Mechanism of microRNA-30b-5p enhancing the response of MDA-MB-231 cells to cisplatin by regulating RAP1B expression

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Abstract. The present study investigated the role of hsa-microRNA (miR)-30b-5p in potentiating cisplatin-induced cytotoxicity in triple-negative breast cancer (TNBC) MDA-MB-231 cells via Ras-related protein Rap-1b (RAP1B) regulation. Expression levels of hsa-miR-30b-5p and RAP1B were examined in multiple breast cell lines, including normal breast epithelial cells (MCF-10A) and breast cancer cell lines (MCF-7, MDA-MB-231 and MDA-MB-468). MDA-MB-231 cells were divided into control, miR-30b-5p mimic, cisplatin (DDP), miR-30b-5p mimic + DDP and miR-30b-5p mimic + overexpressed RAP1B + DDP groups. Reverse transcription-quantitative PCR (RT-qPCR) demonstrated significantly lower miR-30b-5p expression in MDA-MB-231 compared with MCF-10A normal breast epithelial cells (P<0.001), which was rescued by miR-30b-5p mimics. Cell Counting Kit-8 assays revealed that miR-30b-5p overexpression or DDP alone suppressed cell viability (P<0.001), with combined treatment showing synergistic inhibition (P<0.001). Flow cytometry demonstrated increased apoptosis in miR-30b-5p mimic and DDP groups, further amplified by their combination (P<0.001). Luciferase reporter and RNA pull-down-qPCR assays confirmed miR-30b-5p directly targets RAP1B 3'-untranslated region (UTR) via sequence-specific binding. RNA pull-down demonstrated molecular association between miR-30b-5p and RAP1B mRNA, while the luciferase reporter assay confirmed 3'-UTR-specific interaction. Western blotting and RT-qPCR showed miR-30b-5p mimics downregulated RAP1B (P<0.001), reversed by RAP1B overexpression. In the rescue experiment, RAP1B overexpression partially reversed the pro-apoptotic effect of miR-30b-5p under cisplatin treatment, supporting the hypothesis that RAP1B mediates the effect of miR-30b-5p on the cisplatin response (P<0.001). These findings indicate that miR-30b-5p potentiates cisplatin-induced cytotoxicity in TNBC by suppressing RAP1B, suggesting a novel therapeutic strategy to overcome chemoresistance.

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Introduction

Breast cancer (BC) remains one of the most prevalent malignancies worldwide. Primary treatment modalities for BC include surgery, systemic chemotherapy, postoperative radiotherapy, endocrine therapy, targeted therapy and immunotherapy. Despite the continuous development of treatment strategies, notable challenges persist, particularly severe adverse effects and the frequent emergence of drug resistance during late-stage treatment. Recent advances in molecular profiling and signaling pathway research have facilitated the identification of potential therapeutic targets for BC, breakthroughs have been made in the exploration of potential targets of BC. Targeted therapy is a new treatment method after surgery, radiotherapy and chemotherapy, which has the advantages of strong specificity, notable curative effect, reduced toxicity and side effects, and marked improvement in the prognosis of patients (1). Among them, triple negative BC (TNBC) is an aggressive BC subtype with restricted treatment options, primarily dependent on chemotherapy (2-4). As a widely used chemotherapy drug, cisplatin (DDP) blocks DNA replication and transcription by forming DNA cross-links, thus inducing tumor cell death. However, the sensitivity of TNBC to DDP varies considerably among patients and some patients rapidly develop cisplatin resistance, which limits its clinical efficacy. Exploring novel molecular mechanisms that enhance TNBC response to cisplatin is crucial for improving therapeutic outcomes.

In TNBC, the imbalance of microRNA (miRNA/miR) expression is closely associated with tumor development, metastasis and chemosensitivity. miRNAs, short non-coding RNAs, interact with the 3'-untranslated region (UTR) of target mRNA to regulate gene expression by repressing translation or promoting degradation (5,6). Recent studies have identified miRNAs as crucial in modulating tumor cell responses to

chemotherapy (7-10). hsa-miR-30b-5p is frequently dysregulated in breast, prostate and colorectal cancer, among others, but its precise role and mechanisms remain largely unexplored in different cancer types. hsa-miR-30b-5p expression is upregulated in breast cancer tissues and cell lines, and its upregulation promotes cell growth migration and invasion in TNBC cell lines (MDA-MB-231and HCC 1937), while reducing apoptosis, indicating that is serves a tumor-promoting role in TNBC (11). Studies have suggested that hsa-miR-30b-5p influences cell proliferation and migration across multiple malignancies, including breast, prostate and colorectal cancer, by targeting cancer-specific genes such as ASPP2 in breast cancer (11), FLVCR1 and NUAK1 in prostate cancer (12,13), and Rap1b in colorectal cancer (14). However, the mechanism of how hsa-miR-30b-5p affects the sensitivity of TNBC cells to cisplatin remains unclear.

Ras-related protein Rap-1b (RAP1B), a small GTP-binding protein, notably influences cell adhesion, proliferation and survival (15-18). Upregulation of RAP1B expression has been observed in multiple tumor types, where it is closely associated with malignant progression and the acquisition of chemotherapy resistance, as evidenced in glioblastoma (19), esophageal squamous cell carcinoma (20), ovarian cancer (21), prostate cancer (22), and hepatocellular carcinoma (23). Although RAP1B is identified as a potential target of hsa-miR-30b-5p, its precise role in TNBC and the molecular framework by which hsa-miR-30b-5p influences its impact on cisplatin chemotherapy response remain unclear. The present study aimed to investigate how hsa-miR-30b-5p influences the response of MDA-MB-231 cells to cisplatin by targeting RAP1B. The regulation of RAP1B expression by hsa-miR-30b-5p and its potential effects on cisplatin response were assessed, offering a theoretical foundation for molecular targeted therapy in TNBC. The findings may aid in developing new strategies to enhance the response and survival rates of patients with TNBC undergoing cisplatin treatment.

Materials and methods

Reagents. The human mammary epithelial cell line MCF-10A and breast cancer cell lines MCF-7, MDA-MB-231 and MDA-MB-468 were obtained from Wuhan Hengyise Biotechnology Co., Ltd., while cisplatin was acquired from Sigma-Aldrich (Merck KGaA). Cisplatin was dissolved in dimethyl sulfoxide to prepare a stock solution and stored at -20˚C. Working solutions were freshly prepared in the culture medium before each experiment. The overexpression plasmids for the hsa-miR-30b-5p-mimic, hsa-miR-30b-5p-inhibitor and RAP1B were obtained from Suzhou GenePharma Co., Ltd. The sequences of hsa-miR-30b-5p mimic, and their corresponding negative controls (NC) were as follows: miR-30b-5p mimic sense, 5'-UGUAAACAUCCUACACUCAGCU-3'; NC sense, 5'-UUCUCCGAACGUGUCACGUTT-3'. The coding sequence of RAP1B (GenBank accession no. NM_015646.5, 555 bp) was amplified using the forward primer 5'-TT-GGTACC-GAG CTC-GGATCC-ATGCGTGAGTATAAGCTAGT-3'(protective bases-*Kpn*I-*Sac*I-*Bam*HI-RAP1B CDS nt 1-20) and the reverse primer 5'-AC-GGGCCC-TCTAGA-CTCGAG-TTAAAGCAG CTGACATGATG-3' (protective bases-*Apa*I-*Xba*I-*Xho*I-reverse complement of RAP1B CDS nt 536-555), and then cloned into the pcDNA3.1 vector to generate the overexpression plasmid op-RAP1B. The 20-bp RAP1B gene-specific regions at the 3' end of both primers were verified by NCBI BlastN to show 100% identity to the RAP1B CDS (NM_015646.5), and the inserted fragment was confirmed using Sanger sequencing.

miRNA fluorescence quantitative PCR (qPCR) detection kit was provided by Elk (Wuhan) Biotechnology Co., Ltd. The primer sequences used for RT-qPCR were as follows: hsa-miR-30b-5p, forward 5'-GGCCCTGTAAACATCCTA CAC-3', reverse 5'-CTCAACTGGTGTCGTGGAGTC-3'; U6, forward 5'-CTCGCTTCGGCAGCACAT-3', reverse 5'-AAC GCTTCACGAATTTGCGT-3'; RAP1B, forward 5'-TGAAGT AGATGCACAACAGTGTATG-3', reverse 5'-TGGAACATC ATCAGTGTCTTTAACT-3'; and actin, forward 5'-GTCCAC CGCAAATGCTTCTA-3' and reverse 5'-TGCTGTCACCTT CACCGTTC-3'. Lipofectamine® 2000 was obtained from Thermo Fisher Scientific, Inc. The Annexin V/PI Apoptosis Detection Kit and Cell Counting Kit-8 (CCK-8) Kit were obtained from Beyotime Biotechnology. Primary antibodies used for western blotting were anti-RAP1B (1:500; Abcam; cat. no. ab94967) and anti-actin (1:5,000; Proteintech Group, Inc.; cat. no. 60004-1-Ig). Horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (1:1,000; Elk (Wuhan) Biotechnology Co., Ltd.; cat. no. ELK1001) was used for the detection. The Entilink 1st Strand cDNA Synthesis Kit and EnTurbo™ SYBR Green PCR SuperMix were obtained from Elk (Wuhan) Biotechnology Co., Ltd., while the RPMI1640 medium and fetal bovine serum were sourced from Hyclone™ (Cytiva) and Gibco (Thermo Fisher Scientific, Inc.), respectively.

Experimental group. Cultured cells were categorized into three groups: The control group (MDA-MB-231 cells under normal conditions), the hsa-miR-30b-5p-mimic group (cells treated with human miR-30b-5p mimics to simulate miR-30b-5p overexpression) and the cisplatin treatment group (cells treated with cisplatin). The hsa-miR-30b-5p-mimics + DDP group was treated with cisplatin in combination with hsa-miR-30b-5p mimics, whereas the mimics + op-RAP1B + DDP group was treated with cisplatin, miR-30b-5p mimics and RAP1B overexpression. The miR-30b-5p mimic + DDP group was included to enable direct comparison with the miR-30b-5p mimic + op-RAP1B + DDP group, thereby allowing for the proper assessment of whether RAP1B overexpression rescued the effect of miR-30b-5p.

Cell culture conditions and cisplatin administration. MCF-10A, MCF-7, MDA-MB-231 and MDA-MB-468 cell lines were incubated at 37˚C in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a humidified atmosphere containing 5% CO_2. Cells were passaged upon reaching confluence for subsequent experiments. MDA-MB-231 cells underwent a 48-h treatment with 2 μM cisplatin (Sigma-Aldrich; Merck KGaA) at 37˚C. The concentration of 2 μM cisplatin was selected based on previously published literature (24,25). As a negative control, cells were treated with an equivalent volume of the solvent (phosphate-buffered saline) under the same conditions.

Transfection. MDA-MB-231 cells were co-transfected with hsa-miR-30b-5p-mimic, negative control (NC) and RAP1B overexpression plasmids using the Lipofectamine® 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The RAP1B overexpression plasmid was constructed by subcloning the full-length RAP1B gene into the pcDNA3.1 vector backbone (Invitrogen; Thermo Fisher Scientific, Inc.). For each transfection, 1.0 μg total nucleic acids (including plasmids and miRNA mimics) and 2.0 μl Lipofectamine® 2000 were diluted separately in Opti-MEM® I reduced serum medium, mixed and incubated at room temperature for 20 min to allow complex formation. The DNA-lipid complexes were then added to the cells and incubated at 37˚C in a humidified atmosphere containing 5% CO_2 for 48 h. After 48 h of transfection, cell samples were collected for further experiments. Transfection efficiency was verified using RT-qPCR and fluorescence microscopy.

CCK-8 assay. A total of $2x10^4$ MDA-MB-231 cells/well in the logarithmic growth phase were seeded into 96-well plates and transfected for 48 h. Following transfection and drug treatment, each well was incubated for 2 h with CCK-8 reagent. The absorbance was measured at 450 nm using a spectrophotometer. Each experiment was performed in triplicate (three biological replicates) with three technical replicates per biological replicate.

Flow cytometry. Apoptosis was assessed using flow cytometry with an Annexin V/PI detection kit (BD Biosciences). MDA-MB-231 cells were harvested at 48 h post-transfection, stained with Annexin V-FITC and PI, incubated in the dark for 15 min at room temperature, and measurements taken within 1 h. Flow cytometric analysis was performed using a FACSCalibur flow cytometer (BD Biosciences). The gating strategy was as follows: Initial gating on forward scatter (FSC) vs. side scatter (SSC) to exclude debris; doublet exclusion using FSC-area vs. FSC-height; and viable cells were identified as PI-negative populations. Early apoptosis was defined as Annexin V^+/PI^-, and late apoptosis as Annexin V^+/PI^+. A minimum of 10,000 events were recorded per sample. Data were analyzed using FlowJo software version 10.8.1 (BD Biosciences). All experiments were performed with at least three biological replicates.

Double luciferase reporter gene experiment. The relationship and potential binding sites of hsa-miR-30b-5p and RAP1B were predicted by using Starbase v2.0 (starbase.sysu.edu.cn/) and TargetScan (https://www.targetscan.org/vert_80/). RAP1B-wild-type (WT) and RAP1B-mutant (Mut) 3'-UTR sequences were cloned into the pmirGLO dual-luciferase reporter vector (Promega Corporation). For the Mut construct, the predicted miR-30b-5p binding site within the RAP1B 3'-UTR was mutated using site-directed mutagenesis. RAP1B-WT and RAP1B-Mut-luciferase vectors were constructed and co-transfected with hsa-miR-30b-5p-mimic and hsa-miR-30b-5p-inhibitor into MDA-MB-231 cells using Lipofectamine® 2000. Cells were lysed after 48 h, and luciferase activity was assessed with a Dual-Luciferase® Reporter Assay System (Promega Corporation). Firefly luciferase activity was normalized to the *Renilla* luciferase activity. Each experiment was performed in triplicate.

RNA pull-down detection. Biotin-labeled hsa-miR-30b-5p probe and its negative control probe (Biotin-Probe-NC) were biosynthesized by Wuhan Jinkairui Biotechnology Co., Ltd. The probe sequences were as follows: has-miR-30b-5p specific probe, 5'-biotin-GGCCCTGTAAACATCCTACAC-3'; negative control probe, 5'-biotin-AACGCTTCACGAATTTGCGT-3'. Cells were lysed using IP lysis buffer (supplemented with 1X Protease Inhibitor and RNase Inhibitor). The cell lysate was centrifuged at 12,000 x g for 10 min at 4˚C to remove debris, and the supernatant was collected. For each pull-down reaction, 40 μl Nucleic-Acid Compatible Streptavidin Magnetic Beads (B605110; Sangon Biotech, Co., Ltd.) were washed and equilibrated with lysis buffer. The beads were then incubated with the biotin-labeled probe at 4˚C for 2 h with gentle rotation to allow probe immobilization. Following probe immobilization, the beads were mixed with 500 μl cell lysate (containing ~400 μg of total protein) and incubated overnight at 4˚C with gentle rotation. After incubation, the beads were collected using a magnetic rack and washed three times with 500 μl Wash Buffer I, followed by three washes with 500 μl Wash Buffer II. All wash steps were performed at 4˚C with 5 min of gentle rotation per wash, and the beads were separated using a magnetic rack between washes. After washing, the RNA in the pull-down product was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) in the presence of an RNase inhibitor. The enrichment of target gene mRNA in the product was evaluated using reverse transcription (RT)-qPCR to determine the RNA-RNA interaction. Data are presented as relative enrichment normalized to a negative control probe. Each experiment was performed in triplicate.

RT-qPCR. A total of $1x10^6$ MDA-MB-231 cells were seeded per well in a 12-well culture plate and grown to an appropriate confluence. Total RNA was extracted using TRIzol® reagent according to the manufacturer's instructions. The RNA concentration and purity were assessed using a NanoDrop spectrophotometer, ensuring a 260/280 ratio between 1.8 and 2.0. The extracted RNA was used in the RT reaction, and hsa-miR-30b-5p and RAP1B were amplified in a qPCR reaction with specific primers and probes. For miRNA reverse transcription, stem-loop RT primers were used to enhance specificity and sensitivity for mature miRNA detection. Reverse transcription was performed at 42˚C for 60 min, followed by enzyme inactivation at 70˚C for 5 min. For mRNA reverse transcription, oligo(dT) primers were used according to the manufacturer's protocol. The resulting cDNA was then subjected to qPCR amplification. hsa-miR-30b-5p and RAP1B were amplified in a reaction with specific primers and probes. The qPCR thermocycling conditions were as follows: Initial pre-denaturation at 95˚C for 5 min, followed by 40 cycles of denaturation at 95˚C for 20 sec and annealing/extension at 57˚C for 40 sec. U6 small nuclear RNA was used as an internal reference for miR-30b-5p, and GAPDH mRNA was used as an internal reference for RAP1B (both reference genes were confirmed to be stably expressed under the experimental conditions in preliminary tests). The relative expression level of the target gene was quantified by calculating the -ΔΔCq value (26), and the reference gene was used for normalization.

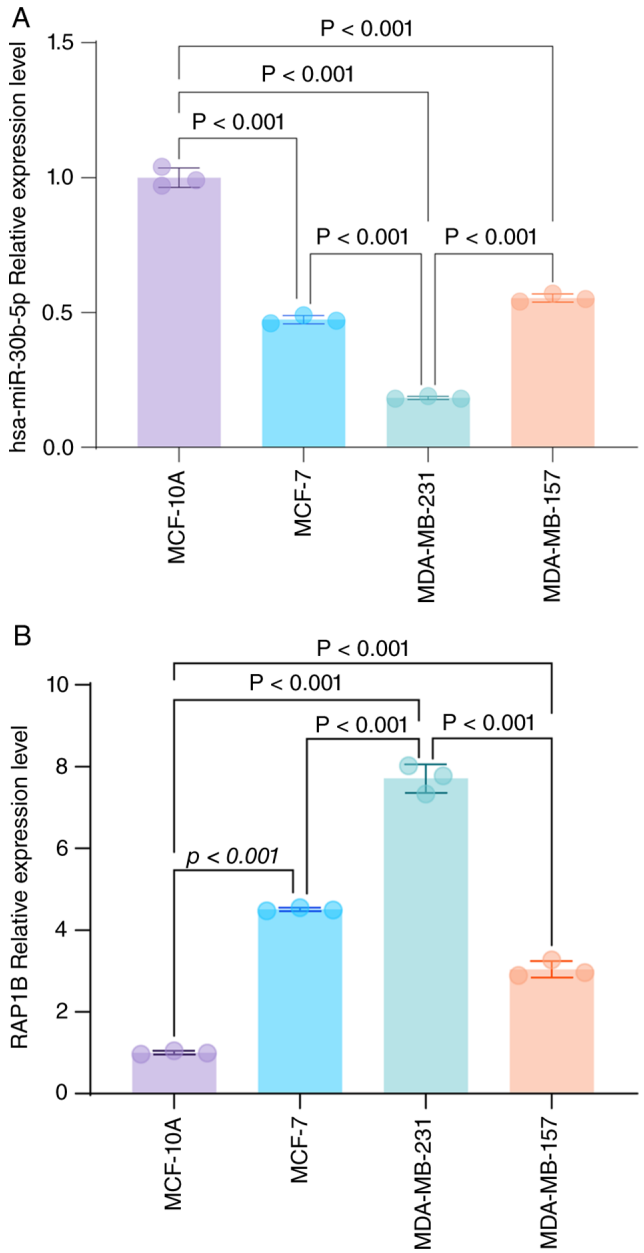

Figure 1. Expression of hsa-miR-30b-5p and RAP1B in different breast cell lines. Total RNA was extracted from normal breast epithelial cells (MCF-10A) and breast cancer cell lines (MCF-7, MDA-MB-231 and MDA-MB-468). Reverse transcription-quantitative PCR was performed to assess the expression levels of (A) hsa-miR-30b-5p and (B) RAP1B mRNA. U6 was used as the internal reference for miR-30b-5p, and actin was used as the internal reference for RAP1B. Data are presented as mean ± SD from three independent experiments (n=3). Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test. RAP1B, Ras-related protein Rap-1b; miR, microRNA.

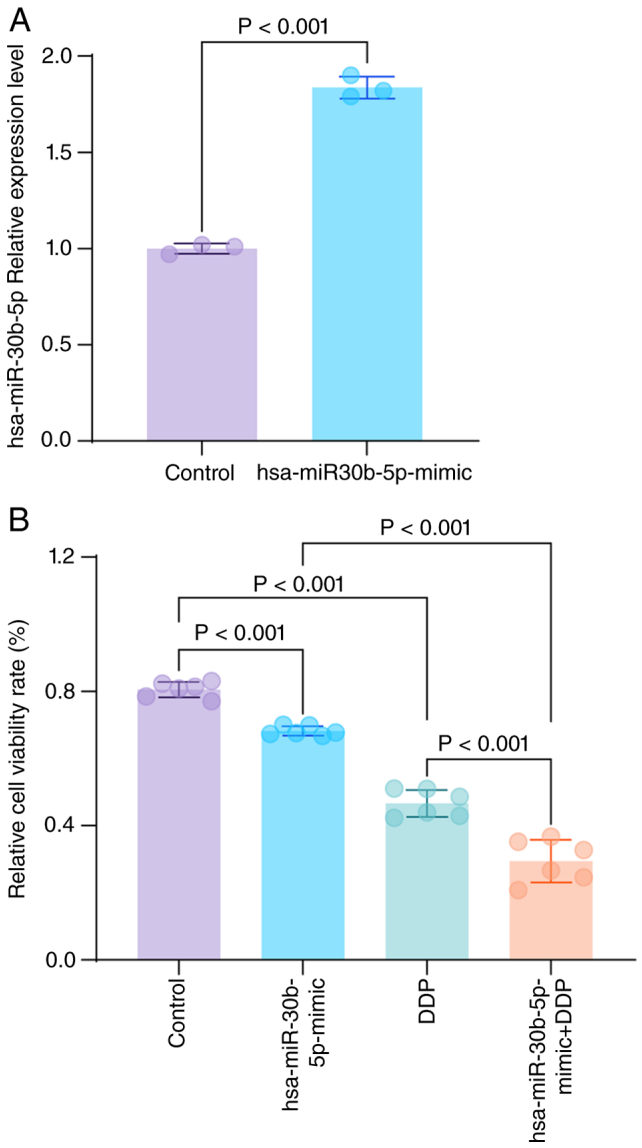

Figure 2. Overexpression efficiency of hsa-miR-30b-5p and its effect on cell viability. (A) MDA-MB-231 cells were transfected with hsa-miR-30b-5p mimic or negative control. After 48 h, reverse transcription-quantitative PCR was used to measure miR-30b-5p expression. (B) Cell viability was assessed using a Cell Counting Kit-8 assay in cells treated with miR-30b-5p mimic, cisplatin (2 μM) or their combination for 48 h. Data are presented as mean ± SD from three independent experiments (n=3), with three technical replicates per experiment. (A) For two-group comparisons, statistical significance was assessed using an unpaired two-tailed Student's t-test. (B) For multiple-group comparisons, one-way ANOVA followed by Tukey's post hoc test was applied. DDP, cisplatin; miR, microRNA.

Each experiment was performed with at least three biological replicates, with three technical replicates per biological replicate.

Western blot analysis. MDA-MB-231 cells were cultured for 24 h, then lysed using RIPA lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS] supplemented with protease and phosphatase inhibitors, and total protein was extracted. Protein concentration was determined using a BCA protein assay kit. Equal amounts of protein (30 μg) were loaded into each lane. The proteins were separated on 12% gels using SDS-PAGE and transferred onto a PVDF membrane. Following washing, the membrane was blocked with 5% non-fat dry milk in TBST (containing 0.1% Tween-20) for 1 h at room temperature and incubated with anti-RAP1B primary antibody (cat. no. ab324733; clone EPR29673-566; rabbit recombinant monoclonal; Abcam) at a 1:1,000 dilution in 5% non-fat dry milk/TBS overnight at 4˚C. After washing, HRP-conjugated secondary antibodies (anti-rabbit IgG-HRP; catalog no. ab6721; Abcam) diluted at 1:1,000

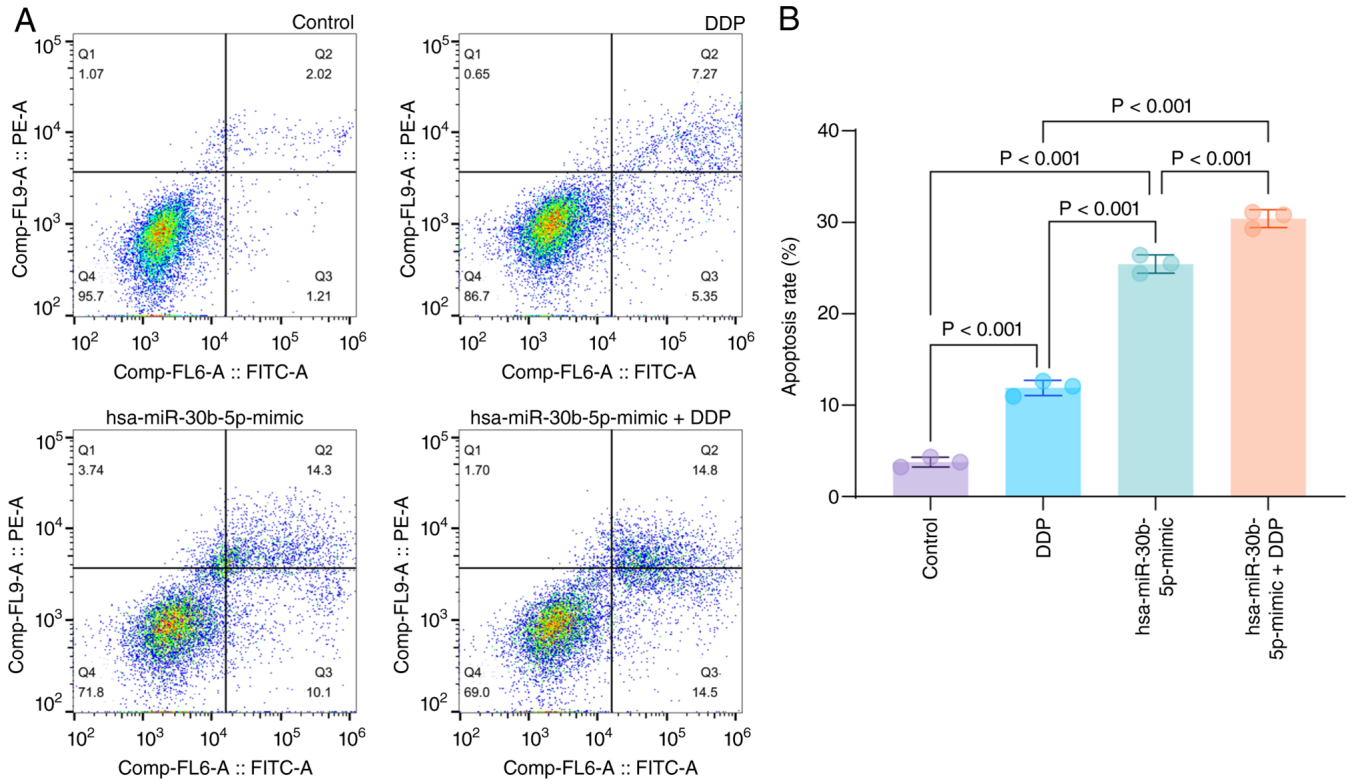

Figure 3. Effect of hsa-miR-30b-5p overexpression on cisplatin-induced apoptosis. MDA-MB-231 cells were transfected with miR-30b-5p mimic a negative control, and treated with cisplatin (2 μM) alone or in combination for 48 h. Apoptosis was assessed using flow cytometry using Annexin V/PI staining. (A) Representative flow cytometric dot plots; (B) quantitative bar graphs of apoptotic rates. Early apoptosis was defined as Annexin V^+/PI^-, and late apoptosis as Annexin V^+/PI^+. A minimum of 10,000 events were acquired per sample. Data are presented as mean ± SD from three independent experiments (n=3). Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test. DDP, cisplatin; miR, microRNA.

were added and incubated for 1 h at room temperature. The protein bands were visualized using an enhance chemiluminescence substrate (Thermo Fisher Scientific, Inc.). β-actin was used as an internal control. Densitometric semi-quantification of the western blot bands was performed using ImageJ software (version 1.53; National Institutes of Health), and the results were expressed as the ratio of RAP1B to β-actin. All western blotting experiments were repeated at least three times.

Statistical analysis. Data analysis was performed using SPSS 26.0 (IBM Corp.) and GraphPad Prism 9.0 (Dotmatics). The results are presented as mean ± standard deviation from at least three independent biological replicates. The normality of the data distribution was assessed using the Shapiro-Wilk test, and the homogeneity of variances was evaluated using Levene's test. Data were analyzed using an unpaired Student's t-test (for two-group comparisons) or one-way ANOVA, followed by pairwise comparisons using Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of hsa-miR-30b-5p and RAP1B in different breast cell lines. The expression levels of hsa-miR-30b-5p and RAP1B mRNA were assessed in normal breast epithelial cells (MCF-10A) and breast cancer cell lines (MCF-7, MDA-MB-231 and MDA-MB-468) using RT-qPCR (Fig. 1A). The expression of hsa-miR-30b-5p in MDA-MB-231 and MDA-MB-468 was significantly lower than the normal mammary epithelial cell line MCF-10A (P<0.001), and the expression of MDA-MB-231 was the lowest (P<0.001). Conversely, RAP1B mRNA expression was significantly higher in MDA-MB-231 cells compared with MCF-10A cells (P<0.001; Fig. 1B). Based on these results, MDA-MB-231 cells were selected for subsequent experiments.

Overexpression efficiency of hsa-miR-30b-5p and its effect on cell viability. The expression of hsa-miR-30b-5p in the hsa-miR-30b-5p mimic group was significantly higher than that in the control group (P<0.001), indicating that an MDA-MB-231 cell line overexpressing hsa-miR-30b-5p was successfully generated (Fig. 2A). CCK-8 assays revealed that miR-30b-5p overexpression alone significantly suppressed cell viability compared with the control group (P<0.001). Cisplatin (2 μM) treatment alone also significantly inhibited cell viability (P<0.001). The combination of miR-30b-5p mimic and cisplatin further reduced the cell viability rate compared with either treatment alone (P<0.001), indicating that hsa-miR-30b-5p enhances cisplatin-induced cell viability inhibition (Fig. 2B).

hsa-miR-30b-5p enhances cisplatin-induced apoptosis. Flow cytometry was used to evaluate the effect of miR-30b-5p overexpression on cisplatin-induced apoptosis. The results showed that the proportion of early apoptotic cells in hsa-miR-30b-5p-mimic group was higher than the control

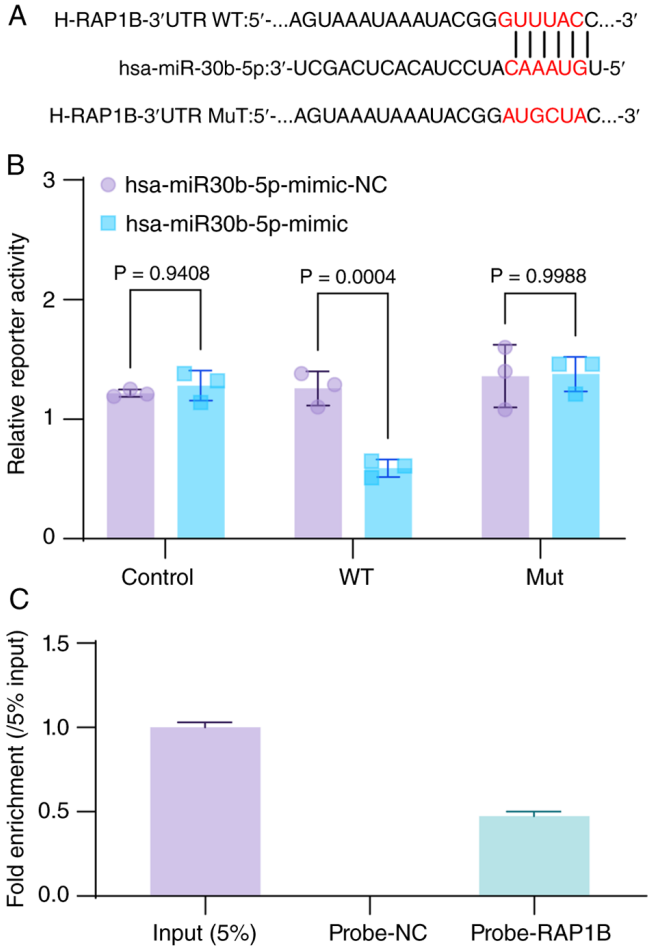

Figure 4. hsa-miR-30b-5p directly targets the 3'-UTR of RAP1B. (A) Predicted binding sites of miR-30b-5p in the WT and Mut RAP1B 3'-UTR. (B) Dual-luciferase reporter assay. MDA-MB-231 cells were co-transfected with WT or Mut reporter vector together with miR-30b-5p mimic or NC. Firefly luciferase activity was normalized to *Renilla* luciferase activity. (C) RNA pull-down assay. Biotin-labeled miR-30b-5p probe (or negative control probe) was incubated with MDA-MB-231 cell lysates, and bound RAP1B mRNA was assessed using reverse transcription-quantitative PCR. Data are presented as relative enrichment normalized to the negative control probe. Data are presented as mean ± SD from three independent experiments (n=3). Statistical analysis was performed using Student's t-test. Mut, mutant; WT, wild-type; miR, microRNA; NC, negative control; RAP1B, Ras-related protein Rap-1b; UTR, untranslated region.

group (P<0.001), and the proportion of early and late apoptotic cells in the DDP group was also higher than the control group (P<0.001). When miR-30b-5p mimic and cisplatin were used in combination, the proportion of apoptotic cells was further increased compared with either treatment alone (P<0.001), which indicated that the overexpression of hsa-miR-30b-5p enhanced cisplatin-induced apoptosis (Fig. 3).

Overexpression of hsa-miR-30b-5p enhances cisplatin-induced apoptosis. The results showed that the proportion of early apoptotic cells in the hsa-miR-30b-5p-mimic group was higher than in control group (P<0.001), and the proportion of early and late apoptotic cells in the DDP alone group was higher than control group (P<0.001). When the miR-30b-5p-mimic and cisplatin were used at the same time, the proportion of apoptotic cells further increased (P<0.001), which indicates that the overexpression of hsa-miR-30b-5p enhances the apoptosis induced by DDP (Fig. 3).

hsa-miR-30b-5p directly targets the 3'-UTR of RAP1B. Bioinformatic analysis using StarBase and TargetScan predicted a putative binding site for hsa-miR-30b-5p within the 3'-UTR of RAP1B mRNA (Fig. 4A). In the dual-luciferase reporter assay, the luciferase activity was significantly decreased in the WT sequence construct (P=0.0004), while in the Mut construct, due to disruption of the binding sites of miR-30b-5p, the inhibitory effect was not significant (P>0.05), indicating a high degree of sequence specificity in the binding and regulation of hsa-miR-30b-5p (Fig. 4B). RNA pull-down assay further supported this discovery, in which the enrichment of RAP1B mRNA in the miR-30b-5p specific probe group was significantly higher than that in the negative control probe group, demonstrating a specific molecular association between miR-30b-5p and RAP1B mRNA. Notably, the RNA pull-down assay demonstrates molecular association between miR-30b-5p and RAP1B mRNA, while the evidence for 3'-UTR-specific interaction is provided by the luciferase reporter assay (Fig. 4C). These results indicate that hsa-miR-30b-5p directly targets the 3'UTR of RAP1B.

hsa-miR-30b-5p suppresses RAP1B expression and RAP1B overexpression reverses its biological effects. RT-qPCR analysis showed that RAP1B mRNA expression was significantly lower in the miR-30b-5p mimic group compared with the control group (P<0.001; Fig. 5A). Western blot analysis with densitometric semi-quantification showed that DDP treatment increased RAP1B protein expression, whereas the miR-30b-5p mimic reduced RAP1B protein expression compared with the control group (P<0.001; Fig. 5B-D). Overexpression of RAP1B reversed this miR-30b-5p-mediated downregulation (P<0.001), confirming that RAP1B is a direct target of miR-30b-5p in the context of cisplatin treatment. The CCK-8 assay showed that compared with the control group, DDP treatment inhibited cell viability (P<0.001). The combination of miR-30b-5p mimic and cisplatin further reduced cell viability (P<0.001; Fig. 5E). Notably, RAP1B overexpression partially reversed the anti-proliferative effect of miR-30b-5p under cisplatin treatment (P<0.001), supporting that RAP1B mediates the functional effects of miR-30b-5p.

RAP1B overexpression partially reverses the pro-apoptotic effect of hsa-miR-30b-5p under cisplatin treatment. To further evaluate whether RAP1B mediates the effect of miR-30b-5p on cisplatin-induced apoptosis, a rescue experiment was performed. The apoptosis rate in the miR-30b-5p mimic group was significantly higher than that in the control group (P<0.001), and DDP treatment alone significantly increased the apoptosis rate (P<0.001) (Fig. 6A and B). The combination of miR-30b-5p mimic and DDP resulted in a further increase in apoptosis compared with either treatment alone (P<0.001). Notably, when RAP1B was overexpressed in combination with miR-30b-5p mimic and DDP treatment (miR-30b-5p mimic + op-RAP1B + DDP group), the apoptosis rate was partially reversed compared with the miR-30b-5p mimic + DDP group

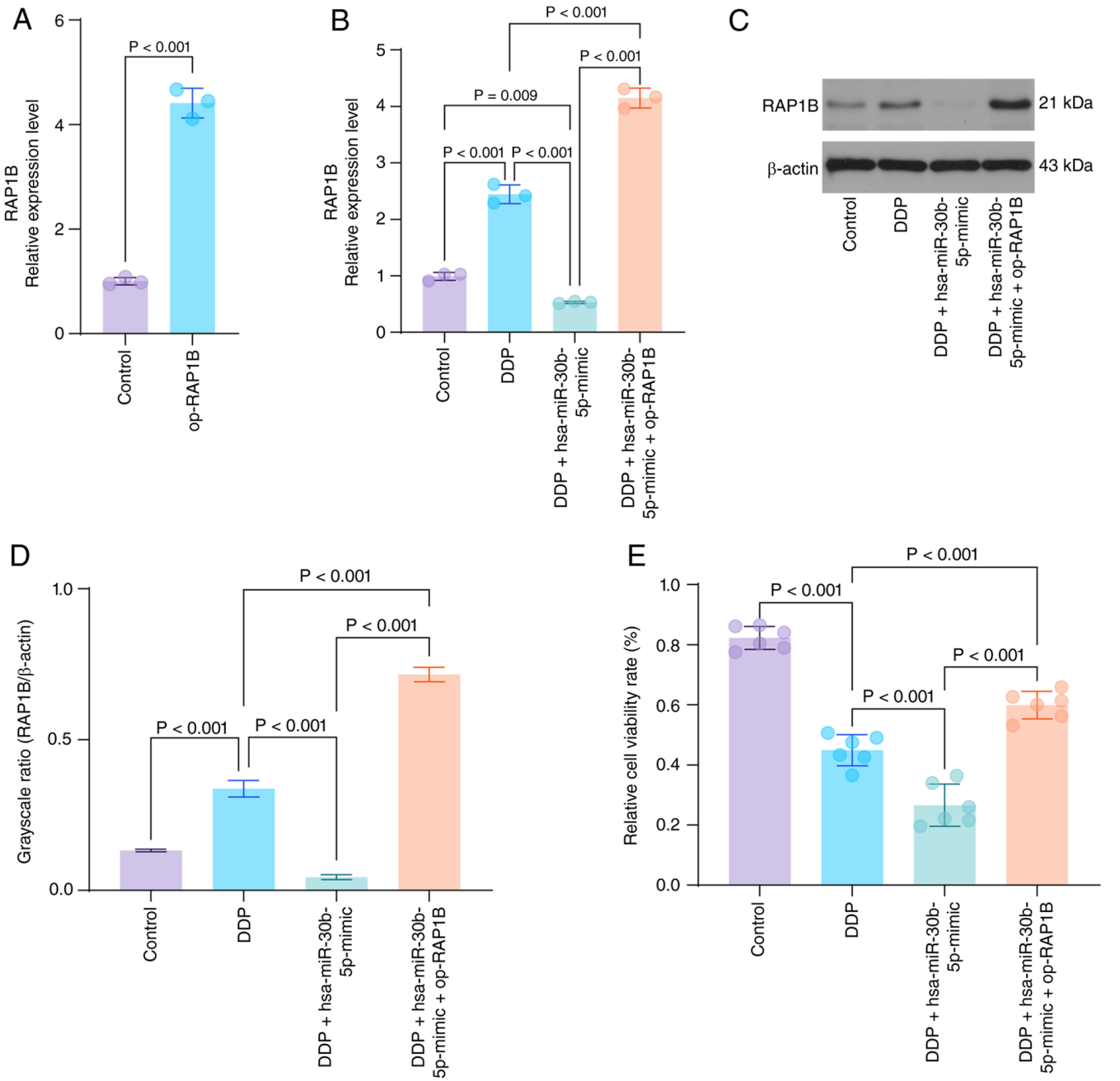

Figure 5. RAP1B mediates the functional effects of hsa-miR-30b-5p on cell viability. (A) RT-qPCR analysis of RAP1B mRNA levels after miR-30b-5p mimic transfection. (B) RT-qPCR detection of RAP1B expression after co-expression of hsa-miR-30b-5p and RAP1B. (C) Western blot analysis of RAP1B protein levels under the same conditions. (D) Densitometric semi-quantification of western blot results normalized to β-actin. (E) Cell Counting Kit-8 assay for cell viability. Data are presented as mean ± SD from three independent experiments (n=3). Statistical analysis was performed using Student's t-test or one-way ANOVA with Tukey's post hoc test. DDP, cisplatin; RT-qPCR, reverse transcription-quantitative PCR; miR, microRNA; RAP1B, Ras-related protein Rap-1b; op, overexpressed.

(P<0.001), consistent with the expected rescue effect. These results indicate that RAP1B mediates, at least in part, the effect of hsa-miR-30b-5p on cisplatin response in TNBC cells (Fig. 6).

Discussion

miRNAs regulate various important cellular activities, such as proliferation and differentiation, by modulating the expression of target genes. Previous studies have demonstrated that miRNAs are aberrantly expressed in various malignant tumors and serve notable roles in the pathogenesis of cancer. miRNA dysregulation also notably influence tumor cell responses to chemotherapy. The present study investigated how hsa-miR-30b-5p potentiates cisplatin cytotoxicity in MDA-MB-231 cells through the regulation of its target gene RAP1B.

The expression of hsa-miR-30b-5p across multiple breast cell lines was examined and there was a notable distinction between MDA-MB-231 cells and MCF-10A cells. MDA-MB-468 cells showed reduced miR-30b-5p expression compared with MCF-10A, supporting the notion

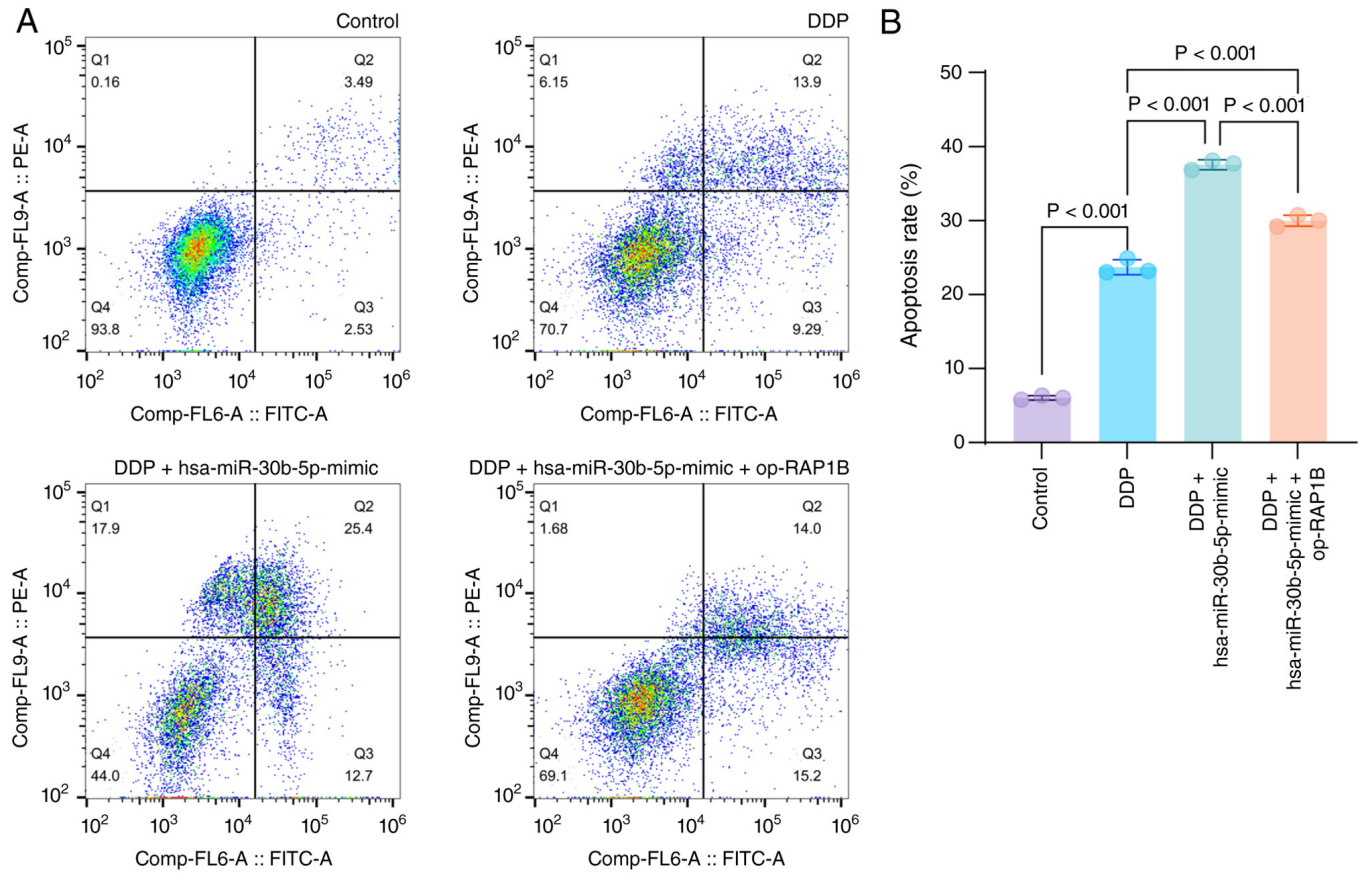

Figure 6. RAP1B overexpression partially reverses the pro-apoptotic effect of hsa-miR-30b-5p under cisplatin treatment. MDA-MB-231 cells were transfected with miR-30b-5p mimic, RAP1B overexpression plasmid or their combination, and treated with cisplatin (2 μM) for 48 h. Apoptosis was assessed using flow cytometry using Annexin V/PI staining. (A) Representative flow cytometric dot plots; (B) quantitative bar graphs of apoptotic rates. A minimum of 10,000 events were acquired per sample. Data are presented as mean ± SD from three independent experiments (n=3). Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test. DDP, cisplatin; miR, microRNA; RAP1B, Ras-related protein Rap-1b; op, overexpressed.

that miR-30b-5p downregulation is a common feature in TNBC. This phenomenon indicates that hsa-miR-30b-5p might contribute to tumor suppression by targeting key signaling molecules such as RAP1B, and its role may be context-dependent in different cellular settings. Consistent with the present findings, previous studies have reported downregulation of miR-30b-5p in various cancers, including gastric cancer and hepatocellular carcinoma, where its low expression was associated with poor prognosis (27,28). The results of the functional experiments showed that the miR-30b-5p-mimic treatment reduced cells viability when compared with the control group. This effect indicates that miR-30b-5p may perform its anticancer function by directly targeting oncogenic regulators such as ASPP2 (11), which are known to modulate cell cycle progression and viability in breast cancer.

The response of MDA-MB-231 cells to cisplatin was also significantly enhanced when treated with miR-30b-5p-mimic followed by DDP exposure. This suggests that hsa-miR-30b-5p serves a role in regulating tumor cell viability and apoptosis and may influence their responsiveness to chemotherapy drugs. The increased drug response could result from hsa-miR-30b-5p's inhibition of signaling pathways associated with chemotherapy resistance or its direct impact on drug target expression. For instance, in lung cancer, miR-30b-5p overexpression enhanced cisplatin sensitivity by directly targeting LRP8 (29). In colorectal cancer, miR-30b-5p reversed oxaliplatin resistance through targeting AVEN, thereby promoting apoptosis (30). In gastric cancer, the long non-coding RNA HNF1A-AS1 acted as a ceRNA for miR-30b-5p, relieving its suppression of EIF5A2 and inducing epithelial-mesenchymal transition, which contributed to 5-FU resistance (31). Moreover, miR-30b-5p has been shown to suppress the PI3K/AKT pathway by targeting multiple oncogenes across various cancer types, further contributing to chemosensitization (32). Collectively, these findings suggest that miR-30b-5p modulates chemotherapeutic responses through both direct regulation of resistance-associated genes and inhibition of pro-survival signaling pathways. DDP is a commonly utilized chemotherapy drug essential for tumor treatment. It can block the replication and transcription process by forming cross-links with DNA, thus inducing the apoptosis of tumor cells (33-35). However, tumor cells often develop resistance to cisplatin through various mechanisms, which limits its therapeutic effect (36,37). Enhancing the response to cisplatin is crucial in this context. The present study found that the miR-30b-5p-mimic independently suppresses TNBC cell proliferation and induces apoptosis, and further amplifies these effects when used in combination with cisplatin.

To investigate the molecular framework by which miR-30b-5p enhances response to cisplatin, its potential target gene, RAP1B was examined. RAP1B, a small GTP-binding protein, participates in diverse cell signal transduction processes such as proliferation, differentiation and migration (38,39). RAP1B is upregulated in various cancers and is associated with malignancy and chemotherapy resistance (21,40,41). Consistent with these reports, the present data showed that RAP1B was upregulated in MDA-MB-231 cells compared with normal breast epithelial cells.

Determining how miR-30b-5p targets RAP1B is crucial for understanding its role in TNBC. By using dual-luciferase reporter gene and RNA pull-down-qPCR experiments, the present study verified that RAP1B mRNA is the direct target of hsa-miR-30b-5p. These experiments showed that hsa-miR-30b-5p can specifically bind to the 3'-UTR of RAP1B mRNA, as mutation of the predicted binding site abolished the inhibitory effect. The RNA pull-down assay further confirmed a specific association between miR-30b-5p and RAP1B mRNA. Transfecting miR-30b-5p mimics into MDA-MB-231 cells significantly reduced RAP1B expression and increased the response to cisplatin. These cells showed lower viability rates and higher apoptosis rates. In the rescue experiments, overexpression of RAP1B partially reversed the pro-apoptotic effect of miR-30b-5p under cisplatin treatment, supporting that RAP1B mediated its effect on cisplatin response. This result indicates that RAP1B may be a key mediator for hsa-miR-30b-5p to regulate the response to chemotherapeutic drugs.

Several limitations of the present study should be acknowledged. First, all functional experiments were performed using MDA-MB-231 cells alone. Due to the heterogeneity of TNBC, conclusions drawn from a single cell line should be generalized to all TNBC subtypes with caution. Future studies utilizing patient-derived organoids or additional TNBC cell lines (such as MDA-MB-468 or HCC1806) for validation will be a priority. Second, the present study used a single cisplatin concentration (2 μM) selected based on previously published literature (15,16). While this concentration is sufficient to demonstrate phenotypic differences, future studies incorporating multiple concentrations and IC_{50} calculations with combination index analysis would provide a more quantitative assessment of the drug interaction. Third, the term 'synergistic' has been replaced with 'enhanced' throughout the manuscript, as formal synergy analysis (such as Chou-Talalay combination index) was not performed. Fourth, the lack of *in vivo* validation limits the translational relevance of the findings; animal studies will be necessary to confirm the therapeutic potential of the miR-30b-5p/RAP1B axis. Finally, incorporation of publicly available datasets (such as The Cancer Genome Atlas or Gene Expression Omnibus) or patient-derived specimens to correlate miR-30b-5p and RAP1B expression with clinical outcomes would substantially strengthen the clinical relevance of the study and should be pursued in future investigations.

The present study elucidated the effect of hsa-miR-30b-5p in TNBC, demonstrating that it enhances the response of MDA-MB-231 cells to DDP by targeting the RAP1B gene. The experimental findings indicated that hsa-miR-30b-5p overexpression markedly reduced RAP1B expression and significantly increased apoptosis in the presence of cisplatin treatment. In the rescue experiments, RAP1B overexpression partially reversed the effects of hsa-miR-30b-5p, supporting the role of RAP1B in cisplatin resistance in TNBC and suggesting that modulating miR-30b-5p and RAP1B expression may represent a strategy to regulate tumor cell response to cisplatin.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

KT contributed to the conception and design of the study, performed data curation and formal analysis, conducted the experimental investigations (including cell culture, transfection and functional assays), validated the reproducibility of the results, prepared the visualisations (figures and graphical data), and drafted the original manuscript. SB contributed to the conception and design of the study, performed data curation and formal analysis, conducted the experimental investigations, validated the reproducibility of the results, prepared the visualisations, supervised the research team, and critically reviewed and edited the manuscript. QZ contributed to the conception and design of the study, acquired funding, performed software-based statistical analysis, validated the data, and reviewed and edited the manuscript. QY contributed to the conception and design of the study, performed formal analysis and investigation, acquired funding, administered the project, validated the data, prepared the visualisations, wrote the original draft, and reviewed and edited the manuscript. SL performed data curation, contributed to the methodology (establishing experimental protocols and optimising assay conditions), performed software-based data analysis, validated the results, and reviewed and edited the manuscript. YT contributed to the methodology (designing and standardising the experimental procedures), provided essential resources (reagents and materials), validated the experimental findings, prepared the visualisations, and reviewed and edited the manuscript. LY conducted the experimental investigations (including cell culture, drug treatments and molecular assays), performed software-based data processing, prepared the visualisations, and reviewed and edited the manuscript. LO provided supervision of the research group, contributed to the interpretation of data, and critically reviewed and edited the manuscript for important intellectual content. All authors have read and approved the final version of the manuscript. KT and SB confirm the authenticity of all the raw data.

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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