ASO-NC group (P=0.0022), the colony number in the shR-AJM1+ASO-NC group was lower than that in the pSilencer-NC+ASO-NC group (P=0.0027), the colony number in the shR-AJM1+ASO-miR-378c group was lower than that in the pSilencer-NC+ASO-miR-378c group (P=0.0065), and the colony number in the shR-AJM1+ASO-miR-378c group was higher than that in the shR-AJM1+ASO-NC group (P=0.0003) Figure 3C. It indicated that knockdown of miR-378c could increase the proliferation ability of SBC-2 cells, and knockdown of AJM1 could reverse the enhancement effect of knockdown of miR-378c on the proliferation of SBC-2 cells. The same trend was obtained through the MTT assay Figure 3D.

The wound healing assay showed that knockdown of miR-378c could increase the migration ability of SBC-2 cells, and knockdown of AJM1 could reverse the enhancement effect of knockdown of miR-378c on the proliferation of SBC-2 cells. Figure 3E.

The transwell assay showed that knockdown of miR-378c could increase the invasion ability of SBC-2 cells, and knockdown of AJM1 could reverse the enhancement effect of knockdown of miR-378c on the proliferation of SBC-2 cells. Figure 3F.

Figure 3: The effects of miR-378c and AJM1 expression changes on the proliferation, migration, and invasion of SBC-2 cells



Note: (A): Upper: The miR-378c and its putative binding sequences in the 3'UTR of AJM1; Lower: The AJM1-WT or AJM1-MUT plasmids were co-transfected with miR-378c mimics into 293T cells, the dual-luciferase reporter system was used to analyze the luciferase activity of each group at 48h post-transfection.

(B): The mRNA levels of AJM1 in SBC-2 cells transfected with pSilencer-NC, shR-AJM1-1, shR-AJM1-2 and shR-AJM1-3 plasmids were detected by RT-qPCR.

(C): Proliferation ability of each group was detected by colony formation assay.

(D): Cell activity of each group was detected by MTT assay.

(E): Migration ability of each group was detected by wound healing assay.

(F): Invasion ability of each group was detected by transwell assay. The above experiments are three independent repeated experiments, and the results are expressed as mean ± SD, *P<0.05, **P＜0.01, ***P＜0.005, ****P<0.001.

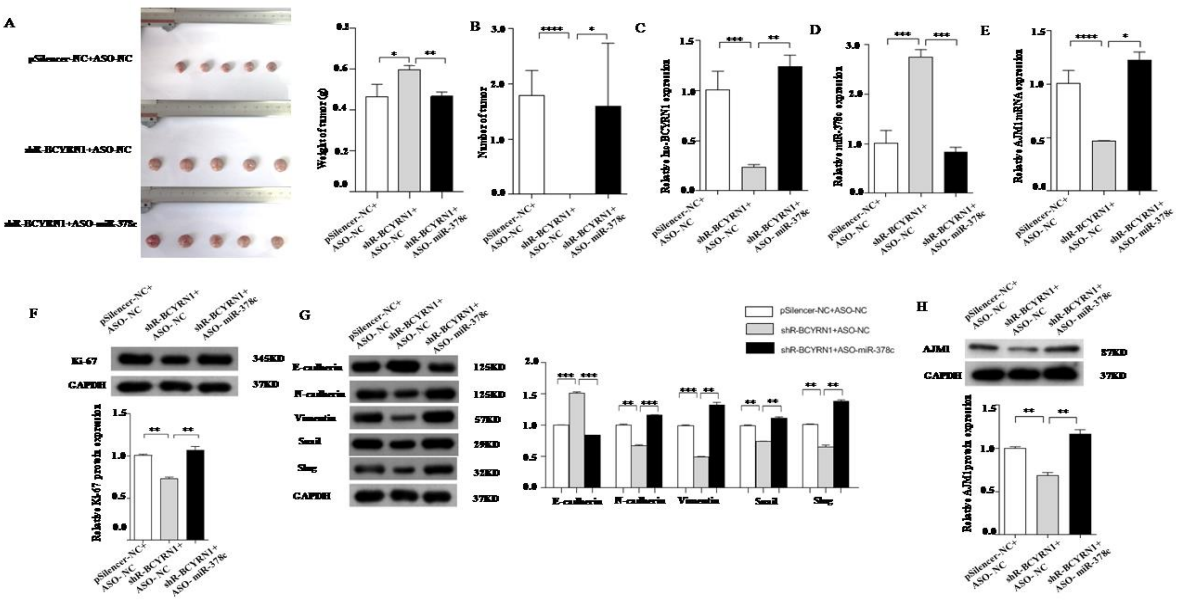
Knockdown of BCYRN1 inhibited EMT by BCYRN1/miR-378c/AJM1 axis on brain metastasis of SCLC cells in nude mice

The SBC-2 cells, stably transfected with pSlience-NC+ASO-NC, shR-BCYRN1+ASO-NC, and shR-BCYRN1+ASO-miR-378c, were injected into carotid artery of nude mice, respectively. The results showed that the tumors derived from cells in the shR-BCYRN1+ASO-NC group were much bigger and weightier than that in the pSlience-NC+ASO-NC group (P=0.0229), and the tumors derived from cells in the shR-BCYRN1+ASO-miR-378c group were much smaller and lighter than that in the shR-BCYRN1+ASO-NC group (P=0.0013) Figure 4A.

The number of tumors in the shR-BCYRN1+ASO-NC group was increased than that in the pSlience-NC+ASO-NC group (P=0.0001), and the number of tumors in the shR-BCYRN1+ASO-miR-378c group was decreased than that in the shR-BCYRN1+ASO-NC group (P=0.0138) Figure 4B. The lnc-BCYRN1 level in the shR-BCYRN1+ASO-NC group was markedly lower than that in the pSlience-NC+ASO-NC group (P=0.0002), and the lnc-BCYRN1 level in the shR-BCYRN1+ASO-miR-378c group was significantly higher than that in the shR-BCYRN1+ASO-NC group (P=0.0059) Figure 4C. The RT-qPCR results showed that the mRNA levels of miR-378c in the shR-BCYRN1+ASO-NC group were higher than that in the pSlience-NC+ASO-NC

group (P=0.0002), and the mRNA levels of miR-378c in the shR-BCYRN1+ASO-miR-378c group were lower than that in the shR-BCYRN1+ASO-NC group (P=0.0001) Figure 4D. The RT-qPCR results showed that the mRNA levels of AJM1 in the three groups had the opposite trend with the mRNA levels of miR-378c Figure 4E. The western blot assay showed that the protein levels of Ki-67, N-cadherin, and AJM1 in the shR-BCYRN1+ASO-NC group were lower than those in the pSlience-NC+ASO-NC group (P=0037; P=0.0019; P=0.0083), and the protein levels of Ki-67, N-cadherin and AJM1 in the shR-BCYRN1+ASO-miR-378c group were higher than those in the shR-BCYRN1+ASO-NC group (P=0.0099; P=0.0004; P=0.0072) Figure 4F-4H. And we got the same trend with Vimentin, Snail, and Slug (Figure 4G). As shown in Figure 4F, the E-cadherin levels in the shR-BCYRN1+ASO-NC group were higher than that in the pSlience-NC+ASO-NC group (P=0.0005), and the protein levels of E-cadherin in the shR-BCYRN1+ASO-miR-378c group were lower than that in the shR-BCYRN1+ASO-NC group (P=0.0003). The above results showed that knockdown of BCYRN1 inhibited the critical protein expression involved in proliferation and EMT of SBC-2 cells, but knockdown of miR-378c could reverse it. These data illustrated that the occurrence of EMT could be inhibited by BCYRN1/miR-378c/AJM1 axis.

Figure 4: The effects of BCYRN1 knockdown on brain metastasis of SCLC cells in nude mice.



Note: SBC-2 cell stable transfected with pSlience-NC+ASO-NC, shR-BCYRN1+ASO-NC, and shR-BCYRN1+ASO-miR-378c, were injected into carotid artery of nude mice, respectively. After 20 days, the mice were sacrificed and examined for metastasis.

(A): Images of tumor size and the weight of each group.

(B): The tumor number of each group.

(C-E): The mRNA levels of lnc-BCYRN1, miR-378c and AJM1 of each group were detected by RT-qPCR, using GAPDH as internal reference.

(F-H): The protein levels of Ki-67, E-cadherin, N-cadherin, Vimentin, Snail, Slug, and AJM1 were detected by western blot assay, using GAPDH as internal reference. The above experiments are three independent repeated experiments, and the results are expressed as mean ± SD, *P<0.05, **P＜0.01, ***P＜0.005, ****P<0.001.

CONCLUSIONS

This study found that BCYRN1 was highly expressed in SCLC cell lines and SCLC patient serum. Knockdown of BCYRN1 in SCLC cell line SBC-2 cells inhibited the levels of proliferation-related gene Ki-67 and EMT, which in turn inhibiting cell proliferation, migration, and invasion. The miR-378c was a direct target of BCYRN1. The miR-378c was downregulated in SBC-2 and was negatively regulated by BCYRN1. Overexpression of miR-378c in BCYRN1 knockdown SBC-2 cells reversed BCYRN1 inhibition of SBC-2 cell proliferation, migration, and invasion. AJM1 was identified as a direct target of miR-378c, and knockdown of AJM1 inhibited the effects of miR-378c on SBC-2 cells, including proliferation, migration, and invasion. Additionally, knockdown of BCYRN1 inhibited the proliferation and EMT by BCYRN1/miR-378c/AJM1 axis on brain metastasis of SCLC cells in nude mice. BCYRN1 was found highly expressed in colorectal cancer,[9] cervical cancer[10] and gastric cancer,[11] which means that expressions of BCYRN1 are tissue specific. Hu[12] found that transcription factors promoted the binding of c-Myc to the BCYRN1 gene promoter to promote its transcription. Barr[13] found c-Myc was highly expressed in SCLC. There probably has a similar mechanism in SCLC cells to promote the transcription of BCYRN1 gene by c-Myc. Its transcriptional regulation mechanism needs to be further elucidated.

Since most SCLC patients had lost the opportunity for surgery when they went to the hospital, this study did not detect the expression of BCYRN1 in SCLC tissues. Our study found that BCYRN1 in the serum of SCLC patients was higher than that in healthy subjects, but there was no significant difference in BCYRN1 between serum of patients with distant metastases and those without distant metastases. The reason may be the limited number of SCLC patients with distant metastasis and without distant metastasis.

Studies in NSCLC found that BCYRN1 was highly expressed in NSCLC tissue and serum. The median progression-free survival time (15 months vs. 21 months) and median overall survival time (19 months vs. 28 months) of patients with high BCYRN1 expression were lower than those of patients with low BCYRN1 expression, suggesting that patients with high BCYRN1 expression had a poor prognosis of NSCLC patients.[14] Moreover, Meng[15] reported that the expression of BCYRN1 in TNM stage II-IV of lung adenocarcinoma and squamous cell carcinoma was higher than that in stage I, and patients with high BCYRN1 expression were more likely to develop lymph node metastasis. Combined with the results of this study, BCYRN1 expression may serve as a potential marker for SCLC and NSCLC diagnosis. In the future, we will further expand SCLC serum samples to analyze the correlation between BCYRN1 expression and the prognosis of serum of SCLC patients.

In this study, we found that upregulated BCYRN1 promoted the proliferation and metasitasis of SCLC cells by sponging miR-387c. Recently, it has been reported that LncRNA-NEF is downregulated in SCLC and inhibits SCLC invasion and metastasis. Colon cancer associated tran-2 (CCAT2), lung adenocarcinoma associated transcript 1 (LAUDT1) and bladder cancer associated transcript 1 (BLACAT1) are all highly expressed in SCLC, which is positively related to the clinical analysis and poor prognosis of SCLC patients, and exerts its effects through different mechanisms. It promotes cancer-promoting products such as SCLC cell proliferation, invasion, metastasis, and chemotherapy resistance.[16] Besides, Kuang[17] performed RNA deep sequencing on tumor tissues of SCLC patients with partial remission and stable/progressive chemotherapy after first-line chemotherapy, and found that HOXA-AS3, cancer susceptibility 9 (CASC9), and KEGG were significantly downregulated in tissues of patients with partial remission. It is suggested that these lncRNAs may regulate cell sensitivity to chemotherapeutics. Further analysis and identification of lncRNAs and molecular mechanisms involved in SCLC metastasis can provide a research basis for a deeper understanding of SCLC. Furthermore, it has been reported that LUADT1 inhibits SCLC cell migration and invasion through sponging of miR-15a-3p and upregulates its target gene Twist-related protein 1 (TWIST1).[18] Linc00173 leads to upregulated of non-receptor tyrosine protein kinase antibody (EKT) by inhibiting miR-218, thereby activating the pathway of Wnt/β-catenin signaling, enhancing SCIC cell proliferation, migration and invasion, and

enhancing the resistance to chemotherapeutic drugs.[19] LncRNA ZFPM2 antisense RNA 1(ZFPM2-AS1) upregulated the expression of TNF receptor associated factor (TRAF4) through sponging of miR-3612, and knockdown of ZFPM2-AS1 could inhibit SCLC cell proliferation, migration, and invasion. Combined with the results of this study, it is suggested that there is a complex lncRNA-miRNA network in SCLC, which is involved in regulating various malignant biological characteristics of SCLC.

This study first demonstrated that miR-378c was hypo-expressed, which acted as a downstream gene of BCYRN1 and upstream gene of AJM1 in SCLC. In NSCLC, miR-378c was also hypo-expressed, sponging by lncRNA PVT1 and upregulating its target gene facilitated glucose transporter (member 1, SLC2A1).[20] These suggested that miR-378c functions as a tumor suppressor of lung cancer, by involving in complex upstream and downstream networks. Previous studies have shown that the levels of miR-378c is remarkably downregulated in cancer tissues of osteosarcoma, intrahepatic cholangiocarcinoma, advanced gastric cancer and colorectal cancer, but no more detailed studies have been done.[21,22,23] Bioinformatics analysis based on microarray and database showed that miR-378c may be involved in proliferation, apoptosis, prognosis and survival of colorectal cancer, cervical squamous cell carcinoma and head and neck squamous cell carcinoma.[24,25,26]

Further analysis of the mechanism, we found that knockdown of BCYRN1 upregulated E-cadherin expression and downregulated the expression of N-cadherin, Snail and Slug in SCLC cells. Our data was supported by the report that Snail and Slug are ranscriptional repressors of E-cadherin, which can bind to the promoter region of E-cadherin to inhibit its transcription, and can also promote the expression of Vimentin through indirect regulation.[27] On the other hand, studies in colon cancer reported that Vimentin was a direct target gene of miR-378. [28] In this study, knockdown of miR-378c resulted in an increase in the expression of Vimentin, indicating that miR-378c probably also targeting Vimentin in SCLC cells.

Apical Junction Molecule-1 (AJM1), one cytosolic apical marker of intestine epithelial cells, is critical to maintaining the epithelial cell junction's integrity.[29-30] The AJM1 gene is predicted to be involved in the cell/cell junction organization.[31] AJM1 is the coiled-coil protein, and it localizes to an apical junctional domain of Caenorhabditis elegans epithelia basilar of the HMR-HMP (cadherin-catenin) complex. Without AJM-1, the domain integrity is damaged.[32]

Taken together, lncRNA BCYRN1 was hyperexpressed in SCLC cells. Knockdown of BCYRN1 could inhibit proliferation and metastasis of SCLC cells in vivo and in vitro, by directly suppressing miR-378c/AJM1 and indirectly suppressing genes involved in proliferation and EMT. Our findings provided a new light on the effects of BYCRN1 and miR-378c in the development of SCLC.

DECLARATIONS

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Authors' contributions

LYF and HWN conceived and designed the study. CWT prepares experimental materials. LD analyzed the data. WZ wrote the manuscript. DGL and LYK confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no competing interests.

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