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ORIGINAL RESEARCH

RETRACTED ARTICLE: Non-coding RNA NEAT1/miR-214-3p contribute to doxorubicin resistance of urothelial bladder cancer preliminary through the Wnt/β-catenin pathway

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Background: Urothelial bladder cancer (UBC) is one of the most lethal urological malignancies in the world. Patients with UBC are routinely given chemotherapy which results in a median survival of 12-15 months. Nuclear-enriched abundant transcript 1 (NEAT1) functions as an oncogene and could be used as a therapeutic target for human UBC. However, the involvement of NEAT1 in doxorubicin (DOX) resistance of UBC has been poorly demonstrated.

Methods: Quantitative Real-time PCR (qRT-PCR) was used to detect the expression levels of NEAT1 and miR-214-3p in UBC tissues and cells. Bioinformatics prediction, RNA pull-down and qRT-PCR were used to assay the regulation manner of NEAT1 and miR-214-3p. Loss/gain function of NEAT1 and miR-214-3p together with western blot, drug resistance assay and flow cytometry were used to explore the influence of NEAT1 in DOX resistance was correlative with miR-214-3p. Finally, luciferase assay system was applied to determine the Wnt/β-catenin signal activity.

Results: NEAT1 was upregulated and miR-214-3p was downregulated in DOX-resistant UBC tissues and cells. NEAT1 knockdown inhibited J82 and T24 cells to DOX chemosensitivity by negatively regulating miR-214-3p expression. NEAT1/miR-214-3p contributed to DOX resistance of UBC preliminary through the Wnt/β-catenin pathway.

Conclusion: NEAT1 contributed to DOX resistance of UBC through the Wnt/β-catenin pathway partly by negatively regulating miR-214-3p expression. Our findings will provide a promising ncRNA targeted therapeutic strategy for UBC with DOX resistance.

Keywords: nuclear-enriched abundant transcript 1, miR-214-3p, urothelial bladder cancer, doxorubicin resistance, Wnt/β-catenin pathway

Introduction

Human bladder cancer, especially urothelial bladder cancer (UBC), is one of the most common urological malignancies in men throughout the world and is characterized by a high rate of early systemic dissemination.[1] Surgery is routinely performed on patients with UBC followed by combined chemotherapy.[2,3] Although tremendous therapeutic strategies including approaches associated with chemo-resistance have been made in recent years, most patients receiving successful chemotherapy initially experienced frequent recurrences, resulting in a median survival of 12–15 months.[4] Resistance to doxorubicin (DOX), a widely used frontline agent in intra-vesical and systemic chemotherapy for UBC, contributes to a barrier, leading to treatment failure. Therefore, it is crucial to elucidate the underlying molecular mechanism of DOX resistance in UBC and identify an effective therapeutic target that can sensitize UBC to DOX.

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Long noncoding RNA (lncRNA), a class of endogenous RNAs, is implicated in carcinogenesis and progression of numerous cancers by acting as an oncogene or tumor suppressor. Moreover, the abnormality of lncRNA has been reported to participate in the development of chemo-resistance in various tumors, including UBC.[5–7] The nuclear-enriched abundant transcript 1 (NEAT1) gene, transcribed from the multiple endocrine neoplasia locus, has been documented acting as a transcriptional regulator and functioning as an oncogene to facilitate tumorigenesis in different types of solid tumors.[8–11] Of note, NEAT1 was reported to be consistently upregulated in UBC and the expression level of NEAT1 in UBC is closely related to its clinical pathologic grade and TNM phase. Meanwhile, NEAT1 contributes to the progression and deterioration of UBC by promoting cells proliferation and migration, inhibiting cells apoptosis.[12] In conclusion, NEAT1 functions as an oncogene and could be a therapeutic target for human UBC. However, the involvement of NEAT1 in UBC DOX resistance is poorly demonstrated.

In this study, we confirmed that NEAT1 was upregulated and miR-214-3p was downregulated in DOX-resistant UBC tissues and cells. Furthermore, mechanism analysis revealed that NEAT1 knockdown negatively regulated miR-214-3p expression and NEAT1/miR-214-3p contributed to DOX resistance in UBC preliminary through the Wnt/β-catenin pathway. This study is the first to establish a NEAT1/miR-214-3p induced DOX resistance regulatory network in UBC, hinting at a promising therapeutic strategy for UBC with DOX resistance.

Materials and methods

Patients and clinical specimens

This study was approved by the ethical committee of China Medical University, and written informed consent was provided by the participants prior to surgery. Sixty-four UBC and matched normal urothelial bladder tissues were collected from patients receiving cystoscope between 2013 and 2014 at Shengjing Hospital, and pathologically examined by two independent pathologists. The samples were stored in liquid nitrogen immediately and divided into: 1) the responsive group (n=39) and 2) the resistant group (n=25) based on their response to DOX or together with other chemotherapeutic drugs. In detail, UBC patients routinely underwent six cycles of chemotherapeutic treatment, then the therapeutic effect was confirmed by both cystoscopy and imaging examination. Patients with reduced tumor volume were classified into the responsive group, otherwise they were classified into the resistant group.

Cell culture

Human UBC cell lines J82 and T24 were obtained from the Chinese Academy of Sciences (Shanghai, People's Republic of China) and stored by our laboratory. All the cells were routinely cultured in DMEM with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) in a 95% air/5% CO_2 incubator under 37°C. The corresponding DOX-resistant UBC cells J82/DOX and T24/DOX were established from the parental cell lines J82 and T24 by stepwise exposure to increasing concentrations of DOX (Sigma-Aldrich Co., St Louis, MO, USA) as before.[13] Finally, [illegible]g/L DOX was additionally added into the medium to maintain the resistance phenotype of J82/DOX and T24/DOX cells.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from the cultured cells and tissues by Trizol reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. After reverse transcribed into cDNA, qRT-PCR was finished by using SYBR® Green Master Mix Kit (QIAGEN, Germany) on a 7500 PCR System (Thermo Fisher Scientific). All reactions were done in triplicate. The expression levels of genes were calculated by the $2^{-\Delta\Delta CT}$ method after normalization with reference controls. The primers used in this study were as below: NEAT1 5′-CTTCCTCCCTTTAACTTATCCATTCAC-3′ and 5′-CTCTTCCTCCACCATTACCAACAATAC-3′; miR-214-3p 5′-GCATCCTGCCTCCACATGCAT-3′ and 5′-GCGCTGAGGAATAATAGAGTATGTAT-3′; GAPDH 5′-TATGATGATATCAAGAGGGTAGT-3′ and 5′-TGTATCCAAACTCATTGTCATAC-3′; snRNAU6 5′-CTCGCTTCGGCAGCACA-3′ and 5′-AACGCTTCACGAATTTGCGT-3′.[14,15]

Vector construction and transient transfection

The specific siRNAs of NEAT1 and scrambled control (si-NEAT1, si-con) were synthesized by RiboBio Corporation (Guangzhou, People's Republic of China). The ectopic vector pcDNA3.1-NEAT1 (pc-NEAT1) and its control (pc-con) were constructed by Thermo Fisher Scientific. The miR-214-3p mimics/inhibitors with corresponding controls (miR-214-3p, anti-miR-214-3p, miR-con and anti-miR-con) were purchased from RiboBio. The Wnt signaling quantitation luciferase reporter plasmids (TOP Flash, FOP Flash) were purchased from BioVector NTCC Inc. (Beijing, People's Republic of China). Transient transfection was carried out using Lipofectamine™ 3,000 (Thermo Fisher Scientific) following the manufacturer's instructions. The reporter activities were determined 48 hours post-transfection by the Dual-Lucy

Assay Kit from Vigorous Biotechnology (Beijing, People's Republic of China), with firefly luciferase as base line and renilla luciferase as internal control described as before.[16]

Western blot

Cells were lysed and protein concentrations were determined as previously described.[17,18] In total, 30 μg protein were processed including protein separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred into a polyvinylidene fluoride membrane. Then after blocking with 5% non-fat milk, the membranes were hybridized with specific antibodies against P-glycoprotein (P-gp) from Santa Cruz Biotechnology Inc., Dallas, TX, USA; Axin2, glycogen synthase kinase 3 beta (GSK-3β), β-catenin and phospho-β-catenin (Ser675) (p-β-catenin) from Cell Signaling Technology, Danvers, MA, USA; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from ProteinTech, USA (Thermo Fisher Scientific). Finally, the bands were detected and analyzed by Image J software (National Institutes of Health, Bethesda, MD, USA) according to the manufacturer's instructions.[17] Protein levels were normalized to GAPDH.

Drug resistance assay

DOX resistance was assessed by using a cell counting kit-8 (CCK-8) method. In brief, cells were cultured 24 hours prior being exposed to various doses of DOX (0.05, 0.1, 0.5, 1, 5, and 10 mg/L) for 48 hours. Then 10 μL of the CCK-8 solution was added, and after incubating at 37°C for 4 hours, the plate was gently mixed on an orbital shaker for 1 minute to ensure homogeneous distribution of color. Then absorbance at 450 nm was recorded using a microplate reader (Tecan, Switzerland). The concentration of DOX causing 50% inhibition of cell growth (IC_{50}) was calculated by the relative dose-response survival curve.

Apoptosis detection

Flow cytometry (BD FACSCanto™ II Flow Cytometry Analyzer Systems from BD Biosciences (San Jose, CA, USA) was used to detect the apoptosis of cells as previously described.[17] In brief, cells were harvested and incubated with FITC Annexin V in a buffer containing propidium iodide (PI) supplied by a FITC Annexin V Apoptosis Detection Kit with PI (BioLegend, San Diego, CA, USA). Then, the Diva 8.0 software (BD Biosciences) was used to analyze the apoptosis rate. Cells undergoing apoptosis were FITC Annexin V positive and PI negative in the right lower quadrant.

RNA pull-down assays

RNA pull-down assays were finished according to the manufacturer's instructions by using the Dynabeads® M-280 Streptavidin (Thermo Fisher Scientific).[17] In detail, probes were marked by biotin using the Biotin RNA Labeling Mix (Roche, Switzerland). Cell lysates were incubated with positive control (Bio-miR-214-3p, Bio-NEAT1), negative control (Bio-miR-214-3p-mut, Bio-NEAT1-mut) and biotinylated RNAs (Bio-NC). Beads were added to the binding reaction at room temperature. The beads were then washed and co-precipitated RNAs were detected by qRT-PCR. The probes used in this study were as follow: Bio-miR-214-3p: 5′-Bio-ACAGCAGGCACAGACAGGCAGT-3′, Bio-miR-214-3p-mut: 5′-Bio-CUCATCUATCTCACTCAATCAG-3′; Bio-NEAT1: 5′-Bio-TTCCCATCTGGACCCTGCUGG-3′, Bio-NEAT1-mut: 5′-Bio-ATGCTTCGTAACTTGATGAA-3′.

Statistical analysis

All statistical analysis was performed using SPSS version 22.0 (IBM Corporation, Armonk, NY, USA) and GraphPad Prism version 5.04 (GraphPad Software, Inc., La Jolla, CA, USA). The data are reported as mean ± standard deviation (SD) of three independent experiments. Unpaired Student's *t*-test and one-way analysis of variance (ANOVA) were used to finish the comparisons. *P*-values less than 0.05 was considered to have statistically significant (*$P<0.05$, ** and # $P<0.01$).

Results

NEAT1 was upregulated and miR-214-3p was downregulated in DOX-resistant UBC tissues and cells

To detect whether NEAT1 and miR-214-3p were associated with UBC DOX resistance, we first detected the expression levels of NEAT1 and miR-214-3p in UBC patients. The qRT-PCR results showed that NEAT1 was upregulated and miR-214-3p was downregulated in the DOX-resistant group compared with that in the DOX-responsive group ($P=0.005$, $P=0.012$) (Figure 1A, B). Then, qRT-PCR was performed to detect the expression of NEAT1 and miR-214-3p in DOX-resistant UBC cells (J82/DOX and T24/DOX). Similarly, higher NEAT1 expression and lower miR-214-3p expression were exhibited in J82/DOX and T24/DOX cells in comparison with their parental cells ($P=0.015$, $P=0.006$; $P=0.012$, $P=0.005$) (Figures 1C, D). These results demonstrated that dysregulation of NEAT1 and miR-214-3p were associated with UBC DOX resistance.

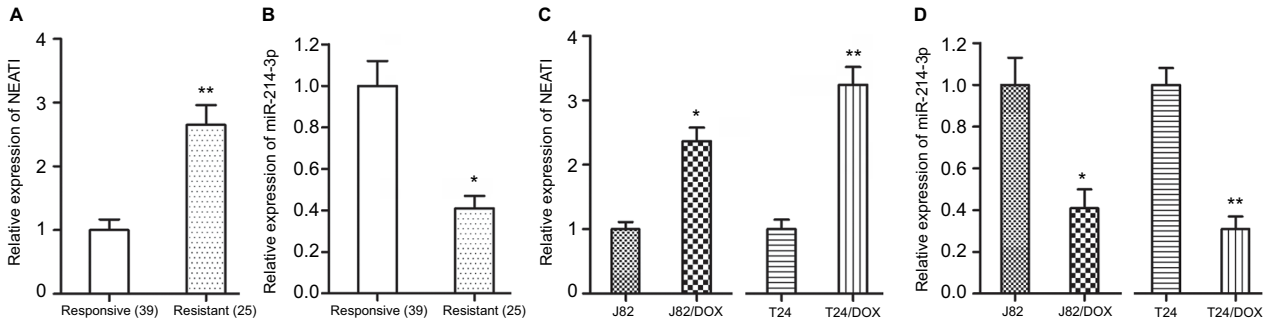

Figure 1 NEAT1 was upregulated and miR-214-3p was downregulated in DOX-resistant UBC tissues and cells.
Notes: (**A**, **B**) qRT-PCR assay shows the expression levels of NEAT1 and miR-214-3p in DOX-resistant UBC tissues; (**C**, **D**) qRT-PCR assay shows the expression levels of NEAT1 and miR-214-3p in DOX-resistant UBC cells. **P*<0.05, ***P*<0.01.
Abbreviations: NEAT1, nuclear-enriched abundant transcript 1; DOX, doxorubicin; UBC, urothelial bladder cancer.

NEAT1 knockdown and miR-214-3p overexpression inhibited J82/DOX and T24/DOX cells occurring DOX resistance

To further explore the effects of NEAT1/miR-214-3p on UBC cells DOX resistance, we incubated J82 and T24 cells with various concentrations of DOX for 48 hours, and then IC_{50} value was detected by the CCK-8 assay. As shown in Figure 2A, B, the IC_{50} value of DOX in J82/DOX and T24/DOX cells was significantly higher than that in J82 and T24 cells, which confirmed the production of DOX resistance in J82/DOX and T24/DOX cells.

The role of NEAT1/miR-214-3p in DOX resistance was evaluated by loss-of and gain-of function methods. qRT-PCR confirmed the transfection efficiency by down-regulation of NEAT1 and upregulation of miR-214-3p in transfected J82/DOX and T24/DOX cells (*P*<0.001, *P*=0.002; *P*<0.001, *P*<0.001) (Figure 2C, D). Multidrug resistance (MDR) is a well-known major obstacle in the successful treatment of multiple cancers, while abnormal expression of P-glycoprotein (P-gp) encoded by the MDR1 gene is the most common reason. Thus we secondly investigated the effect of NEAT1 knockdown and miR-214-3p overexpression on the protein expression level of P-gp by Western blot. The results showed that the protein level of P-gp was remarkably reduced in corresponding transfected J82/DOX and T24/DOX cells (*P*=0.008, *P*=0.021; *P*=0.011, *P*=0.006) (Figure 2E, F). In addition, IC_{50} determination showed that NEAT1 knockdown and miR-214-3p overexpression significantly decreased the DOX resistance in J82/DOX and T24/DOX cells (*P*=0.012, *P*=0.008; *P*=0.013, *P*=0.016) (Figure 2G, H).

The J82/DOX and T24/DOX cells were then treated with 0.5 mg/L DOX for 48 hours, and flow cytometry was used to observe whether NEAT1 and miR-214-3p-mediated alteration of DOX resistance was related to apoptosis. The results showed that the DOX-induced apoptosis rate was obviously enhanced after the introduction with si-NEAT1 and miR-214-3p in J82/DOX and T24/DOX cells (*P*=0.017, *P*=0.020; *P*=0.023, *P*=0.01) (Figure 2I). Taken together, these data suggested that NEAT1 knockdown and miR-214-3p overexpression inhibit the resistance of J82 and T24 cells to DOX.

NEAT1 suppressed miR-214-3p expression in J82/DOX and T24/DOX cells

To determine whether the influence of NEAT1 on DOX resistance was correlative with miR-214-3p, we firstly used the web-based tool Starbase 2.0 (http://starbase.sysu.edu.cn/mirLncRNA.php); miR-214-3p was predicted to have complementary bases pairing with NEAT1 (Figure 3A). Then the co-expression patterns analysis showed a negative correlation between NEAT1 and miR-214-3p in UBC (Figure 3B). Furthermore, RNA pull-down assay showed that NEAT1 could be specifically pulled down by biotinylated miR-214-3p probe, while miR-214-3p could be specifically pulled down by biotin-labeled NEAT1 probe (Figures 3C, D). In addition, qRT-PCR showed that miR-214-3p was significantly downregulated in pc-NEAT1-transfected J82/DOX and T24/DOX cells, while si-NEAT1 transfecion could significantly reverse miR-214-3p expression (*P*=0.009, *P*=0.014; *P*=0.016, *P*=0.011) (Figure 3E, F). These data indicated that NEAT1 suppressed miR-214-3p expression in DOX-resistant UBC cells.

NEAT1 knockdown improved DOX sensitivity in UBC J82/DOX and T24/DOX cells by negatively regulating miR-214-3p

To determine whether the NEAT1-induced inhibition on DOX resistance was mediated by miR-214-3p, J82/DOX and

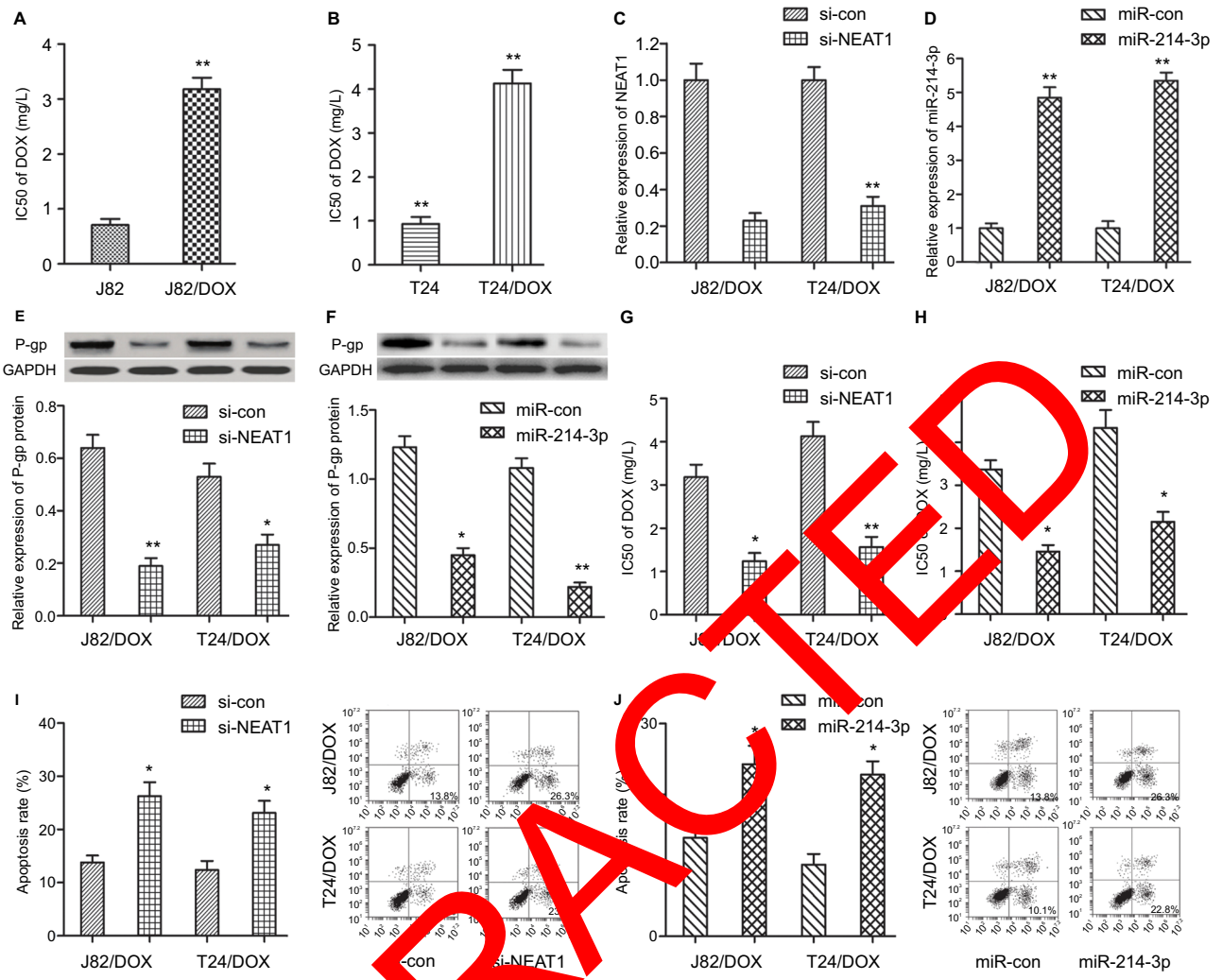

Figure 2 NEAT1 knockdown and miR-214-3p overexpression inhibited DOX resistance in UBC J82/DOX and T24/DOX cells.
Notes: (**A**, **B**) IC50 values of DOX in J82/DOX and T24/DOX cells with their parental cells; (**C**, **D**) qRT-PCR analysis of the knockdown and overexpression efficiency of NEAT1 and miR-214-3p in J82/DOX and T24/DOX cells; (**E**, **F**) Western blot assay shows the expression level of P-gp in J82/DOX and T24/DOX cells; (**G**, **H**) CCK-8 assay shows the IC50 value of DOX in J82/DOX and T24/DOX cells; (**I**, **J**) Flow cytometry assay shows the apoptotic rate of J82/DOX and T24/DOX cells. *P<0.05, **P<0.01.
Abbreviations: NEAT1, nuclear-enriched abundant transcript 1; DOX, doxorubicin; UBC, urothelial bladder cancer; si-con, scrambled control; si-NEAT1, siRNAs of NEAT1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

T24/DOX cells were transfected with si-NEAT1, anti-miR-214-3p and related controls. Western blot analysis demonstrated that NEAT1 knockdown led to an obvious reduction of P-gp expression in J82/DOX and T24/DOX cells, while anti-miR-214-3p transfection could significantly reverse the si-NEAT1-mediated P-gp reduction (P=0.031; P=0.024) (Figure 4A, B). Drug resistance assay showed that NEAT1 deficiency effectively enhanced DOX sensitivity in J82/DOX and T24/DOX cells. However, anti-miR-214-3p introduction greatly abolished the si-NEAT1-triggered DOX sensitivity increase (P=0.021; P=0.017) (Figure 4C, D). Meanwhile, flow cytometry analysis revealed that NEAT1 silence dramatically promoted DOX-induced apoptosis in J82/DOX and T24/DOX cells, whereas anti-miR-214-3p treatment markedly abated the promotive effect of si-NEAT1 on DOX-induced apoptosis (P=0.025; P=0.021) (Figure 4E, F). These results illustrated that miR-214-3p downregulation partially overturned NEAT1 knockdown-induced DOX sensitivity in DOX-resistant UBC cells.

NEAT1/miR-214-3p regulated Wnt/β-catenin pathway to promote the UBC cells occurring DOX resistance

To investigate the mechanism of NEAT1/miR-214-3p on DOX resistance, Pathway and GO analysis revealed that both NEAT1 negative-associated and miR-214-3p positive-associated genes are enriched in the Wnt/β-catenin pathway. To validate these correlations in UBC J82/DOX and T24/DOX cells, the impact of NEAT1 and miR-214-3p overexpression on the

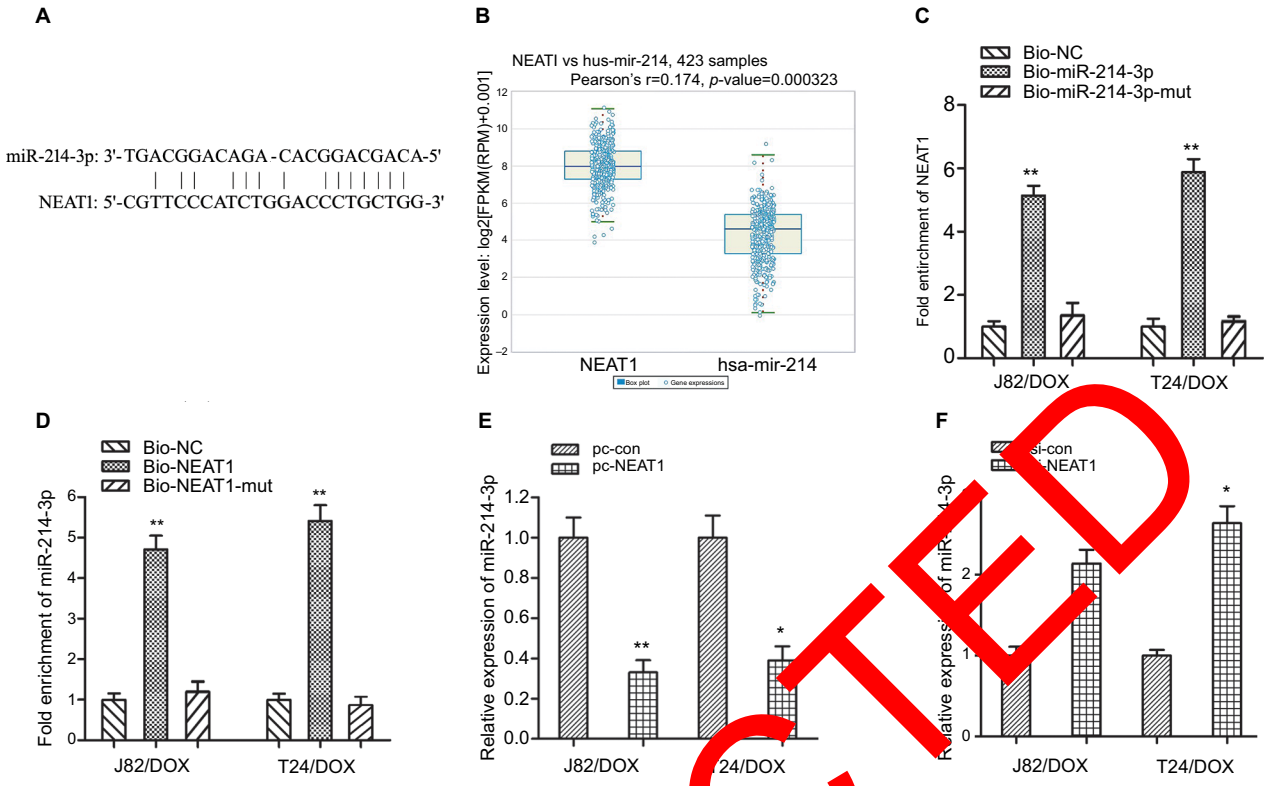

Figure 3 NEAT1 suppressed the expression of miR-214-3p in UBC J82/DOX and T24/DOX cells.
Notes: (A) The complementary bases between miR-214-3p and NEAT1 were predicted using the web-based tool Starbase 2.0; **(B)** The co-expression pattern between NEAT1 and miR-214-3p in UBC was searched using the online server ChIPBase; **(C, D)** qRT-PCR assay shows the RNA levels of NEAT1 and miR-214-3p in the substrate of pull-down. MiR-214-3p- and NEAT1-mut probes were used as negative controls, respectively; **(E, F)** qRT-PCR assay shows the expression of miR-214-3p in J82/DOX and T24/DOX cells treated with pc-NEAT1 and si-NEAT1 or matched controls. *$P<0.05$, ** $P<0.01$.
Abbreviations: NEAT1, nuclear-enriched abundant transcript 1; DOX, doxorubicin; UBC, urothelial bladder cancer; si-con, scrambled control; si-NEAT1, siRNAs of NEAT1.

Wnt/β-catenin pathway activity was examined. Through the Western blot assay, we found that miR-214-3p overexpression could significantly increase the Axin2 and GSK3B expression levels, and reduce the nuclear β-catenin and p-β-catenin (Ser675) levels, as well as the MDR1-encoded protein P-gp (direct target of the Wnt/β-catenin pathway), while NEAT1 overexpression reversed these effects (Figure 5A, B). Also, the TCF-LEF reporter system indicated that overexpressed miR-214-3p could attenuate Wnt/β-catenin signaling activity, while NEAT1 overexpression reversed this effect (P=0.012, P=0.017; P=0.[illegible], P=0.014) (Figure 5C, D). Together with the IC_{50} results that NEAT1 knockdown dramatically attenuated DOX resistance in J82/DOX and T24/DOX cells, and the negative regulatory manner between NEAT1 and miR-214-3p, we concluded that NEAT1/miR-214-3p abnormal expression (NEAT1 upregulation and miR-214-3p downregulation) regulated Wnt/β-catenin preliminary through repressing Axin2/GSK3B expression and helping β-catenin occur nuclear transport, which further led to UBC cells occurring DOX resistance.

Discussion

LncRNAs have been well documented to participate in the development of chemo-resistance in various solid cancers, including UBC. For example, enrichment of lncRNA LINP1 was found in doxorubicin- and 5-fluorouracil-resistant cells and induced chemo-resistance in breast cancer.[19] LncRNA LUCAT1 knockdown decreased the proliferation, invasion and methotrexate resistance in osteosarcoma.[20] NEAT1 dysregulated in ovarian cancer, lung cancer, gastric cancer and leukemia were reported to contribute to the chemotherapy resistance of paclitaxel, cisplatin, adriamycin, alisertib and bortezomib.[14,21–23] LncRNA PVT1, TUG1 and UCA1 were reported to upregulate in UBC, especially in the doxorubicin and cisplatin-resistant UBC tissues and cell lines. Forced lncRNA-LET expression delayed gemcitabine-induced tumor recurrence. Moreover, knockdown of these lncRNAs promoted UBC carcinogenesis and drug resistance.[5–7,24,25] Our results indicated that NEAT1 knockdown inhibited the sensitivity of J82 and T24 cells to DOX, which might provide a promising therapeutic target for UBC with DOX resistance.

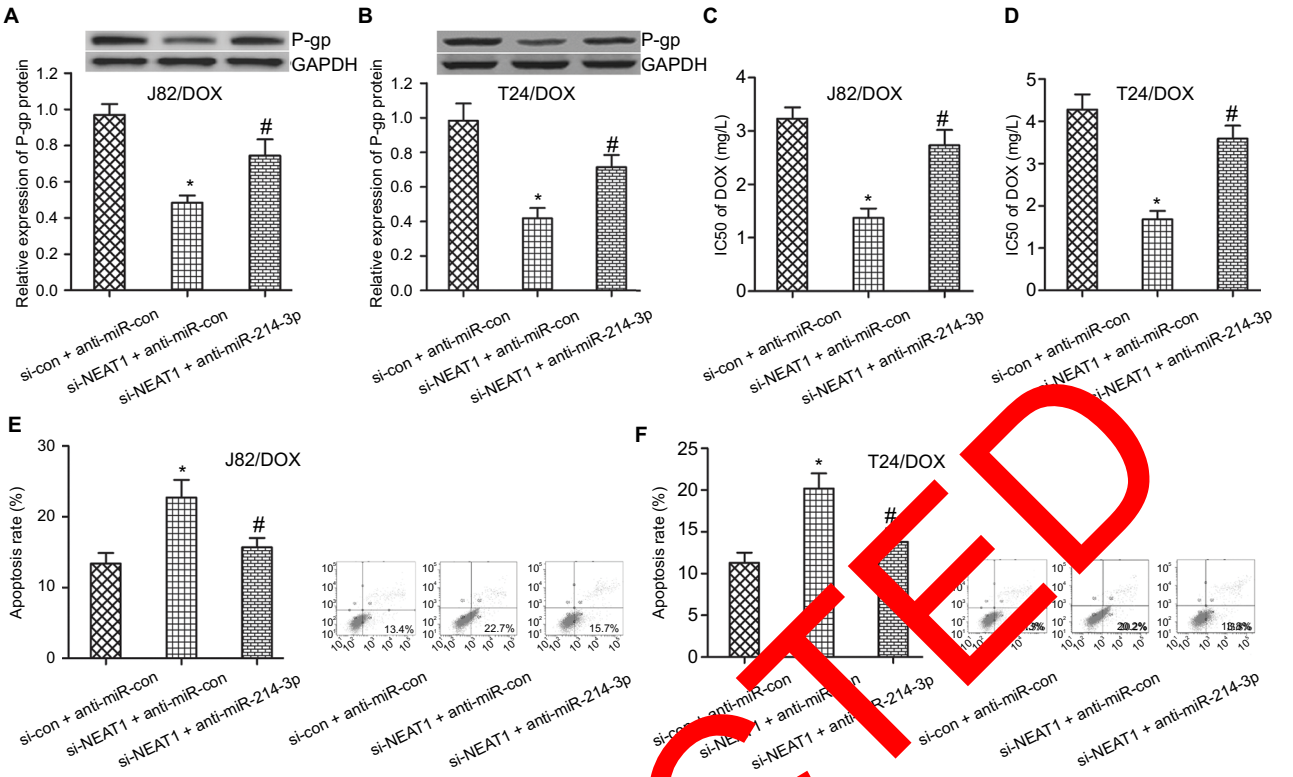

Figure 4 NEAT1-induced inhibition on DOX resistance in UBC was mediated by miR-214-3p.
Notes: J82/DOX and T24/DOX cells were transfected with si-NEAT1 and anti-miR-214-3p or matched controls. (**A**, **B**) Western blot analysis shows the expression level of P-gp in J82/DOX and T24/DOX cells; (**C, D**) CCK-8 assay shows the IC50 value of DOX in J82/DOX and T24/DOX cells; (**E**, **F**) Flow cytometry assay shows the apoptotic rate of J82/DOX and T24/DOX cells. *P<0.05. #P<0.01.
Abbreviations: NEAT1, nuclear-enriched abundant transcript 1; DOX, doxorubicin; UBC, urothelial bladder cancer; si-con, scrambled control; si-NEAT1, siRNAs of NEAT1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Recently, lncRNAs have been proposed to act as miRNA sponges or competitive endogenous RNAs (ceRNAs), forming extensive regulatory networks thereby negatively regulating miRNA expression.[26,27] For example, lncRNA LUCAT1 modulated methotrexate resistance in osteosarcoma through sponging miR-200c[20]; lncRNA GACAT3 acting as a miRNA sponge, could modulate gastric cancer multidrug resistance by regulating miR-4[illegible] expression.[28] Using bioinformatics databases StarBase 2.0 and PANCAN, we predicted the negative regulation model between NEAT1 and miR-214-3p in UBC. As miR-214-3p has been shown to be closely associated with chemotherapy resistance in breast cancer, ovarian cancer, cervical cancer and tongue squamous cell carcinomas, little attention has been paid to the miR-214-3p-associated chemotherapy effect in UBC.[29–32] Together with a previous study showing that miR-214-3p downregulated in muscle-invasive bladder cancer patients could improve the prognosis of patients after radical cystectomy, we set out to explore whether the influence of NEAT1 on DOX resistance is mediated by miR-214-3p in UBC.[33] In detail, we confirmed the regulation model between NEAT1 and miR-214-3p based on the following: 1) RNA pull-down assay revealed that NEAT1 functions via interaction with miR-214-3p; 2) Overexpression/knockdown of NEAT1 in J82/DOX and T24/DOX cells significantly decreased/increased miR-214-3p expression; 3) miR-214-3p overexpression/NEAT1 knockdown obviously reduced P-gp expressions in J82/DOX and T24/DOX cells, while miR-214-3p knockdown significantly reversed NEAT1 knockdown mediated reduction of P-gp expressions; and 4) miR-214-3p overexpression/NEAT1 knockdown effectively enhanced DOX sensitivity in J82/DOX and T24/DOX cells, while miR-214-3p knockdown greatly abolished NEAT1 knockdown triggered increase in DOX sensitivity.

In addition, flow cytometry analysis revealed dysregulation of NEAT1/miR-214-3p influence on UBC cells resistant to DOX by occurring apoptosis. These data strongly suggested that NEAT1 knockdown improves DOX sensitivity in UBC J82/DOX and T24/DOX cells by negatively regulating miR-214-3p.

MDR is one of the prominent obstacles causing chemotherapeutic resistance of patients in various solid tumors such as breast cancer, colon cancer and lung cancer.[34,35] Wnt/β-catenin signaling is crucial in the regulation of MDR1 transcription.[36] In the canonical Wnt/β-catenin, β-catenin, Axin and GSK-3β are the main factors coupling with lymphoid-

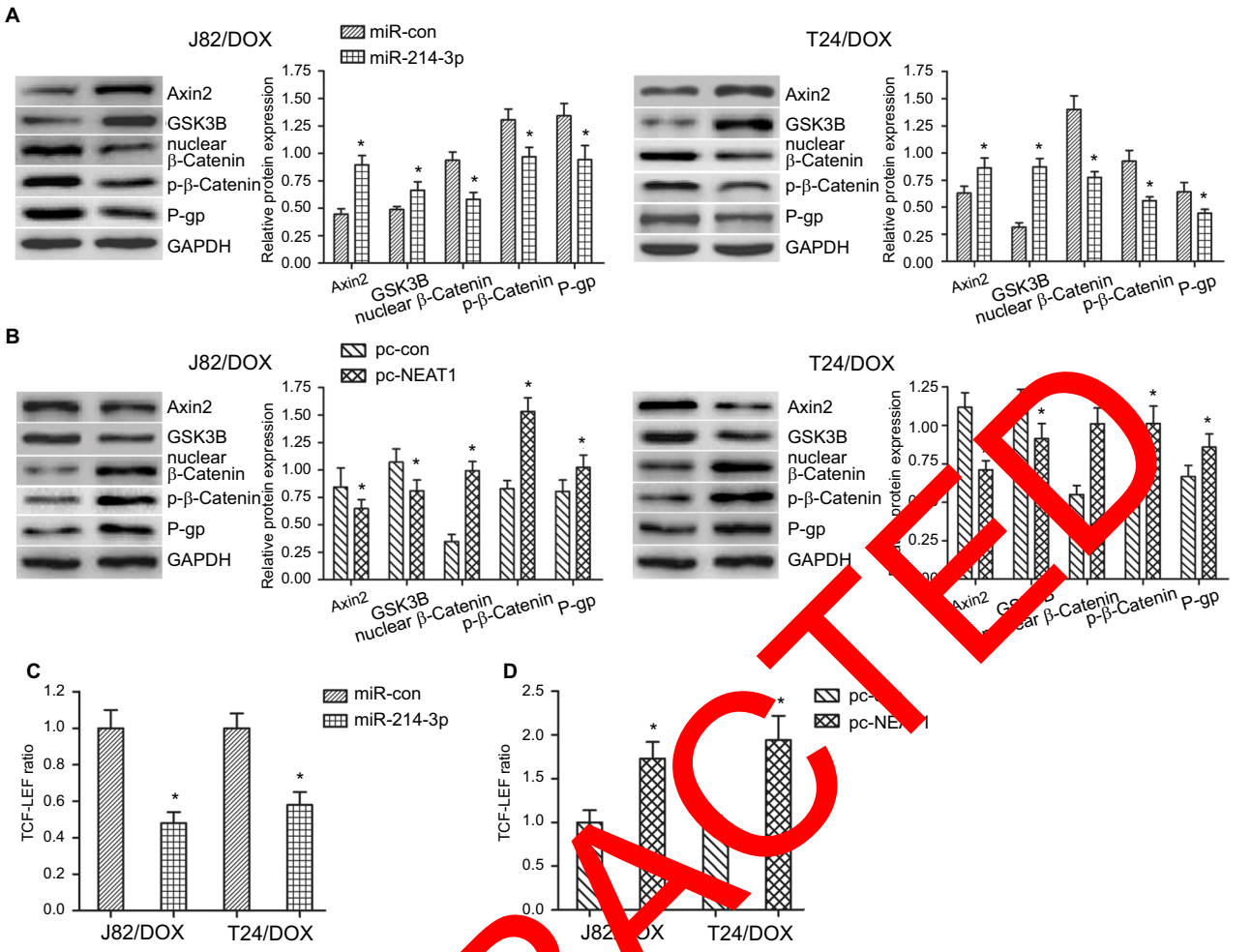

Figure 5 NEAT1/miR-214-3p promoted the UBC cells occurring DOX resistance by the Wnt/β-catenin pathway.
Notes: J82/DOX and T24/DOX cells were transfected with miR-214-3p, NEAT1 or matched controls. (**A**, **B**) Western blot analysis shows the expression level of Axin2, GSK3B, β-catenin, p-β-catenin and P-gp in J82/DOX and T24/DOX cells; (**C**, **D**) Wnt/β-catenin signaling activity was assayed in J82/DOX and T24/DOX cells. *P<0.05.
Abbreviations: NEAT1, nuclear-enriched abundant transcript 1; DOX, doxorubicin; UBC, urothelial bladder cancer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; pc-NEAT1, ectopic vector pcDNA3.1-NEAT1; pc-con, ectopic vector pcDNA3.1 control.

enhancing factor/T-cell factor (LEF/TCF) family, thereby driving target gene transcription. Accumulating evidence showed that the "lncRNA–microRNA" pair abnormal expression associated with Wnt/β-catenin signal activation, which in turn modulated the chemo-resistance of cancers. For instance, lncRNA CRNDE/miR-181a-5p regulated the progression and chemo-resistance of colorectal cancer via activating the Wnt/β-catenin signaling[38]; ncRNA MALAT1/miR-101 and SOX9 enhanced the chemo-resistance of lung cancer cells to DDP through the Wnt signaling pathway.[39] However, the interaction between NEAT1/miR-214-3p-related DOX resistance and Wnt/β-catenin signaling in UBC has not been explored. By a series of studies, we found that miR-214-3p overexpression could significantly increase the Wnt/β-catenin signaling-associated genes expression, thereby activating the pathway; while NEAT1 overexpression could reverse these effects. Together with the drug resistance assay and loss/gain function of NEAT1/miR-214-3p in J82/DOX and T24/DOX cells, we believe we have obtained a novel regulation model that NEAT1/miR-214-3p abnormal expression modulates UBC cells occurring DOX resistance preliminary via the Wnt/β-catenin pathway.

There are still some limitations in our study, for example, whether NEAT1 negatively regulated miR-214-3p expression by acting as a miRNA sponge or ceRNA? Whether other pathways like PTEN influence NEAT1/miR-214-3p-related DOX resistance in UBC?[40] How the regulation model of NEAT1/miR-214-3p acts in DOX dependent multi-chemotherapy? Precise molecular mechanisms of NEAT1/miR-214-3p in UBC chemo-resistance will be explored in the future.

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Disclosure

The authors report no conflicts of interest in this work.

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