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Original Article

MiR-34a functions as a tumor suppressor in oral cancer through the inhibition of the Axl/Akt/GSK-3β pathway

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KEYWORDS
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Abstract *Background/purpose:* Oral cancer is a prevalent malignancy affecting men globally. This study aimed to investigate the regulatory role of miR-34a in oral cancer cells through the Axl/Akt/glycogen synthase kinase-3β (GSK-3β) pathway and its impact on cellular malignancy. *Materials and methods:* We examined the effects of miR-34a overexpression on the malignancy of oral cancer cells. Multiple oral cancer cell lines were assessed to determine the correlation between endogenous miR-34a and Axl levels. Transfection experiments with miR-34a were conducted to analyze its influence on Axl mRNA and protein expression. Luciferase reporter assays were performed to investigate miR-34a's modulation of Axl gene transcription. Manipulation of miR-34a expression was utilized to demonstrate its regulatory effects on oral

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cancer cells through the Axl/Akt/GSK-3β pathway.
Results: Overexpression of miR-34a significantly suppressed the malignancy of oral cancer cells. We observed an inverse correlation between endogenous miR-34a and Axl levels across multiple oral cancer cell lines. Transfection of miR-34a resulted in decreased Axl mRNA and protein expression, and luciferase reporter assays confirmed miR-34a-mediated modulation of Axl gene transcription. The study revealed regulatory effects of miR-34a on oral cancer cells through the Axl/Akt/GSK-3β pathway, leading to alterations in downstream target genes involved in cellular proliferation and tumorigenesis.
Conclusion: Our findings highlight the significance of the miR-34a/Axl/Akt/GSK-3β signaling axis in modulating the malignancy of oral cancer cells. Targeting miR-34a may hold therapeutic potential in oral cancer treatment, as manipulating its expression can attenuate the aggressive behavior of oral cancer cells via the Axl/Akt/GSK-3β pathway.

Introduction

Oral cancer is ranked as the third most common malignancy in men in Taiwan. The limited availability of effective drugs for oral cancer patients hampers prognosis. Clinically used chemotherapy drugs include cisplatin and fluorouracil, which often lead to severe side effects. Regarding targeted therapy, only cetuximab is used as a first-line treatment. However, after the failure of frontline treatment for oral cancer, the prognosis for recurrent patients is poor, and there is a lack of effective drugs.[1] Therefore, the search for new biomarkers or the development of novel treatment modalities is urgently needed. MicroRNAs (miRNAs) are a class of small non-coding RNAs that bind to the 3′ untranslated region (UTR) of target mRNAs, thereby regulating the expression of associated proteins.[2] Growing evidence suggests that miRNAs participate in various cancer processes, including cell growth, apoptosis, invasion, and metastasis.[3] Among them, miR-34a functions as a potential tumor suppressor gene and is frequently lost or down-regulated in multiple cancers, such as prostate cancer,[4] breast cancer,[5] osteosarcoma,[6] acute myeloid leukemia,[7] and oral cancer.[8]

MiR-34a is known to play a crucial role in regulating diverse biological processes and diseases by modulating multiple signaling pathways.[9] Notably, miR-34a acts as a transcriptional target of p53, contributing to cellular apoptosis, cell cycle regulation, and anti-cancer activities.[10] Furthermore, it influences the Wnt pathway by suppressing downstream effector genes, impacting cellular proliferation, differentiation, and stem cell self-renewal.[11] Additionally, miR-34a negatively regulates the phosphoinositide 3-kinase (PI3K)/Akt pathway by targeting Akt expression, thereby reducing cell survival and proliferation.[12] A comprehensive understanding of the intricate network of signaling pathways regulated by miR-34a provides valuable insights into its multifaceted roles in various physiological and pathological processes.

Axl is a receptor tyrosine kinase (RTK) and one of the potential targets of miR-34a.[13] Upon binding with the growth arrest-specific 6 (Gas6) protein, Axl activation modulates downstream signaling, including the PI3K/Akt pathway. The Gas6/Axl pathway upregulates various pro-tumorigenic functions, such as epithelial-to-mesenchymal transition (EMT), cell growth, immune evasion, and drug resistance. MiR-34a's regulation of Axl has been previously reported in various cancer types. For instance, in lung cancer cells, miR-34a negatively regulates Axl expression, and it has been observed that Axl can, in turn, regulate miR-34a expression through the c-Jun N-terminal kinase (JNK)/ETS-like gene 1 (ELK1) pathway.[14] In breast cancer cell studies, miR-34a was found to inhibit Axl, impacting the formation of vessel-like structures by tumor cells.[15] Previous study identified a significant negative correlation between Axl protein levels and miR-34a expression in a comprehensive panel of cancer samples.[16] This growing body of evidence underscores the importance of investigating the interplay between miR-34a and Axl in various malignancies and highlights the potential significance of this regulatory axis in cancer pathogenesis.

In oral cancer, Li et al. had showed that cancer-associated fibroblasts contributed to oral cancer cell proliferation and metastasis through exosome-mediated paracrine miR-34a-5p signaling.[17] Our prior investigations have further validated the substantial involvement of Axl in oral cancer progression.[18,19] Base on these reports, the current study aims to comprehensively explore the relevant mechanisms involving miR-34a and Axl in the context of oral cancer.

Materials and methods

Cell lines and cell culture

Five human oral cancer cell lines, namely YD-38, OEC-M1, SAS, HSC-3 and HSC-4, were employed in this study. YD-38, OEC-M1, and SAS cells were cultured in Roswell Park Memorial Institute 1640 medium while HSC-3 and HSC-4 cells

were cultured in Dulbecco's modified Eagle's medium. All cancer cell lines were carefully screened to ensure they were free of mycoplasma contamination.

Reagents and antibodies

The p-GSK-3β inhibitor TWS-119 was obtained from MedChemExpress (Monmouth Junction, NJ, USA). The p-Akt inhibitor LY294002 was purchased from Cell Signaling Technology (Danvers, MA, USA). For miRNA experiments, all miRNAs were obtained from Dharmacon (Lafayette, CO, USA). Antibodies used in the experiments included anti-Axl and cyclin D1, both obtained from Santa Cruz Biotechnology (Dallas, TX, USA), as well as p-Axl (Tyr702), Akt, p-Akt (Ser473), GSK-3β, p-GSK-3β (Ser9), and GAPDH, all sourced from Cell Signaling Technology. The β-catenin antibody was obtained from BD Biosciences (San Jose, CA, USA), and the cellular Myc protein (c-Myc) antibody was sourced from Abcam (Cambridge, UK). The α-tubulin antibody came from Proteintech (Wuhan, China). Anti-rabbit or anti-mouse HRP-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA), and Alexa Fluor 555 anti-mouse immunoglobulin G (IgG) was obtained from Invitrogen (Carlsbad, CA, USA).

Plasmid construction and transfection

We utilized miR-34a mimic, miR-34a inhibitor, mimic control miRNA, and inhibitor control, and all transfections were performed following the manufacturer's recommended protocols to ensure effective and reliable modulation of miR-34a expression levels in the oral cancer cells under investigation. Human Axl cDNA was amplified and was then subjected to digestion with HindIII restriction enzymes. Subsequently, the digested product was cloned into the mammalian expression vector with the cytomegalovirus promoter (pCMV)-Tag 2A, following a previously established protocol.[20] The Axl 3′-UTR reporters were synthesized in Professor Shuang-En Chuang's laboratory by constructing them into the pMIR-Report vector. This was achieved by inserting full-length or deletion forms of the Axl 3′-UTR sequences into the SpeI/SacI restriction site of the pMIR-Report vector, following a previously described method.[14]

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and colony formation assay

Cell proliferation was assessed using MTT assay. Purple crystals formed were dissolved in dimethyl sulfoxide (DMSO), and their absorbance was measured at 570 nm using an enzyme-linked immunosorbent assay reader. For the colony formation assay, oral cancer cells were seeded at a density of 1000 cells per well in 6-well plates and incubated in a cell culture incubator for 7 days. After fixation, the cells were stained with a 1% crystal violet solution for 30 min on a shaker. Colony visualization and quantification were performed using ImageJ software. Detailed procedures can be found in a previously published methods article.[21]

Cell invasion and migration assay

Cell invasion was assessed using a transwell chamber with a Matrigel-coated membrane insert. Invaded cells were stained with propidium iodide for 30 min, and counted in 5 randomly selected fields. Cell migration was evaluated using a wound-healing assay. The initial wound area was recorded by capturing images under a microscope, and subsequent changes in the wound area were analyzed using ImageJ software to quantify cell migration.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated and purified using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions. Subsequently, the purified RNA was converted to cDNA. For the PCR step, 10 μM of both sense and antisense primers were employed.[21] RT-qPCR was performed using SYBR Green I Master mix (Bioline, London, UK) and a Roche LightCycler 480 RT-PCR system (Roche, Basel, Switzerland).

Western blot analysis

For Western blot analysis, cellular proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes via electrophoresis. The subsequent procedures followed previously described methods.[21–23]

Luciferase assay

Oral cancer cells were co-transfected with miR-34a mimic or control and either full-length or deletion mutant constructs of the Axl 3′-UTR sequences in the pMIR-Report vector. The transfection was carried out using the polyjet transfection reagent. After transfection, the relative luciferase activity was assessed using a dual luciferase assay system, following a previously established protocol.[21]

Immunocytochemistry

The cells were incubated overnight at 4 °C with an anti-β-catenin antibody. Subsequently, the cells were further incubated with the secondary antibody, Alexa Fluor 555 anti-mouse IgG (Invitrogen), which was conjugated with a red fluorescent dye, at a dilution of 1:1000. Afterward, the cells were treated with 4′,6-diamidino-2-phenylindole (DAPI) for nuclear localization. Confocal fluorescence images were captured using a Zeiss LSM 510 Inverted Confocal Microscope (Oberkochen, Germany).

Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego,

CA, USA). Pearson's correlation coefficient was employed for the linear regression analysis. For comparisons between multiple groups, a one-way or two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was used. Student's *t*-test was employed for comparing two groups. A *P*-value of less than 0.05 was considered statistically significant. All data were presented as mean ± standard error of the mean (SEM).

Results

MiR-34a overexpression suppresses oral cancer cell malignancy

To investigate the role of miR-34a in oral cancer, YD-38 and SAS oral cancer cells were transfected with miR-34a mimic for 24 h, followed by MTT assays to evaluate cell viability and growth. The results demonstrated a significant decrease in cell survival rate compared to the control group (Fig. 1A and B). Similarly, colony formation assays revealed a reduction in the number of colonies following transfection

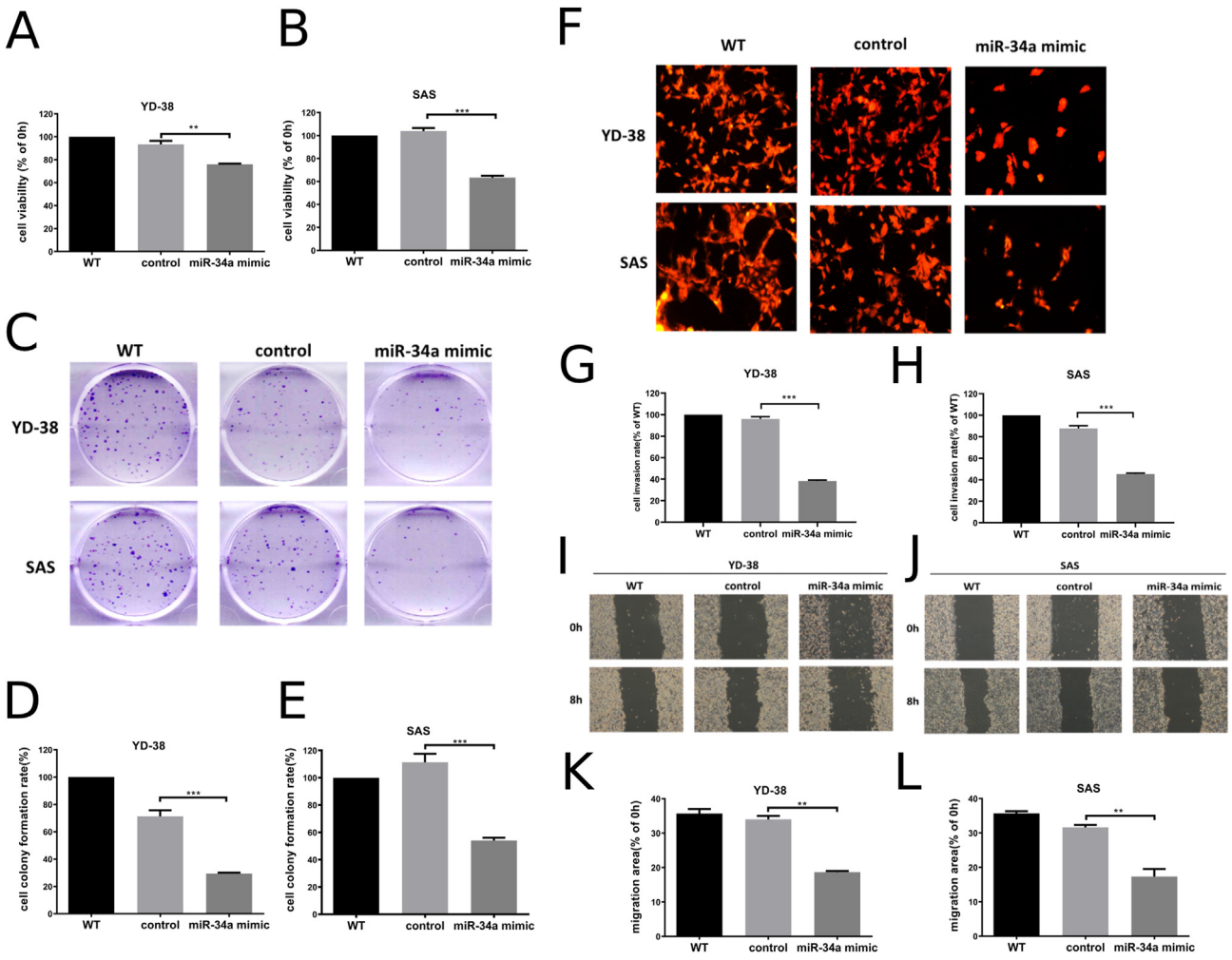

Figure 1 The effect of miR-34a on proliferation, invasion, and migration of human oral cancer cells. YD-38 and SAS cells were transfected with 10 nM of miR-34a. (A, B) The MTT assay results revealed a significant reduction in cell viability compared to the scramble control. (C) The colony formation assay demonstrated a noticeable decrease in cell proliferation relative to the scramble control. (D, E) The quantification results of the colony formation assay. (F) The invasion assay displayed a substantial decrease in the number of invading cells compared to the scramble control. (G, H) The quantitative results of the invasion assay. (I, J) The migration assay exhibited a significant decline in the crawling rate of cells compared to the scramble control. (K, L) The quantification of the migration assay results confirmed that miR-34a mimic attenuated cell crawling function. Data are shown as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$. WT, wild-type; MTT assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

with miR-34a mimic (Fig. 1C–E). The invasion experiment showed a significant decrease in the number of invaded cells in the miR-34a mimic-transfected group compared to the control (Fig. 1F–H). In the wound-healing migration assay, a slower closure rate was observed in the miR-34a mimic-transfected cells (Fig. 1I-L).

MiR-34a inversely regulates Axl expression in oral cancer cells

Utilizing TargetScan and miRDB,[24,25] bioinformatics tools, we identified Axl as a target gene of miR-34a. To assess the relationship between miR-34a and Axl, we measured their relative expression levels in five oral cancer cell lines using RT-qPCR (Fig. 2A). Regression analysis revealed an inverse association between miR-34a expression levels and Axl expression in oral cancer cells ($P = 0.0538$) (Fig. 2B). Furthermore, transfection of miR-34a mimics significantly increased miR-34a and decreased Axl expression levels in YD-38 and SAS cells compared to control (Fig. 2C and D). To further explore the regulation of Axl mRNA by miR-34a, we conducted luciferase reporter assays using intact pMIR-Report-Axl 3′ UTR and deletion mutant pMIR-Report-Axl 3′ UTR constructs.[14] MiR-34a was found to down-regulate the activity of the Axl 3′ UTR reporter, and this effect was dependent on the presence of the putative miR-34 target site in the Axl 3′ UTR (Fig. 3A). Furthermore, transfected with miR-34a mimics reduced expression of p-Akt and p-GSK-3β in cells (Fig. 3B).

MiR-34a overexpression reduces tumor cell malignancy via regulation of the Axl/Akt/GSK-3β pathway

To further validate the influence of miR-34a on cellular functions through the Axl/Akt/GSK-3β pathway, we transfected oral cancer cells with different conditions: control, miR-34a mimic, Axl overexpression, and a combination of miR-34a mimic with Axl overexpression. Western blotting analysis was performed to assess the protein expression levels. The results revealed a significant reduction in the protein levels of p-Axl, p-Akt, and p-GSK-3β in the miR-34a

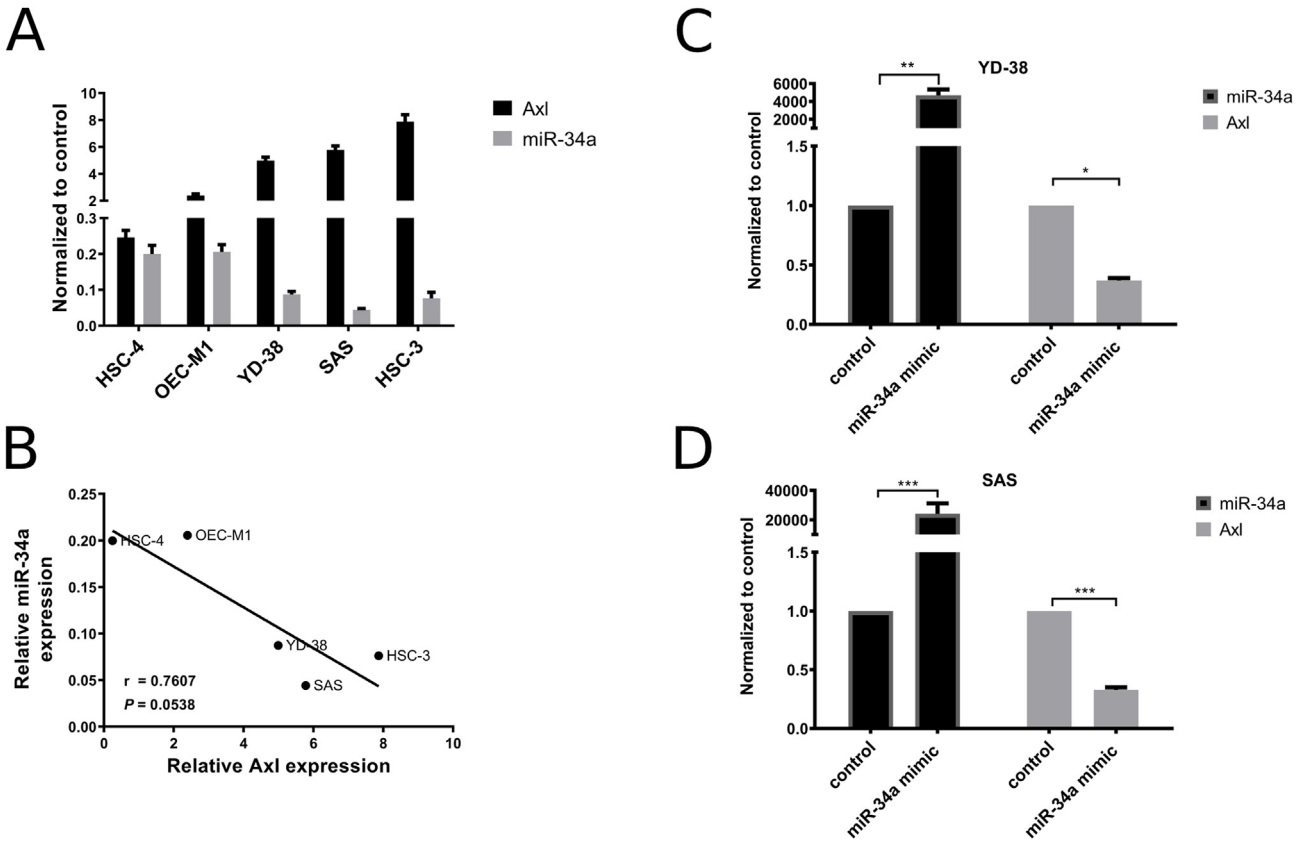

Figure 2 Inverse correlation of miR-34a and Axl in oral cancer cells. (A) RT-qPCR analysis was performed to assess the endogenous RNA expression levels of miR-34a and Axl in five oral cancer cell lines, namely HSC-4, OEC-M1, YD-38, SAS, and HSC-3. The expression levels of miR-34a and Axl were sequentially analyzed for each cell line. (B) The RNA data obtained from (A) were utilized to determine the correlation between miR-34a and Axl in the five cell lines. Linear regression analysis revealed a *P*-value of 0.0538, indicating a borderline significant inverse correlation. (C, D) YD-38 and SAS cell lines, known for their higher transfection efficiency, were selected. Following transfection with miR-34a mimic or control, RT-qPCR analysis was performed to evaluate the expression levels of Axl. The results demonstrated a significant decrease in Axl expression upon transfection with miR-34a mimic compared to the control. Data are shown as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

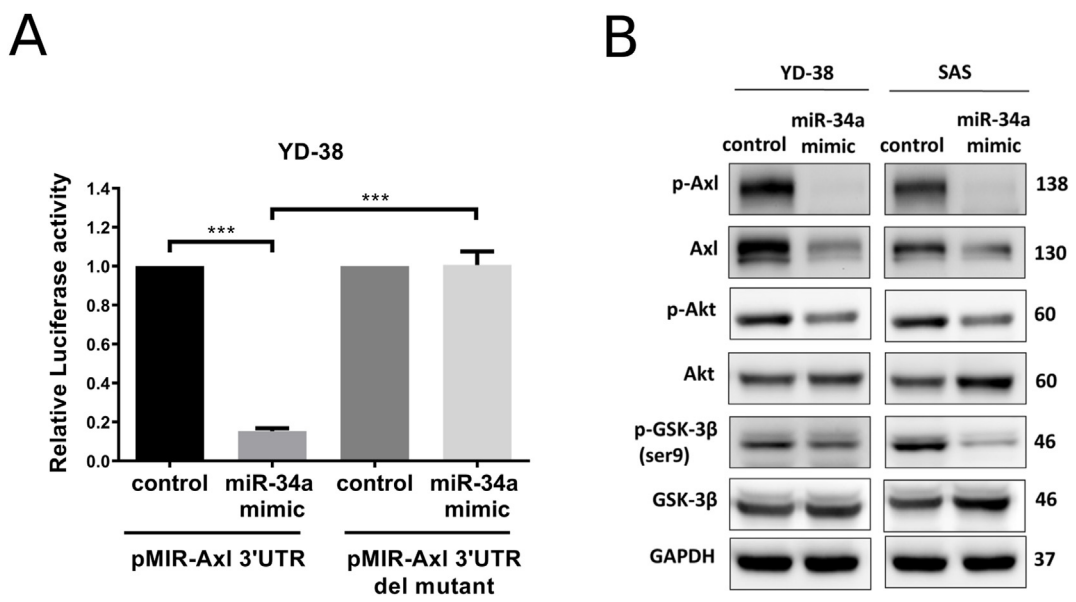

Figure 3 Regulation of Axl expression by miR-34a: luciferase assays and protein analysis. (A) Luciferase reporter assays were conducted in YD-38 cells to validate the binding of miR-34a to the Axl 3′ UTR and its subsequent impact on luciferase activity. Cells were co-transfected with pMIR-Axl 3′ UTR or pMIR-Axl 3′ UTR del mutant constructs, along with control or miR-34a mimic. The assays were performed to assess any changes in luciferase activity resulting from the interaction between miR-34a and the Axl 3′ UTR. (B) YD-38 and SAS cells were transfected with miR-34a mimic, and protein was extracted for Western blotting analysis. The results demonstrated a significant decrease in p-Axl protein levels following miR-34a mimic transfection compared to the control. Moreover, downstream proteins including p-Akt and p-GSK-3β also showed a significant reduction. GPADH was used as the loading control. Data are shown as mean ± SEM. ***$P < 0.001$. UTR, untranslated region; del, deletion; GSK-3β, glycogen synthase kinase-3β.

mimic group compared to the control (Fig. 4A). In the Axl overexpression group, the levels of p-Akt and p-GSK-3β are increased (Fig. 4A). In the combined transfection group of miR-34a mimic and Axl overexpression, a restoration of the protein expression levels of p-Axl, p-Akt, and p-GSK-3β was observed compared to the miR-34a mimic group (Fig. 4A). Through dose-dependent experiments with miR-34a mimic, we observed a gradual decrease in the expression levels of β-catenin, c-Myc, and cyclin D1 as the concentration of miR-34a mimic increased (Fig. 4B). Immunofluorescence staining revealed a decrease in both the total and nuclear levels of β-catenin in oral cancer cells transfected with miR-34a (Fig. 4C). Additionally, a partial restoration of c-Myc and cyclin D1 protein levels was observed in the combined transfection group compared to the miR-34a mimic group (Fig. 4D). MTT assays revealed a restoration of cell viability in the combined transfection group compared to the miR-34a mimic group (Fig. 4E and F). Similar results were also observed in the invasion assay (Fig. 4G—I).

MiR-34a-mediated regulation of the Axl/Akt/GSK-3β pathway involved in oral cancer malignant behaviors

To further examine the potential signal pathways involved in regulating miR-34a mimic and Axl, we performed rescue experiments using miR-34a inhibitor, p-Akt inhibitor, and p-GSK-3β inhibitor. Oral cancer cells were transfected with different conditions: control, miR-34a inhibitor, combined treatment of miR-34a inhibitor with p-Akt inhibitor, and combined treatment of miR-34a inhibitor with p-GSK-3β inhibitor. The results revealed that the miR-34a inhibitor group showed an increase in p-Axl expression (Fig. 5A). Furthermore, when miR-34a inhibitor was combined with p-Akt inhibitor, a decrease in the protein levels of p-Akt and p-GSK-3β was observed (Fig. 5A). Similarly, when miR-34a inhibitor was combined with p-GSK-3β inhibitor, a reduction in p-GSK-3β expression was observed (Fig. 5A). In the MTT assay, the miR-34a inhibitor combined with p-Akt inhibitor and miR-34a inhibitor combined with p-GSK-3β inhibitor exhibited significantly decreased cell viability compared to the miR-34a inhibitor group (Fig. 5B and C). In the invasion assay, the groups treated with p-Akt inhibitor or p-GSK-3β inhibitor showed reduced cell invasion compared to the group transfected with miR-34a inhibitor alone (Fig. 5D—F). In addition, the migration assay demonstrated similar trends after an 8-h period of creating a cell wound (Fig. 5G—J).

Discussion

In the present study, we transfected oral cancer cells with miR-34a, leading to the suppression of oral cancer cell malignancy. Through gain-of-function and loss-of-function experiments, we demonstrated that miR-34a regulates the Axl/Akt/GSK-3β pathway, affecting downstream genes like β-catenin, c-Myc, and cyclin D1 (Fig. 6).

Previous studies utilizing mRNA microarray analysis have identified Axl, mitogen-activated protein kinase kinase 1 (MAP2K1), and fucosyltransferase 1 (FUT1) as important

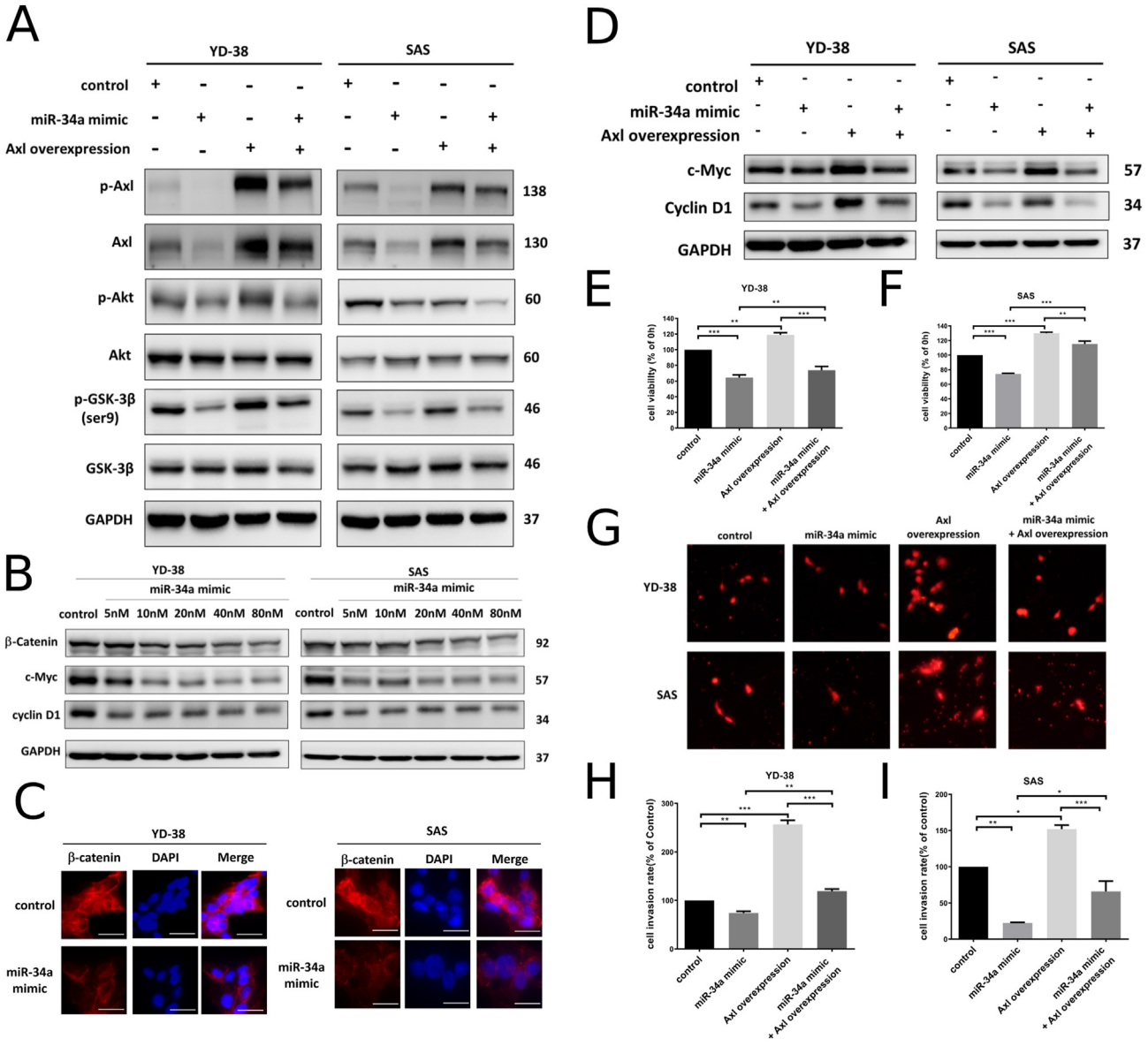

Figure 4 Modulation of cell function via the Axl/Akt/GSK-3β pathway by miRNA-34a mimic and overexpression of Axl. (A) YD-38 and SAS cells were transfected separately with miR-34a mimic, overexpressed Axl, or a combination of both. Total protein was extracted and subjected to Western blotting analysis. The results revealed a decrease in the protein expression levels of p-Axl, p-Akt, and p-GSK-3β following transfection of miR-34a mimic, while overexpression of Axl increased the expression levels of p-Axl, p-Akt, and p-GSK-3β. Notably, when miR-34a mimic and overexpressed Axl were combined, the protein expression levels exhibited an increase compared to the sole miR-34a mimic. (B) The protein lysates from oral cancer cells transfected with different concentrations of miR-34a mimic (5, 10, 20, 40, and 80 nM) were subjected to Western blotting analysis. The results showed a decrease in the expression levels of β-catenin, c-Myc, and cyclin D1 with increasing concentrations of miR-34a mimic. (C) Immunocytochemical staining of β-catenin in oral cancer cells after transfection with miR-34a control and miR-34a mimic. β-catenin was detected using goat anti-mouse Alexa Flour 555 (Red), and nuclei were counterstained with DAPI (Blue). Scale bars: 20 μm. (D) Similar observations were made for downstream genes c-Myc and cyclin D1. (E, F) MTT assays were performed on YD-38 and SAS cells transfected separately with miR-34a mimic, overexpressed Axl, or a combination of both. The results indicated an increase in cell viability following the combined transfection compared to sole miR-34a mimic. (G) In the invasion experiment, transfected with miR-34a mimic, overexpressed Axl, or a combination of both resulted in a consistent increase in the number of invading cells compared to sole miR-34a mimic. (H, I) Quantification of the invasion assay depicted in Figure G. Data are shown as mean ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. GSK-3β, glycogen synthase kinase-3β; c-Myc, cellular Myc protein; DAPI, 4′,6-diamidino-2-phenylindole; MTT assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

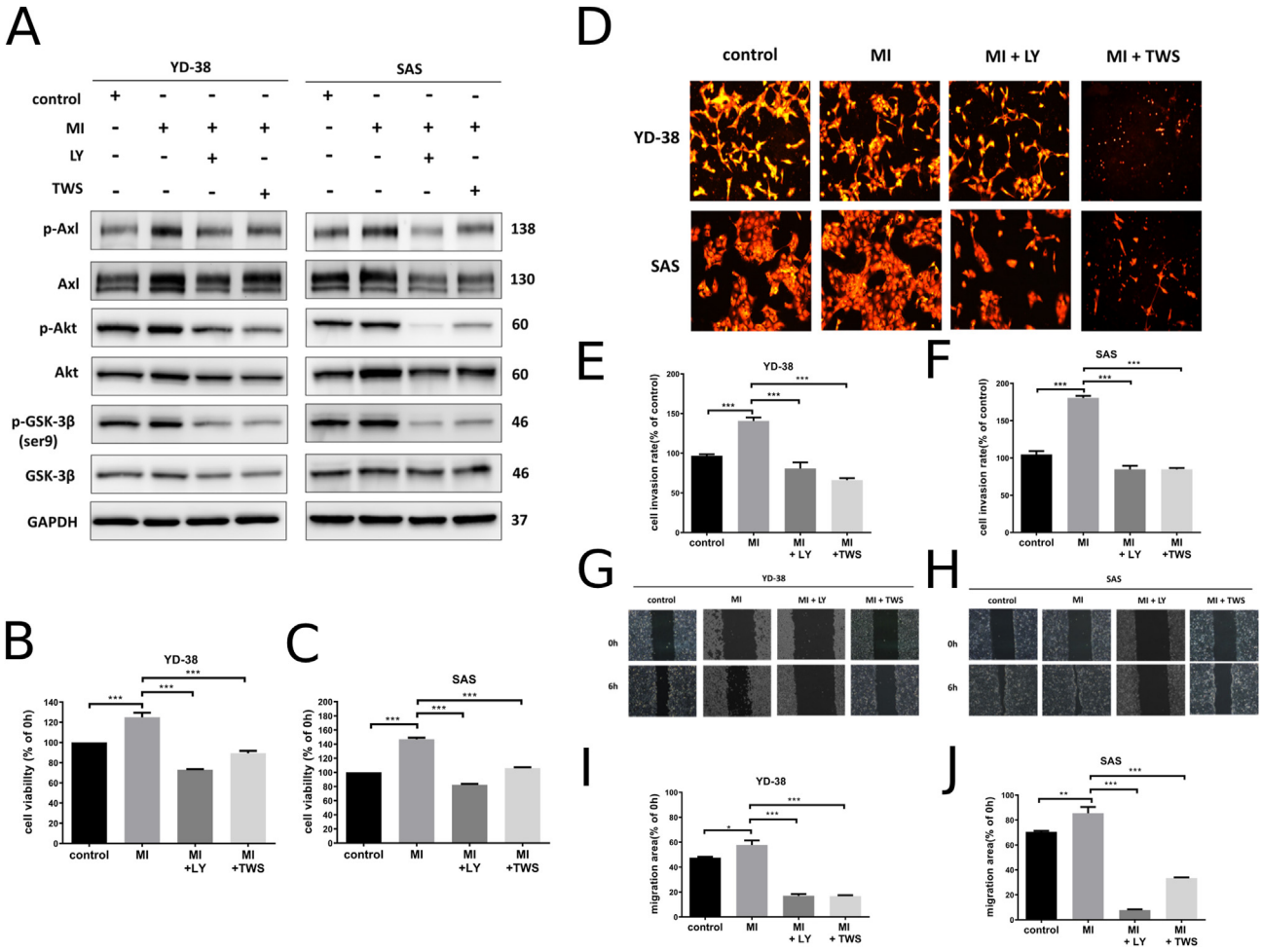

Figure 5 Combined inhibition of miR-34a and Axl signaling modulates cell function in oral cancer. (A) YD-38 and SAS cells were transfected with miR-34a inhibitor, a combination of miR-34a inhibitor and p-Akt inhibitor, or a combination of miR-34a inhibitor and p-GSK-3β inhibitor. Western blotting analysis revealed increased Axl expression following miR-34a inhibitor transfected, while the addition of p-Akt and p-GSK-3β inhibitors resulted in altered downstream protein expression. (B, C) MTT assays demonstrated reduced cell viability following the combined treatment compared to sole miR-34a inhibitor transfected in YD-38 and SAS cells. (D) Invasion assays showed a decrease in the number of invading cells following the combined treatment compared to sole miR-34a inhibitor transfected. (E, F) Quantification of the invasion assay depicted in Figure D. (G, H) Invasion and migration assays revealed a slower migration rate of cells following the combined treatment compared to sole miR-34a inhibitor transfected. (I, J) Quantification of the results depicted in Figure G and H. Data are shown as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. MI: miR-34a inhibitor; LY: LY294002, p-Akt inhibitor; TWS: TWS-119, p-GSK-3β inhibitor; GSK-3β, glycogen synthase kinase-3β; MTT assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

downstream targets of miR-34a.[26] Our study further elucidates the comprehensive signaling pathway through which miR-34a acts on Axl. In line with Jiang et al. study on nasopharyngeal carcinoma, miR-34a modulates cell invasion and EMT through the Axl/p-Akt signaling pathway.[27] However, in the context of lung cancer, Cho et al. had identified an Axl regulation feedback loop involving ELK1-mediated up-regulation of miR-34a through the JNK pathway.[14] In the breast cancer cells, Lim et al. reported that miR-34a suppresses Axl expression and governs the formation of vessel-like structures by tumor cells.[15] In our study, we demonstrated that miR-34a may exert its effects on malignant cellular behavior in oral cancer by influencing β-catenin. These divergent findings highlight the context-dependent nature of miR-34a's regulatory mechanisms, which can vary across different cancer types and molecular pathways. The identification of miR-34a's involvement in the modulation of β-catenin in oral cancer adds to the growing understanding of the diverse roles played by miR-34a in various malignancies and underscores its potential as a therapeutic target for oral cancer treatment. In line with previous report,[17] our results demonstrated the regulatory role of miR-34a through the Axl/Akt/GSK-3β pathway in altering the malignancy of oral cancer cells. These findings underscore the importance of the miR-34a-mediated Axl/Akt/GSK-3β pathway in oral cancer progression and provide additional insights into its functional significance.

The etiology of oral cancer may vary, leading to different molecular mechanisms for therapeutic targeting.

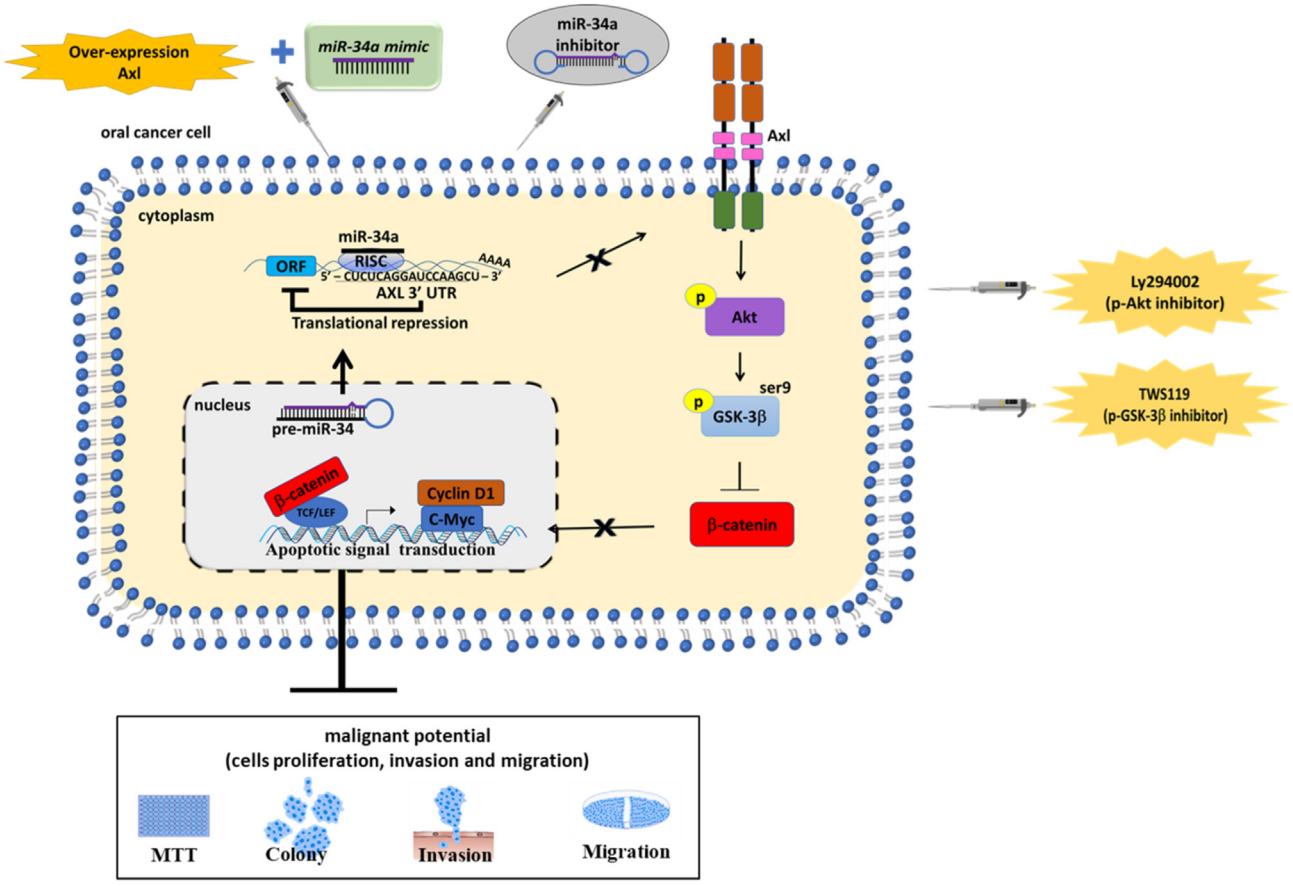

Figure 6 Impact of miR-34a on the malignancy of oral cancer cells through the Axl/Akt/GSK-3β pathway.

In Taiwan, the primary cause of oral cancer is attributed to betel nut chewing, which has a significant association with the presence of arecoline.[28] Previous studies have indicated a substantial upregulation of β-catenin expression in the epithelium of oral cancer patients with a history of betel nut chewing, and arecoline has been shown to directly induce an increase in β-catenin protein expression.[29] Shiah et al. also showed that arecoline can modulate the Wnt-β-catenin signaling pathway.[30] Additionally, a study from Thailand showed that arecoline can regulate the growth of oral cancer cells through c-Myc.[31] These studies underscore the close association between arecoline and the β-catenin signaling pathway in oral cancer in the Southeast Asian region. In combination with our results, these findings shed light on the potential therapeutic implications of targeting the miR-34a-mediated pathway and its interconnected signaling network for the treatment of betel nut-associated oral cancer.

In conclusion, our study contributes to a better understanding of the regulatory mechanisms underlying oral cancer and provides valuable insights into potential targeted therapies for improving patient outcomes. Future studies focusing on nanoparticle-based delivery of miR-34a and investigating its impact on the Axl/Akt/GSK-3β pathway hold promise for advancing oral cancer treatment strategies.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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