Tumor suppressive functions of hsa-miR-34a on cell cycle, migration and protective autophagy in bladder cancer

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Abstract. Bladder cancer (BC) cells exhibit a high basal level of autophagy activity, which contributes to the development of a protective mechanism for cellular survival against current treatments. Hsa-microRNA-34a (miR-34a) presents anti-tumor function in several types of cancer. However, the functional mechanism of miR-34a in regulating tumor aggressiveness and protective autophagy of BC remains largely unknown. First, transfected BC cells with miR-34a mimic exhibited LC3-II and p62 accumulation through immunofluorescence staining. It was demonstrated that syntaxin 17 (STX17), which is required for autophagosome-lysosome fusion, was downregulated upon miR-34a mimic treatment. Mechanistically, miR-34a reduced the expression of STX17 proteins that directly bind on STX17 3'-untranslated regions and thus suppressed STX17 mRNA translation to eventually inhibit protective autophagy in BC. Cell viability and colony formation assays revealed that overexpression of miR-34a in BC cells enhances the chemosensitivity of cisplatin, doxorubicin, epirubicin and mitomycin C. Furthermore, miR-34a inhibited cell proliferation and triggered G0/G1 cell cycle arrest by inhibiting cyclin D1 and cyclin E2 protein expression. Moreover, miR-34a suppressed cell motility through the downregulation of epithelial-mesenchymal transition. In summary, miR-34a inhibits cell proliferation, motility and autophagy activity in BC, which can benefit BC treatment.

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Introduction

Bladder cancer (BC) is a common urinary system cancer worldwide (1). Among cases of BC, 90% are of urothelial carcinoma, 5% are of squamous cell carcinoma, and the remaining 5% are of adenocarcinoma and undifferentiated carcinoma (2). Although the overall survival of patients with BC has significantly improved with current therapies of transurethral resection, intravesical chemotherapy, and Bacillus Calmette-Guérin (BCG)-based immunotherapy, a large proportion (~60-70%) of patients with BC have recurring non-invasive or *in situ* tumors (3), which progress into muscle invasive BC (MIBC; in ~50% of all patients with BC) (4). Therefore, the development of novel therapeutic agents is required to advance BC treatment.

Hanahan and Weinberg in 2011 proposed ten hallmarks of cancer that are crucial for the transformation from normalcy to neoplastic growth states and their ability to form malignant tumors and distant metastatic focus (5). This substantially affects the development of new therapeutic strategies for the treatment of various cancers (6). One of the major features of cancer is invasion and metastasis, which was demonstrated to be controlled by the epithelial-mesenchymal transition (EMT) state (7). EMT is a dynamic process in which an epithelial phenotype converts into a mesenchymal phenotype to enable the invasion and metastasis of cancer cells (8). Autophagy also plays a critical role in several features of cancer cells; most importantly, autophagy is implicated at various stages of tumorigenesis (9). Autophagy is a double-edged sword; it not only promotes tumor progression but also serves as a survival mechanism in cancer cells to evade apoptotic cell death against therapeutic agents (10). Notably, BC cells were reported to exhibit a higher basal level of autophagic flux with increased LC3-II expression compared with cells of breast, prostate and kidney cancer (11). The inhibition of autophagy activity using pharmacological inhibitors-such as chloroquine, hydroxychloroquine, and bafilomycin A1-blocks BC cell invasiveness (12), triggers cell apoptosis *in vitro*, and decreases tumor growth

in vivo (13). Therefore, the targeting of autophagic flux may be an efficient new strategy for overcoming autophagy-related resistance to current therapies against BC.

MicroRNA (miRNA or miR), a small molecule of noncoding RNAs (~21-25 nucleotides), acts in conjunction with RNA-induced silencing complex to bind on the 3'-untranslated region (UTR) of target messenger RNA. It prevents the formation of the translation initiation complex, and this results in translation inhibition (14). Previous studies have reported that up to 60% of protein-coding genes can be regulated by miRNAs at the translational level (15). In fact, miRNAs are involved in the regulation of numerous cellular and developmental processes (16). Furthermore, the dysregulation of miRNAs is associated with cancer (17,18), Alzheimer's disease (19), rheumatoid arthritis (20), and cardiovascular diseases (21). miRNAs can be divided into oncogenic (i.e., tumor-promoter) miRNA and tumor-suppressor miRNA (22). Both oncogenic and tumor-suppressor miRNAs play roles in most features of cancer, including proliferation, apoptosis, angiogenesis, invasion and metastasis (23). MiR-34a was reported to be a tumor-suppressor miRNA by the majority of studies in the literature (24,25). For instance, miR-34a epigenetically and negatively regulates the functional properties of cancer stem cells by targeting stemness factors including NOTCH, MYC, BCL-2, and CD44 (26). MiR-34a suppresses tumor progression by inhibiting cell cycle (27), invasiveness (28), metastasis (29) and cancer-specific immune evasion (30), but how it regulates autophagic flux is not completely understood in BC.

In the present study, the role of miR-34a was investigate in numerous cancer-related activities, including cell cycle arrest, cell motility inhibition and autophagy activity suppression, and the emergence of miR-34a treatment as a novel therapeutic strategy for BC was discussed.

Materials and methods

Cell culture. The BC cell lines 5637 and T24 were obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan) and cultured in RPMI-1640 and McCoy's 5A medium, respectively (Gibco; Thermo Fisher Scientific, Inc.). Both culture media were supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.).

Transfection of miRNA mimic. MISSION synthetic negative control mimic and hsa-miR-34a mimic were purchased from Sigma-Aldrich; Merck KGaA. ViaFect transfection reagent (Promega Corporation) was used to transfect 25 nM control mimic or hsa-miR-34a into BC cells at 37˚C for 24 h. Cell samples were then analyzed for p62, LC3-II and STX17 expression.

Establishment of hsa-miR-34a stable cells. The pLenti-III-mir-GFP vector inserted with hsa-miR-34a sequence was provided by Applied Biological Materials. The procedure for establishing the miR-34a-expressing stable cells was identical to that of a previous study (18).

Western blot analysis. The immunoblotting method was identical to that in a previous study by the authors (31). Primary antibodies anti-p62 (1:3,000; cat. no. GTX100685), anti-LC3-II (1:3,000; cat. no. GTX127375) (both from GeneTex, Inc.), anti-STX17 (1:1,000; cat. no. 31261), anti-cyclin D1 (1:1,000; cat. no. 2978), anti-cyclin E2 (1:1,000; cat. no. 4132) (all from Cell Signaling technology, Inc.), or anti-GAPDH (1:5,000; cat. no. ab8245; Abcam) were used to detect indicated protein levels.

Gap closure assay. A three-well silicone insert (Ibidi GmbH) was used to perform wound-like scratches in each well. Detached miR-34a stable cells were removed with phosphate-buffered saline wash and cultured in the serum-free medium for 24 h. Microscope images of migratory cells were captured (Zeiss AG) and the width of the gap in each scratch was measured using ImageJ software v1.44p (National Institutes of Health).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNAs were isolated using easy-BLUE™ Total RNA Extraction Kit. The mRNA and miRNA were reverse transcribed to cDNA using the M-MLV Reverse Transcriptase kit (Invitrogen; Thermo Fisher Scientific, Inc.) and Mir-X™ miRNA First-Strand Synthesis kit (Clontech Laboratories, Inc.) according to the manufacturer's instructions, respectively. RT-qPCR analyses were conducted using a SYBR Green Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) and conducted on a StepOnePlus sequence detection system (Thermo Fisher Scientific, Inc.). The thermal cycling conditions were identical to those in previous studies (32,33). RT-qPCR primers for indicated mRNAs detection were listed in Table I. For miR-34a detection, the forward primer of miR-34a was 5'-TGGCAGTGTCTTAGC TGGTTGT-3' (mRQ 3 as reverse primer supplied with the Mir-X™ miRNA First-Strand Synthesis kit). The relative gene expression was analyzed by using the comparative Ct ($2^{-\Delta\Delta Cq}$) method with genes normalized to GAPDH (mRNA), β-Actin (mRNA) or snRNA U6 (miRNA) (34).

Colony formation assay. The miR-34a stable cells were seeded on a six-well plate at a density of $3x10^3$ cells/well. A colony consists of at least 50 stable cells. After 7 days of colony formation, colonies were fixed with 3.7% formaldehyde for 20 min and then stained with 0.05% crystal violet (w/v) for another 20 min at room temperature. Colonies were indicated by the absorbance of crystal violet extraction with 10% acetic acid.

Luciferase reporter assay. A DNA fragment containing 3'-UTRs of STX17 targeted by miR-34a were constructed into a pmirGLO vector (Promega Corporation). BC cells seeded on a 12-well plate were transfected with empty vector or 3'-UTR reporter constructs (1 $\mu g/\mu l$) using a ViaFect transfection reagent (Promega Corporation) for 24 h. Subsequently, cells were treated with control or miR-34a mimic for another 24 h. Finally, luciferase activity was measured using a Dual-Luciferase kit (Promega Corporation). Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Flow cytometry. The miR-34a stable cells ($3x10^5$) were seeded in six-well plates. Cell samples were then stained with propidium iodide (Cell Signaling Technology, Inc.) at room temperature

Table I. Sequences of primers used for reverse transcription-quantitative PCR.

Gene name	Species	Primer sequence (5'→3')
KIAA1632 (EPG5)	Human	F: CCTTCTGTATCTTCACCGTCCG
	Human	R: GAAGTCAGCCACCTCGGTCAAA
SNAP29	Human	F: CAGCCACCCAAACCTTAGAAAGC
	Human	R: CGATCTTCTGGTGATAGGCTCG
Syntaxin 17	Human	F: TCGTGGGAAACCTTAGAAGCGG
	Human	R: GCAGCACTGTTGACATGGTCTG
RAB7L1	Human	F: TGCTCTGAAGGTTCTCCAGTGG
	Human	R: GGCAGAGGCATCCCGATAATAC
Cyclin D1	Human	F: TCTACACCGACAACTCCATCCG
	Human	R: TCTGGCATTTTGGAGAGGAAGTG
Cyclin E2	Human	F: CTTACGTCACTGATGGTGCTTGC
	Human	R: CTTGGAGAAAGAGATTTAGCCAGG
Snail	Human	F: ACTGCAACAAGGAATACCTCAG
	Human	R: GCACTGGTACTTCTTGACATCTG
Twist	Human	F: GCCAGGTACATCGACTTCCTCT
	Human	R: TCCATCCTCCAGACCGAGAAGG
ZEB1	Human	F: GGCATACACCTACTCAACTACGG
	Human	R: TGGGCGGTGTAGAATCAGAGTC
ZEB2	Human	F: AATGCACAGAGTGTGGCAAGGC
	Human	R: CTGCTGATGTGCGAACTGTAGG
E-cadherin	Human	F: CGAGAGCTACACGTTCACGG
	Human	R: GGGTGTCGAGGGAAAAATAGG
N-cadherin	Human	F: TGCGGTACAGTGTAACTGGG
	Human	R: GAAACCGGGCTATCTGCTCG
CDH11	Human	F: ACCCTCACCATCAAAGTCTG
	Human	R: TCAGGGTCACAAACAATACT
Vimentin	Human	F: AGTCCACTGAGTACCGGAGAC
	Human	R: CATTTCACGCATCTGGCGTTC
β-actin	Human	F: CACCATTGGCAATGAGCGGTTC
	Human	R: AGGTCTTTGCGGATGTCCACGT
GAPDH	Human	F: GTCTCCTCTGACTTCAACAGCG
	Human	R: ACCACCCTGTTGCTGTAGCCAA

F, forward; R, reverse.

for 30 min. Cell cycle distribution analysis was assessed using an Accuri C5 flow cytometer (BD Biosciences) and quantified using the CellQuest Pro software (BD Biosciences).

Immunofluorescence staining. The BC cells were placed on a chamber slide (Sigma-Aldrich; Merck KGaA) for treatment. The immunofluorescence staining methods were identical to those in a previous study by the authors (33). Primary anti-p62 and anti-LC3-II antibodies (both 1/100 dilution) were used to detect p62 and LC3-II proteins expression in BC cells. In addition, an acridine orange (AO) staining assay (Sigma-Aldrich; Merck KGaA) was used to visualize autophagic vacuoles after miR-34a mimic treatment. Lysosome alteration in miR-34a mimic-treated BC cells was detected using LysoTracker Red DND-99 (Thermo Fisher Scientific, Inc.). All fluorescent imaging was performed using a Nikon Ti2 microscopy system (Nikon Corporation).

Statistical analysis. All experiments were performed in triplicate and analyzed by using GraphPad Prism 9 (GraphPad Software, Inc.). Differences were analyzed using unpaired t-test. One-way analysis of variance followed by Bonferroni's post hoc comparison tests were used to compare the means of three or more groups. The results are presented in terms of the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

MiR-34a blocks autophagic flux in human BC cells. Autophagy has been reported to exert a protective mechanism in BC (35). First, it was aimed to determine whether miR-34a suppresses protective autophagy in human BC cells (5637 and T24). The treatment of 5637 and T24 cells with miR-34a mimic induced autophagy marker LC3-II and p62 protein expression

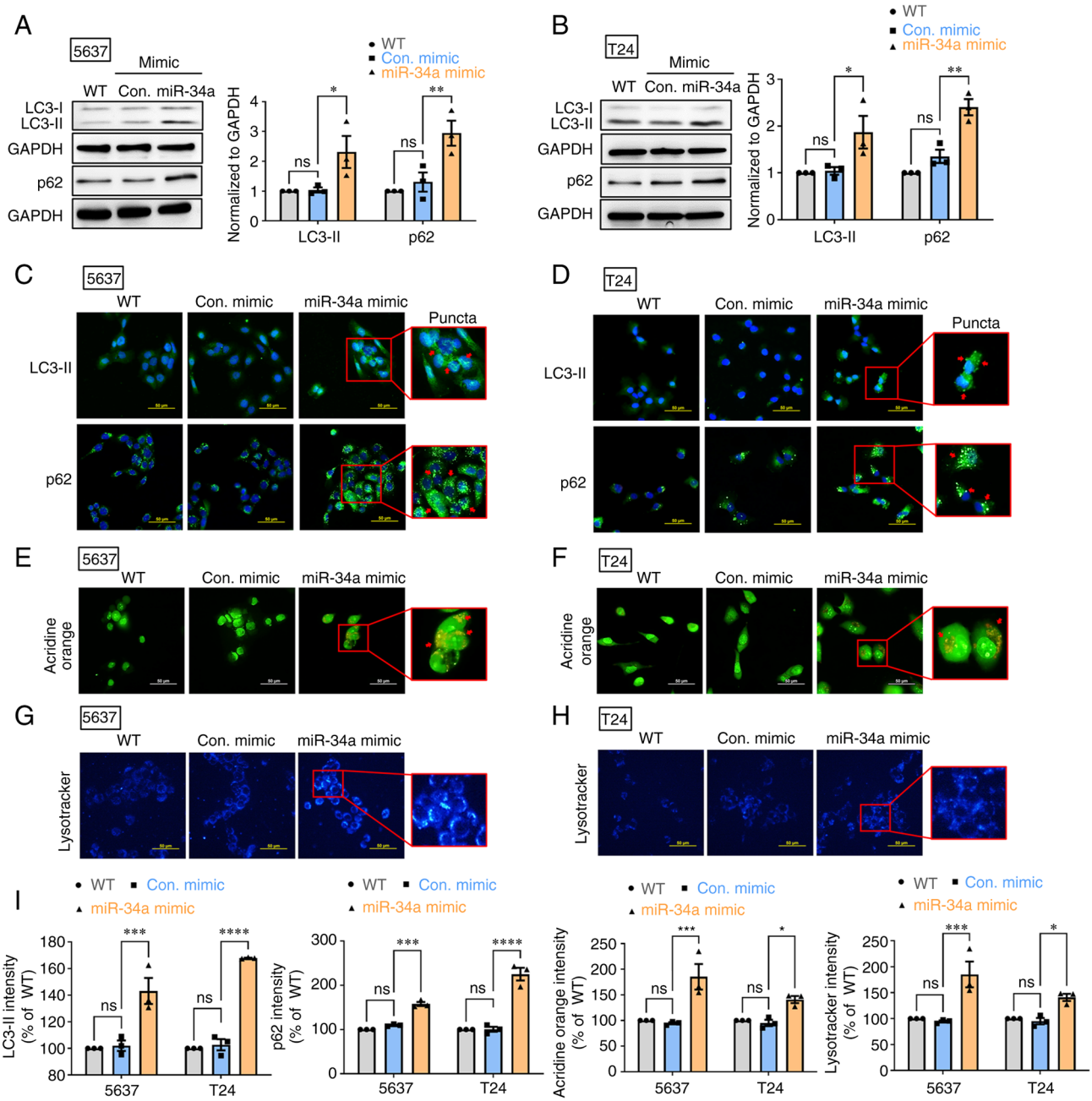

Figure 1. MiR-34a interrupts autophagic flux in BC cells. (A and B) Human BC cells (5637 and T24) were transfected with negative control (Con.) or miR-34a mimic (100 nM) for 24 h, then protein levels of LC3-I/II and p62 were examined using western blot assay. Protein expression values were quantified using UN-SCAN-IT gel 6.1 software. (C and D) Detection of LC3-II and p62 puncta used immunofluorescence analysis (Red arrow: puncta). Nuclei were counter-stained with 40,6-diamidino-2-phenylindole. (E and F) The acidic vesicular organelle presented in BC cells were stained with acridine orange (Red arrow: acidic vesicular organelle). (G and H) The BC cells were incubated with LysoTracker Blue to detect the lysosome in cytoplasm, wherein blue signals indicated the lysosomes. (I) Fluorescence intensity was quantified by ImageJ software. All data are expressed as the means ± SD in triplicate samples. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 relative to the control group. miR, microRNA; BC, bladder cancer; WT, wild-type.

(Fig. 1A and B). The successful transfection of miR-34a in mimic was confirmed by RT-qPCR assay (Fig. S1A). It was found that miR-34a promoted LC3-II and p62 accumulation in cytoplasm to form aberrant LC3-II and p62-positive puncta (Fig. 1C and D). Subsequently, AO staining was performed to visualize autophagic vacuoles after miR-34a treatment. The findings indicated that miR-34a increased the number of autophagic vacuoles in BC cells (Fig. 1E and F). The results from LysoTracker Blue staining confirmed the accumulation of lysosomes after miR-34a treatment in BC cells (Fig. 1G and H). Fluorescence intensity of LC3-II, p62, AO and LysoTracker Blue in BC cells is represented in Fig. 1I. These findings jointly suggested that miR-34a causes defects in autophagic flux, which leads to LC3-II and p62 accumulation in the cytoplasm of BC cells.

MiR-34a inhibits STX17 expression by integrating with the 3'-UTR regions of STX17 mRNA. The autophagosome-lysosome fusion step is indispensable to autophagic flux and helps cells degrade components in the cytoplasm (36).

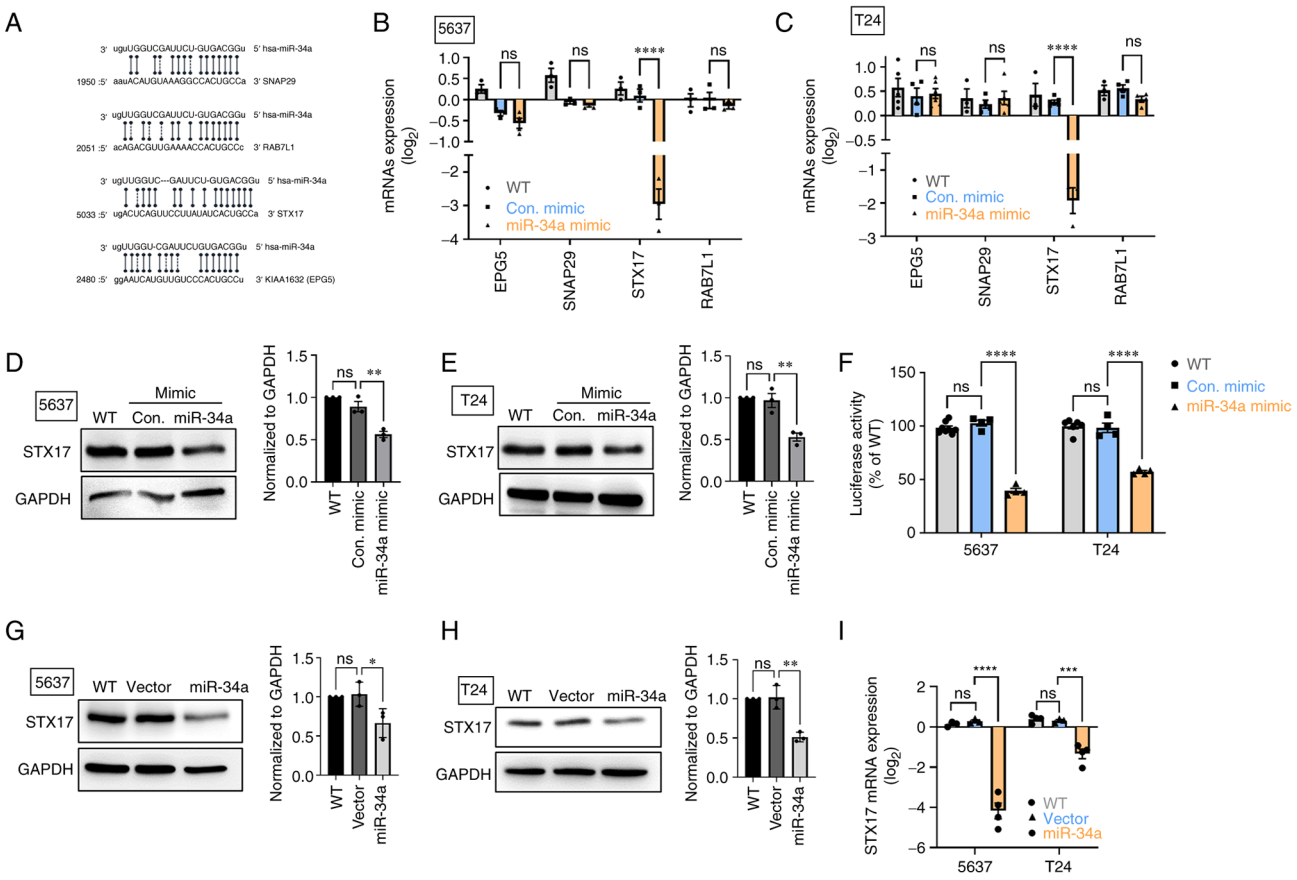

Figure 2. STX17 is involved in the mechanism of miR-34a-mediated autophagy activity. (A) Positions of the putative miR-34a binding sites in 3'UTR of SNAP29, RAB7L1, STX17 and EPG5. (B and C) RT-qPCR assay revealed SNAP29, RAB7L1, STX17 and EPG5 mRNA expression after miR-34a mimic treatment. (D and E) The STX17 protein expression was detected using a western blot assay. (F) Transfection of bladder cancer cells with STX17-3'-UTR plasmid (1 $\mu g/\mu l$) and miR-34a mimic (100 nM) for 24 h, then relative luciferase/Renilla activities were measured. (G-I) The basal levels of STX17 protein and mRNA expression in control stable and miR-34a stable cells were detected using western blotting and RT-qPCR assay, respectively. All data are expressed as the mean ± SD in triplicate samples. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 relative to the control group. STX17, syntaxin 17; miR, microRNA; UTR, untranslated region; RT-qPCR, reverse transcription-quantitative PCR; WT, wild-type; Con, control; ns, not significant.

Analyzing a miRNA database, four candidates target of miR-34a were identified: Ectopic P-granules 5 autophagy tethering factor (EPG5 or known as KIAA1632), synaptosome associated protein 29 (SNAP29), syntaxin 17 (STX17), and a member of RAS oncogene family-like 1 (RAB7L1; Fig. 2A), which are all implicated in the process of autophagosome-lysosome fusion (37). The transfection of BC cells with miR-34a mimic was observed to decrease STX17 mRNA and protein expression (Fig. 2B-E); however, no such effect was identified in mRNA levels of EPG5, SNAP29 and RAB7L1 expression (Fig. 2B and C). Subsequently, a luciferase reporter assay was used to verify that miR-34a directly binds on 3'-UTR regions of STX17 mRNA. As indicated in Fig. 2F, miR-34a strongly inhibited luciferase activity; this indicated that miR-34a directly binds on 3'-UTR of STX17 to inhibit STX17 mRNA translation.

Stable cells that constitutively express miR-34a were established to verify the effect of miR-34a overexpression in regulating STX17 expression. The resulting data indicated that miR-34a exhibited lower mRNA expression and a lower STX17 protein level compared with an empty vector (Fig. 2G-I). Therefore, miR-34a serves as a novel autophagic inhibitor through the inhibition of the autophagosome-lysosome fusion step in BC.

MiR-34a inhibits STX17-mediated autophagy activity and promotes chemosensitivity. STX17 overexpression was used to verify the function of STX17 in the miR-34a-inhibited autophagic flux in BC cells. The overexpression efficiency of STX17 was verified by comparisons with a vector at protein levels (Fig. 3A). Upregulated expression of LC3-II and reduced p62 expression was observed, confirming autophagic activity during STX17 overexpression (Fig. S1B). It was found that miR-34a promoted LC3-II and p62 accumulation in both 5637 and T24 cells; however, STX17 overexpression inhibited such accumulation (Fig. 3B and C). To explore the therapeutic potential of miR-34a, miR-34a and first-line chemotherapeutic drugs were jointly administered for BC. Cell viability results indicated that miR-34a promotes the chemosensitivity of cisplatin, doxorubicin, epirubicin, and mitomycin C in BC cells; while STX17 overexpression suppresses this phenomenon (Fig. 4A-D). Colony formation assay showed that the combinatory treatment of each chemotherapeutic drug and miR-34a further reduces cell survival as compared with chemotherapeutic drug alone (Fig. 4E-H). Therefore, the inhibition of STX17-regulated autophagy by miR-34a sensitizes BC to chemotherapeutic drug-induced cell death.

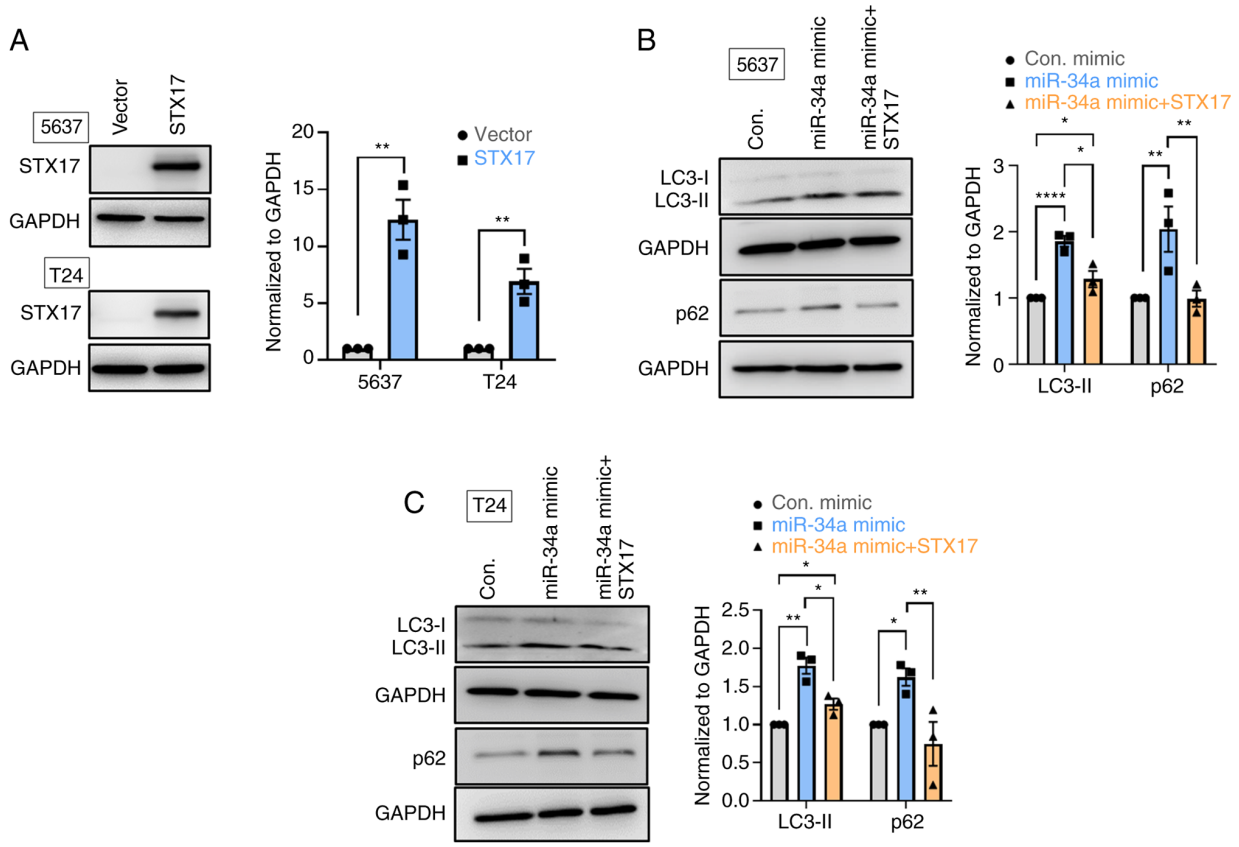

Figure 3. STX17 overexpression rescues miR-34a-inhibited autophagy activity. (A) BC cells were transfected with vector or STX17 plasmid (1 μg/μl) for 24 h. The overexpressing efficiency of STX17 was verified using western blot assays in 5637 and T24 cells. (B and C) Co-transfection of BC cells with miR-34a mimic and STX17 plasmid for 24 h, the LC3-I/II and p62 protein expression were then analyzed through western blotting. All data are expressed as the mean ± SD in triplicate samples. *P<0.05, **P<0.01 and ****P<0.0001 relative to the control group. STX17, syntaxin 17; miR, microRNA; BC, bladder cancer.

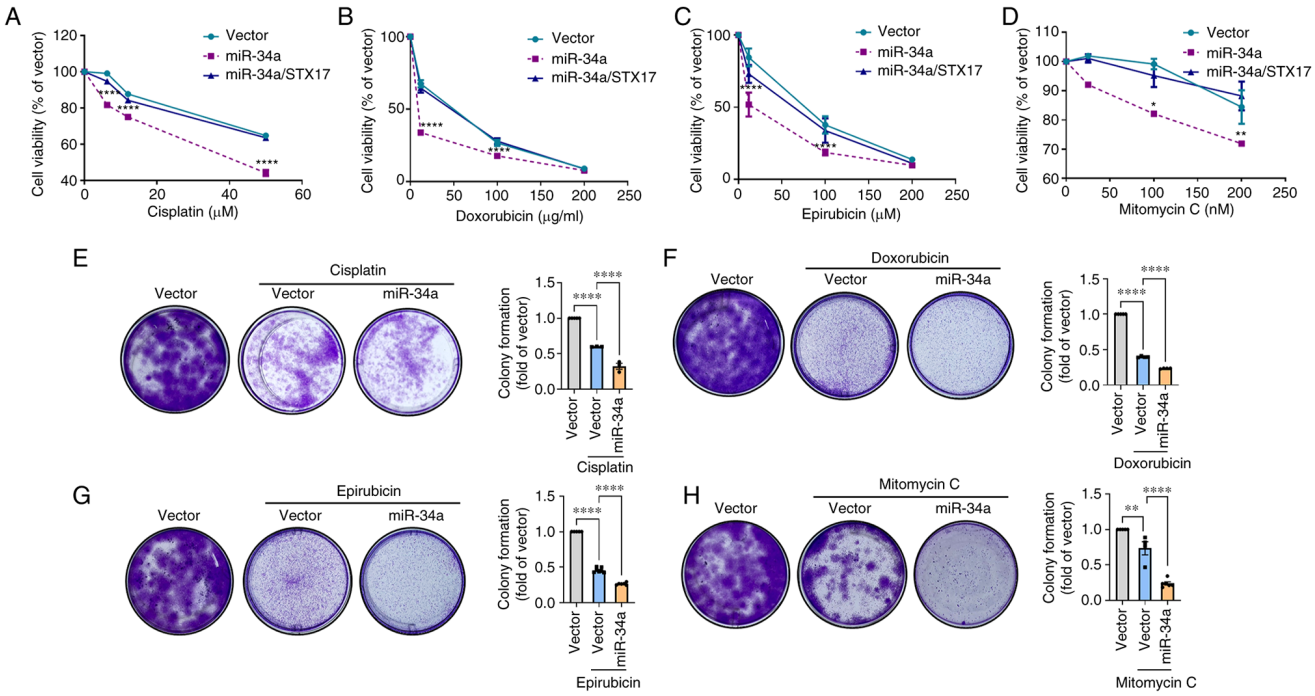

Figure 4. MiR-34a confers chemosensitivity in BC cells. (A-D) The miR-34a stable cells were transfected with or without STX17 plasmid (1 μg/μl) for 24 h followed by exposing to various concentrations of chemotherapeutic drugs, including cisplatin (0-50 μM), doxorubicin (0-200 μg/ml), epirubicin (0-200 μM) and mitomycin C (0-200 nM). After 24 h of treatment, resazurin-based cell viability was measured. (E-H) The miR-34a stable cells were treated with chemotherapeutic drugs, including cisplatin (1.5 μM), doxorubicin (0.1 μg/ml), epirubicin (0.1 μM) and mitomycin C (12.5 nM) for 7 days. Cell survival was detected by colony formation assay. All data are expressed as the mean ± SDs in triplicate samples. *P<0.05, **P<0.01, and ****P<0.0001 relative to the control group. miR, microRNA; BC, bladder cancer; STX17, syntaxin 17.

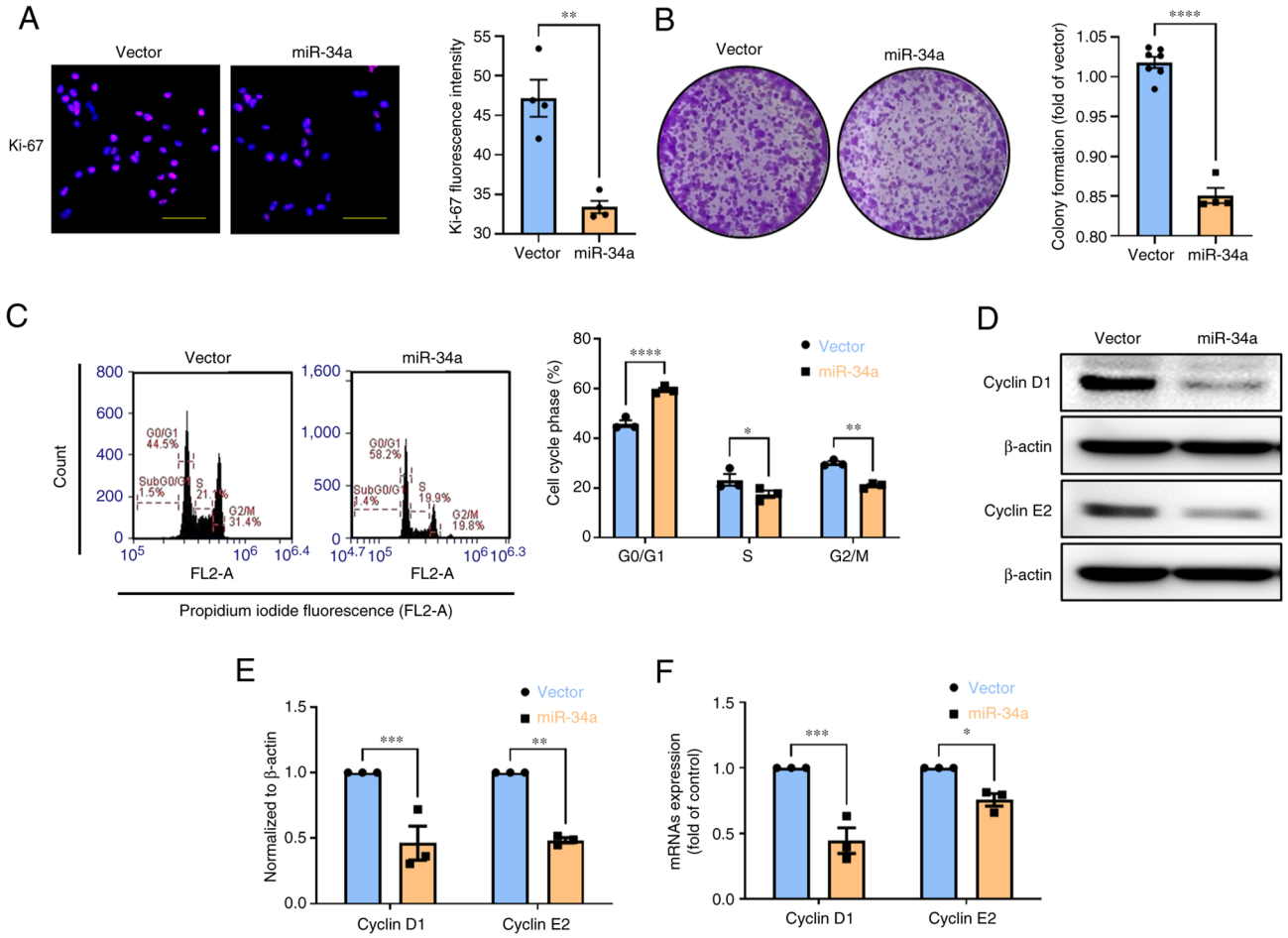

Figure 5. MiR-34a promotes G0/G1 cell cycle arrest in BC cells. (A) The miR-34a stable cells were stained with DAPI for nucleus (blue) and Alexa Fluor 546 for Ki-67 (red). Ki-67 fluorescence intensity was quantified by ImageJ software. (B) Colony formation assay was used to measure cell survival in miR-34a stable cells. (C) Effects of miR-34a on cell cycle by flow cytometry in miR-34a stable cells. (D-F) The protein and mRNA levels of cyclin D1 and cyclin E2 were detected by western blot assay and reverse transcription-quantitative PCR assay in miR-34a stable cells, respectively. All data are expressed as the means ± SD in triplicate samples. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 relative to the control group. miR, microRNA; BC, bladder cancer.

MiR-34a triggers G0/G1 cell cycle arrest in BC cells. Chronic proliferation and cell migration and invasion play a critical role in the features of cancer (5), therefore, the antitumor function of miR-34a on these mechanisms deserves to be thoroughly investigated in BC. Overexpression of BC cells with miR-34a significantly decreased proliferation marker Ki-67 expression and colony formation (Fig. 5A and B), while STX17 overexpression suppresses this phenomenon (Fig. S1C). Compared with the vector, miR-34a induced an increase in the G0/G1-phase population from 45.86-59.45%. A decrease in the S-phase and G2/M-phase population from 23.26-17.48% and from 30.06-20.95%, respectively, was observed (Fig. 5C). Mechanistically, miR-34a suppresses the protein and mRNA expression of cyclin D1 and cyclin E2; this affects G0/1 arrest in BC cells (Fig. 5D-F). Collectively, these data revealed that miR-34a inhibits cell proliferation through G0/G1 cell cycle arrest in BC.

Mir-34a suppresses cell motility through EMT inhibition. EMT is associated with migration and invasion in various cancers (38,39). Given the importance of these mechanisms, it was investigated whether miR-34a inhibits cell motility through EMT suppression. As revealed in the gap closure assay, miR-34a group presented the inhibitory action on cell motility, and the vector group had no such effect (Fig. 6A). However, STX17 overexpression impeded miR-34a-inhibited cell motility (Fig. S1D), indicating that autophagy is involved in miR-34a-regulated cell motility of BC. It was found that miR-34a strongly suppressed the levels of EMT-transcription factors expression, including snail1, twist, and ZEB1/2 (Fig. 6B-E). Further results indicated that the expression of the epithelial marker (E-cadherin) was upregulated (Fig. 6F), whereas the mesenchymal markers (N-cadherin and cadherin-11) was reduced in BC (Fig. 6G and H). Vimentin exhibited no such effect (Fig. 6I). Hence, the present results suggested that miR-34a plays a critical role in inhibiting cell motility through EMT suppression.

Discussion

The findings of the present study indicated that miR-34a exerts potent anticancer effects through three mechanisms: i) MiR-34a triggers G0/G1 arrest and thus inhibits cell proliferation; ii) miR-34a suppresses cell motility by EMT downregulation; and iii) miR-34a directly inhibits STX17 expression to block autophagic flux in BC, which in turn confers chemosensitivity.

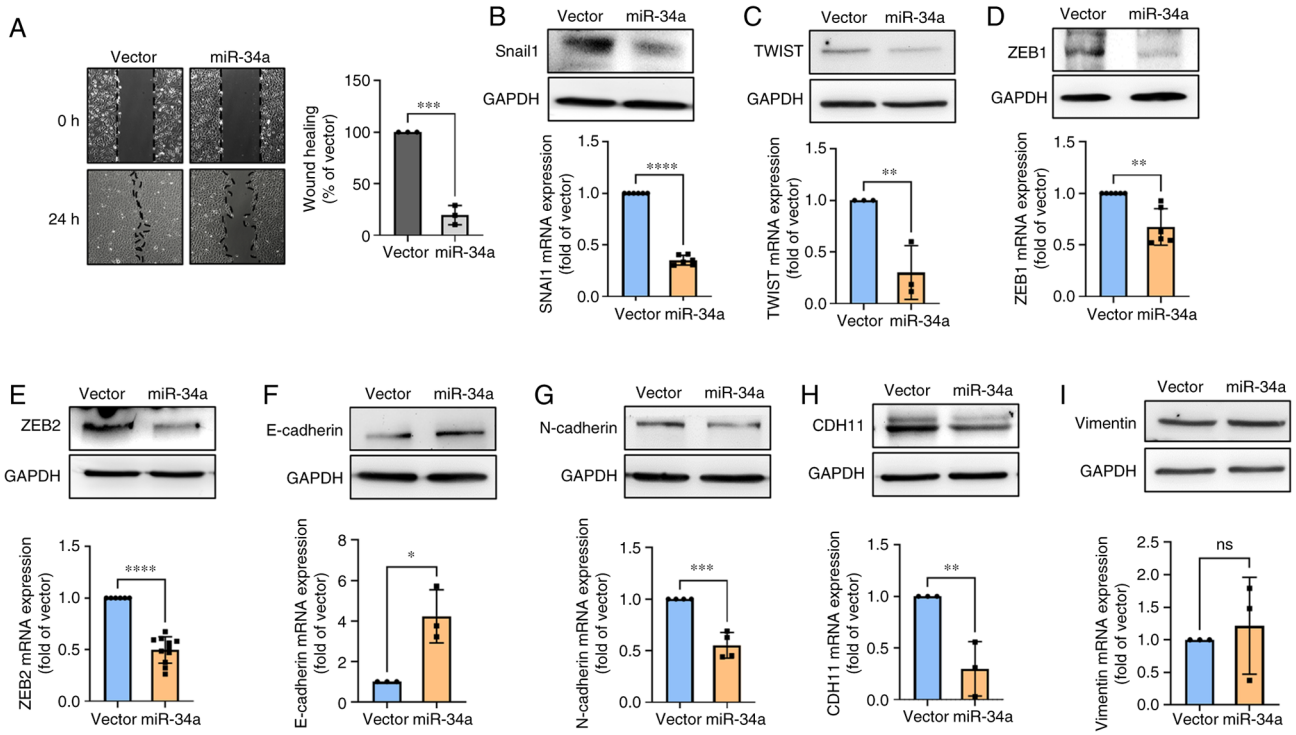

Figure 6. MiR-34a inhibits cell motility through EMT suppression. (A) The cell migratory capacity was assessed using a gap closure assay and quantified by counting the migratory cells. (B-I) The levels of EMT-TFs and EMT markers protein and mRNA expression were evaluated using western blot assay and reverse transcription-quantitative PCR assay, respectively. All data are expressed as the mean ± SDs in triplicate samples. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 relative to the control group. miR, microRNA; EMT, epithelial-mesenchymal transition; ns, not significant.

Multiple targets of miR-34a are verified in human cancers (25). For example, miR-34a leads to cell cycle arrest by targeting cyclins and cyclin dependent-kinases (CDK1, CDK4, CDK6, cyclin D1 and cyclin E2) (27,40). MiR-34a also negative regulates EMT activity by targeting EMT-transcription factors ZEB1 (41), Snail (42) and Twist (43) in various cancers, showing the broad potency of miR-34a as a tumor suppressor. Moreover, miR-34a has been reported to be involved in tumor-mediated immunosuppression (44,45). Mechanistically, the overexpression of miR-34a in cells from acute myeloid leukemia blocks PD-L1 surface expression, which interrupts PD-L1-specific T cell apoptosis. This indicates that miR-34a may serve a similar function as anti-CTLA-4 and anti-PD-1/PD-L1 immune checkpoint inhibitors do (45). Furthermore, miR-34a inhibits cancer cell proliferation, migration and invasion by upregulating the tumor-suppressor gene PTEN in BC cells (46) and downregulating proto-oncogene c-Met in uveal melanoma cells (47). A previous study by the authors highlights that miR-34a interacts with the 3'-UTR regions of MMP-2 mRNA to silence cell invasiveness in BC (24). Clinically, miR-34a is also associated with BC based on our analysis of The Cancer Genome Atlas database-regardless of overall survival, ethnicity, tumor histology, molecular subtype, clinical stage and nodal metastasis status-and can thus serve as a BC biomarker (35). These findings considerably advance our understanding of miR-34a's function as a potential therapeutic drug for or diagnostic marker of human BC or other types of cancer.

Protective autophagy induced by antitumor agents was found to function as a survival pathway to promote therapeutic resistance in various cancers (35). Numerous preclinical studies have demonstrated that lysosomal inhibitors of autophagy, such as chloroquine and hydroxychloroquine (which have already been approved for clinical use by the US Food and Drug Administration), sensitize cancer cells to conventional therapy (48-50). In addition, autophagy has been reported to facilitate focal adhesion and cell motility through the collaboration of LC3 with paxillin (51). Autophagy supports to regulate cell cycle by the degradation of a number of cell cycle proteins and other signaling adaptors, including cyclins, cyclin-dependent kinase, hypoxia-inducible factor 1-alpha and senescence associated protein (52). Therefore, interrupting autophagy activity by a combination of autophagy inhibitors in cancer therapy to improve clinical outcomes for patients has become a potential strategy for future cancer treatments (53). The present findings demonstrated that miR-34a can serve as a novel autophagic inhibitor by suppressing STX17 expression in BC cells. The cellular function of STX17 is known for promoting autophagosomal fusion with endosome or lysosome (54); however, miR-34a can directly bind on STX17 3'-UTR to inhibit its protein translation, which in turn blocks autophagic flux in cancer cells. It was also observed that miR-34a mimic enhanced the efficacy of chemotherapeutic drugs, including cisplatin, doxorubicin, epirubicin and mitomycin C, against BC cells. However, STX17 overexpression rescued cell survival and motility inhibited by miR-34a (Fig. S1C and D), showing that autophagy is involved in miR-34a-regulated antitumor functions of BC. A similar study reported that miR-30a-3p enhances cisplatin-based chemosensitivity through the inhibition of critical autophagy-related proteins (ATG5, ATG12 and Beclin1) in BC and has high efficacy against MIBC through the suppression

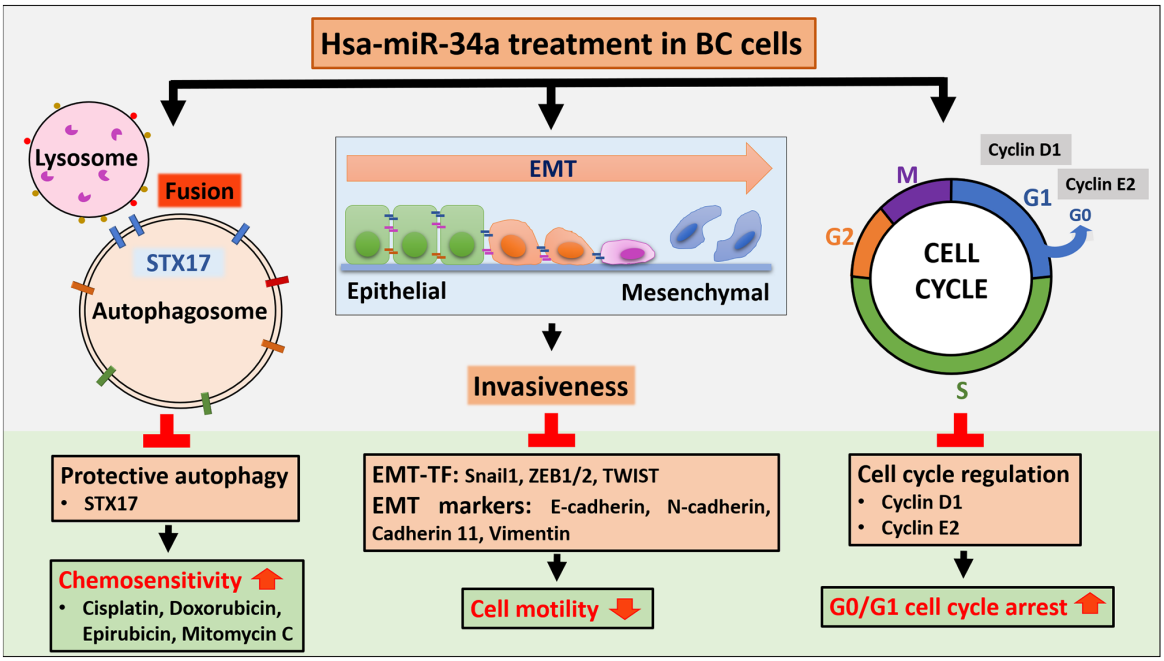

Figure 7. Inhibition of protective autophagy, cell motility, and promotion of cell cycle arrest by hsa-miR-34a treatment in BC. miR, microRNA; BC, bladder cancer.

of matrix metalloproteinases-mediated cell aggressiveness (18). These preclinical results demonstrated the potential of protective autophagy-targeting miRNA therapy.

In 2013, the first and the only clinical trial (NCT01829971) of liposomal mimic of miR-34a (MRX34) was conducted to evaluate its safety when used against several solid tumors, including primary liver cancer, small cell lung cancer, lymphoma, melanoma, multiple myeloma, renal cell carcinoma and non-small cell lung cancer. Unfortunately, the clinical trial was terminated due to the development of five serious immune-related adverse events upon MRX34 treatment in patients (55). In addition, numerous clinical trials have been conducted to identify miRNAs as novel diagnostic and prognostic biomarkers of i) non-Hodgkin's lymphoma and acute leukemia (NCT05477667), ii) hormone-sensitive breast cancer (NCT01612871) (56), and iii) non-MIBC (NCT03591367). Similar clinical trials are ongoing. Such extensive research efforts suggest the potential of miRNA therapy for cancer.

In conclusion, the present findings revealed new clinical potential for miR-34a as an antitumor agent in the treatment of human BC through blocking the cell cycle, inhibiting motility and impairing protective autophagy. MiR-34a can be administered with anti-BC chemotherapeutic drugs to improve their efficacy (Fig. 7).

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Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Authors' contributions

TIH and ACC conceptualized the study. TFT and KYC curated data. TFT and CYH performed formal analysis. TIH, Ye-CC and ACC conducted investigation. KYC, Yu-CC and ACC developed methodology. Yu-CC, HEC and CYH were involved in project administration. Ye-CC, PCC and TFT managed resources. PHC, Yu-CC and PCC performed software analysis. HEC and TIH supervised the study. PCC and ACC wrote the original draft. ACC and TIH wrote, reviewed and edited the manuscript. ACC and TIH confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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