Pseudogene KRT17P3 Drives Cisplatin Resistance of Human NSCLC Cells by Modulating miR-497-5p/mTOR

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Research

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Abstract

Background: Chemoresistance is a major obstacle in non-small-cell lung cancer (NSCLC) treatment. The pseudogene KRT17P3 has been shown to be up-regulated in lung cancer tissues from patients with cisplatin resistance. However, its molecular mechanism in promoting chemoresistance has not been elucidated.

Methods: Real-time PCR was performed to evaluate KRT17P3 levels in plasma samples collected from 30 cisplatin-resistance patients and 32 cisplatin-sensitive patients. KRT17P3 was overexpressed or knocked down in A549 and SK-MES-1 NSCLC cells to evaluate the effects of KRT17P3 on the in vitro and in vivo resistance of NSCLC to cisplatin. Cell Counting Kit 8, Annexin V/propidium iodide staining, and PARP-1 cleavage assays were performed to evaluate cell viability and apoptosis. Dual luciferase reporter, RNA immunoprecipitation, Western blot assays, and rescue experiments were used to evaluate the functional interaction of KRT17P3, miR-497-5p and mTOR.

Results: Our results demonstrate that KRT17P3 overexpression in cultured NSCLC cells increases cell viability and decreases apoptosis upon cisplatin treatment, while KRT17P3 knockdown has the opposite effect. KRT17P3 overexpression promotes NSCLC tumor growth upon in cisplatin-treated xenografted mice. Mechanistically, KRT17P3 acts as a molecular sponge for miR-497-5p and relieves the binding of miR-497-5p to its target gene mTOR. Rescue experiments validated the functional interaction among KRT17P3, miR497-5p, and mTOR. Moreover, the plasma level of KRT17P3 is up-regulated in cisplatin-resistance patients.

Conclusions: Our findings indicate that KRT17P3/miR497-5p/mTOR regulates the chemosensitivity of NSCLC, suggesting a potential therapeutic target for cisplatin-resistant NSCLC patients. KRT17P3 may be a potential peripheral blood marker of NSCLC patients resistant to cisplatin.

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide in both males and females, which can be attributed to its high incidence and mortality [1]. Non-small-cell lung cancer (NSCLC) accounts for 85% of lung cancer cases. Platinum-based combination chemotherapy is currently recommended as a standard treatment for patients with advanced NSCLC [2]. Treatment with cisplatin plus one of the new agents remains the most widely employed first line chemotherapeutic regimen for the treatment of NSCLC. However, the individual response to drug therapy varies, and the emergence of drug resistance greatly limits long-term curative effects. Therefore, it is important to identify molecular mechanisms involved in cisplatin chemoresistance of lung cancer.

There are almost 15,000 human pseudogenes, according to GENCODE Release annotations (version 33) (http://www.gencodegenes.org/stats/current.html). Pseudogenes were initially regarded as non-functional genomic junk resulting from inactivating gene mutations during evolution. However, recent studies have implicated dysregulation of pseudogenes in diverse physiological and pathological
processes, including cancer [3, 4]. Pseudogenes can interact with parental genes or other gene loci, leading to alterations in sequences and/or transcriptional activities. Pseudogene-derived RNAs play multifaceted roles in posttranscriptional regulation as antisense RNAs, endogenous small-interference RNAs, and competing endogenous RNAs that govern gene expression related to cancer progression, metastasis, and drug resistance in multiple tumors [5-9]. For example, recent research has revealed that the pseudogene KRT19P3 suppresses gastric cancer growth and metastasis through the COPS7A-mediated NF-κB pathway [7]. NANOGP8 expression is correlated with cell proliferation, invasion, metastasis and oxaliplatin chemo-resistance in gastric cancer [6]. Additionally, the pseudogene-derived long non-coding RNA SFTA1P promotes apoptosis and increases cisplatin sensitivity by regulating the hnRNP-U-GADD45A axis in lung squamous cell carcinoma [9].

Our previous work demonstrated that pseudogene KRT17P3 (Keratin 17 Pseudogene 3, ENSG00000231870; previously named lncRNA AC006050.3-003) is significantly downregulated in Partial Response (PR) compared to Progressive Disease (PD) tumor tissue samples in lung squamous cell carcinoma patients. This result was obtained by using lncRNA expression microarray data and was confirmed in another 60 lung squamous cell carcinoma (LUSC) carcinoma patients by quantitative real-time polymerase chain reaction (RT-qPCR) [10]. Moreover, receiver operating characteristic curve analysis revealed that KRT17P3 constitutes a valuable biomarker for differentiating PR patients from PD patients. KRT17P3 is located in Chromosome 17: 30,567,700-30,571,748 reverse strand in GRCh38 coordinates. To date, no other studies have detailed the expression pattern, biological functions, or regulatory mechanisms of KRT17P3 in human cancer. Furthermore, the biological function and underlying molecular mechanism of KRT17P3 in cisplatin resistance has not been characterized.

In the current study, we demonstrate that dysregulation of KRT17P3 confers cisplatin sensitivity in A549 and SK-MES-1 NSCLC cells in vitro and in a mouse model in vivo. KRT17P3 modulates cisplatin sensitivity via direct binding of miR-497-5p, which regulates the target gene mTOR. Moreover, KRT17P3 expression is up-regulated in peripheral blood samples of PD NSCLC patients. Taken together, these findings provide new insights into the role of KRT17P3 in cisplatin resistance.

**Materials And Methods**

**Cell culture and reagents**

The A549 and SK-MES-1 cell lines were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences and were maintained in RPMI 1640 (Gibco, Grand Island NY, USA; for A549) or EMEM (ATCC 30-2003, Manassas, VA, USA; for SK-MES-1); they were supplemented with 10% FBS (Gibco, Grand Island NY, USA), 100 U/mL penicillin and 0.1 mg/mL streptomycin (Gibco); and were cultured in 5% CO₂ at 37°C. The cells were treated with cisplatin (Sigma-Aldrich, St. Louis, MO, USA) for indicated periods of time. Hsa-miR-497-5p mimic, miR-497-5p inhibitor and control mimic or inhibitor were purchased from RiboBio (Guangzhou, China).

**Clinical samples**
Plasma was collected from 62 NSCLC patients at the Jinling Hospital, Nanjing University School of Medicine from January 2016 to January 2019. The clinicopathological characteristics of the NSCLC patients are summarized in Table 1. The entry standards and standards for objective tumor response for target lesions used in this study were identical to those in our previous study [10]. Front-line chemotherapy for lung Adenocarcinoma (LUAD) comprised cisplatin 75 mg/m² on day 1, plus gemcitabine 1000 mg/m² on days 1 and 8, or plus Docetaxel 75 mg/m² on days 1, every 21 days for 4 cycles. Front-line chemotherapy for LUSC comprised cisplatin 75 mg/m² on day 1, plus pemetrexed 500 mg/m² on day 1, or plus Docetaxel 75 mg/m² on days 1, every 21 days for 4 cycles. Up to 5 ml of blood was collected from patients in a K₂EDTA plasma tube (BD Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and immediately centrifuged (2000g for 10 minutes) within 30 minutes after collection. After separation, plasma samples were transferred to RNase Dnasefree tubes and stored at -80°C until total RNA extraction. All samples were obtained after obtaining written informed consent of patients, in accordance with the Declaration of Helsinki. The study was approved by the Ethics Committee of Jinling Hospital, Nanjing University School of Medicine.
Table 1
Clinicopathological characteristics of participants in this study.

|                        | Resistant (n=30) | Sensitive (n=32) | P value |
|------------------------|------------------|------------------|---------|
| **Gender**             |                  |                  |         |
| female                 | 5                | 9                | 0.367   |
| male                   | 25               | 23               |         |
| **Age (year)**         |                  |                  | 0.459   |
| ≥65                    | 17               | 15               |         |
| <65                    | 13               | 17               |         |
| **Smoking**            |                  |                  | 1.000   |
| No                     | 13               | 13               |         |
| Yes                    | 13               | 19               |         |
| **Histological type**  |                  |                  | 0.789   |
| Adenocarcinoma         | 21               | 21               |         |
| Squamous cell carcinoma| 9                | 11               |         |
| **Tumor size (cm)**    |                  |                  | 0.069   |
| <4                     | 14               | 23               |         |
| ≥4                     | 16               | 9                |         |
| **TNM stage**          |                  |                  | 0.492   |
| I+II                   | 0                | 2                |         |
| III+IV                 | 30               | 30               |         |

**RNA extraction and RT-qPCR**

RNA extraction from cultured cells was performed using TRIzol reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer’s protocol. Plasma RNA was extracted using miRNeasy Serum/Plasma Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer’s protocol. cDNA was
synthesized with the PrimeScript 1st Strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan) according to the user’s manual. RT-qPCR analysis with gene-specific primers was performed to determine the relative expression of KRT17P3 using SYBR green reagents (Takara Bio) in an ABI 7300 sequence detector (Applied Biosystems, Foster City, CA, USA). GAPDH mRNA was used for normalization. The PCR primers used in this study were: KRT17P3: Forward: 5’-GCAAAACCTGAGCTGGAGGTGAAG-3’ and Reverse: 5’-CAATCTGAGCAGAGTGGTGGC-3’; and ACTIN: Forward: 5’-TCATGAAGTGTGACGTGGACAT-3’ and Reverse: 5’-CTCAGGAGGAGCAATGATCTTG-3’.

**Cell Counting Kit 8 (CCK8) assay**

Cells transfected with plasmid or infected with lentivirus were seeded into 96-well plates at a density of 5×10^3 cells. After cellular adhesion, medium containing cisplatin at distinct concentrations (0–14 μg/ml) was added to the corresponding cells. After 72h, cell viability was determined by the CCK8 (Donjindo, Kumamoto, Japan) according to the manufacturer’s protocol. Absorbance at 450 nm was measured using a microplate reader (Bio-Tek, Winooski, VT, USA). The IC50 was calculated with Graphpad software.

**Cell apoptosis analysis**

Cells were treated with different concentration of cisplatin for 24h and were stained with FITC Annexin V and propidium iodide (BD Biosciences) in accordance with the manufacturer’s instructions, and then analyzed by FACScan using CellQuest software (BD Biosciences).

**Generation of KRT17P3 knockdown or overexpression cells**

Lentivirus harboring shRNA-KRT17P3 and KRT17P3 expression plasmids were constructed by Genechem (Shanghai, China). After 72h infection, the cells were subjected to further studies. Cells infected with lentivirus expressing scrambled shRNA or transfected with a puromycin resistance plasmid were used as control. The targeting sequence of the KRT17P3 shRNA was 5’-TGAACGAGATGCATGAGTA-3’. For the tumorigenicity studies, stable A549 cells with KRT17P3 overexpression were selected in puromycin.

**Xenograft animal model**

BALB/c nude mice (4-5 weeks old) were maintained on a 12-h light/12-h dark cycle under pathogen-free conditions. After anesthetization, stable KRT17P3-overexpressing A549 cells and control cells (4 × 10^6) were injected subcutaneously in the back flanks of mice. On day 7 after tumor cell implantation, cisplatin was intraperitoneally injected intravenously (i.p) twice a week. Tumor growth was examined every 7 days for 4 weeks, and the tumor volume was assessed according to the equation: volume = length × width^2 × 0.5. All mice were sacrificed, and the xenografts were dissected and weighed for further studies. Animal welfare and experimental procedures were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

**Western blot analysis**
Cells were lysed with RIPA, and 30 μg proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were blotted with 5% nonfat milk and probed with primary antibodies against anti-PARP-1 (Abcam, Cambridge, MA, USA), anti-mTOR (Abcam) or anti-GAPDH (Santa Cruz, CA, USA), followed by the appropriate secondary antibodies and chemiluminescent detection.

**Luciferase reporter assay**

The KRT17P3 sequence with a predicted miR-497-5p binding site and the respective mutated sequence were amplified by PCR and inserted into the pmiR-RB-Report vector (Genechem) to construct plasmids KRT17P3-WT and KRT17P3-MUT. These plasmids were then co-transfected into HEK293T cells with miR-497a mimic or mimic-NC. Dual-luciferase assays (Promega, Madison, WI, USA) were performed according to the manufacturer’s instructions.

**RNA immunoprecipitation (RIP)**

RIP was performed using an EZ-Magna RIP Kit (Millipore, MA, USA) according to the manufacturer’s instructions. Briefly, A549 cells were cotransfected with pKRT17P3 (Genechem) or control plasmid in the presence or absence of pMS2-GFP (Genechem) plasmid and were lysed with RIP lysis buffer. The lysates were incubated with magnetic beads conjugated with anti-GFP (Abcam) or nonspecific control IgG with rotation overnight at 4°C. Then the proteins in the immunoprecipitants were digested with protein K and removed. Finally, the purified RNA was detected by RT-qPCR to measure the level of miR-497-5p. For anti-AGO2 RIP, cells were transfected with pmiR-497 plasmid or control plasmid and were subjected to RIP assays using an AGO2 antibody (Abcam).

**Statistical analysis**

Statistical analysis was performed with SPSS 20.0 (Abbott Laboratories, IL, USA). All data are presented as means ± SEM. The Student’s t-test was used to analyze data between two groups. The Chi-Square test was applied to examine the clinicopathological characteristics between chemoresistant and chemosensitive patients. The Student’s t-test was applied to the examination of relationship between KRT17P3 levels and clinicopathological characteristics. A value of $P<0.05$ was considered statistically significant.

**Results**

**Dysregulation of KRT17P3 confers chemosensitivity to cisplatin in A549 and SK-MES-1 cells.**

To evaluate the effect of KRT17P3 on the chemosensitivity of NSCLC cancer cells to cisplatin, we transfected specific shRNA-KRT17P3 and control shRNA-NC into A549 and SK-MES-1 cells to knock down KRT17P3 expression (Fig. 1A). Cell counting kit-8 (CCK-8) assay results show that shRNA-KRT17P3 cells exhibited significantly increased cisplatin sensitivity (Fig. 1B). Calculation of the IC50 for shRNA-KRT17P3 and sh-NC in A549 cells (1.518 ± 0.187 μM versus 3.194±0.336 μM; P<0.05) and SK-MES-1 cells
(6.92±0.203 μM versus 9.936 ± 0.232; P<0.05) verified the effect of shRNA-KRT17P3 in increasing cisplatin sensitivity (Fig. 1C).

As apoptosis is one of the important characteristics of chemosensitivity, we evaluated the effect of KRT17P3 on apoptosis. The results show that for both A549 and SK-MES-1 cells, the apoptotic rate of shRNA-KRT17P3-expressing cells with cisplatin treatment was significantly higher than that of shRNA-NC-expressing cells (both P<0.01) (Fig. 1D). Interestingly, even in cells without cisplatin treatment, there was a difference between sh-KRT17P3 and sh-NC cells in terms of the fraction of Annexin V-positive cells (Fig. 1D). To confirm these findings, we further observed the levels of cleaved PARP-1 in shRNA-KRT17P3 and shRNA-NC cells with or without cisplatin treatment. Consistent with the results from Fig. 1C, KRT17P3 shRNA enhanced cleaved PARP-1 levels in the presence of cisplatin exposure (Fig. 1E). These results suggest that the chemosensitivity of A549 and SK-MES-1 cells is increased by KRT17P3 knockdown, which is also associated with decreased cell proliferation.

We also investigated the effect of overexpressing KRT17P3 on chemosensitivity using KRT17P3-expressing lentivirus (Lv-KRT17P3) (Fig. 2A). CCK-8 assay results show that overexpression of KRT17P3 significantly increased the cisplatin-induced cell viability (Fig. 2B). We measured the IC50 and found that KRT17P3-overexpressing cells were more resistant to cisplatin than the control cells (6.139±0.18 μM versus 3.887±0.16 μM for A549, P < 0.01; 14.44 ± 0.136 μM versus 11.28 ± 0.136 μM for SK-MES-1, P < 0.05) (Fig. 2C). Conversely, to the KRT17P3-knockdown results, overexpression of KRT17P3 reduced the amount of cell apoptosis and cleaved PARP-1, both in cisplatin-treated and untreated cells (Fig. 2D-E). These results suggest that overexpression of KRT17P3 contributes to cisplatin chemoresistance in A549 and SK-MES-1 cells.

**Dysregulation of KRT17P3 confers chemosensitivity to cisplatin in vivo.**

To further determine whether KRT17P3 has an effect on the in vivo resistance of NSCLC to cisplatin, we injected KRT17P3-overexpressing or control A549 cells subcutaneously into the flanks of 5-week-old nude mice. Starting at 1 week after inoculation, cisplatin was administered by intraperitoneal injection twice a week for 3 weeks to half of the mice. Compared to the cisplatin-treated control xenograft tumors, the tumors grown from Lv-KRT17P3 A549 cells in cisplatin-treated mice had larger mean volumes, faster growth and heavier weights (Fig. 3). Therefore, these results verify that KRT17P3 reduces the effect of cisplatin in suppressing NSCLC cell growth.

**KRT17P3 contributes to cisplatin resistance of NSCLC cells by negatively regulating miRNA-497-5p**

A growing volume of literature has proposed that pseudogene transcripts can regulate transcription by sequestering shared microRNAs (miRNAs), thus acting as competing endogenous RNAs (ceRNAs)[11]. We performed a search for miRNAs that have complementary base pairing with KRT17P3 (ENSG00000231870), using the online software program miRcode (http://www.mircode.org). Furthermore, to identify putative functions for the miRNAs in chemoresistance, we performed miRNA target prediction and evaluation using online software program bibiserv (https://bibiserv.cebitec.uni-
The search results identified 9 miRNA families that may form complementary base pairing with KRT17P3 (Supplementary Fig. S1, Supplementary Fig. S2). Given its reported involvement in cisplatin resistance [12, 13], we focused on miR-497-5p in subsequent studies. The binding site between KRT17P3 and miRNA-497-5p is shown in Fig. 4A.

To evaluate the role of miRNA-497-5p in KRT17P3-mediated chemoresistance, we constructed KRT17P3 luciferase reporter plasmids containing wild type and mutant-binding sites for miR-497-5p and transfected them into 293T cells together with miR-497-5p mimic or a control mimic. The miR-497-5p mimic reduced the luciferase activity of the wild-type but not the mutant plasmid (Fig. 4B). To further verify the direct interaction between KRT17P3 and miR-497-5p, we over-expressed MS2-GFP-tagged KRT17P3 in A549 cells and performed GFP-RIP. The precipitated miRNAs were analyzed by real-time PCR. The results suggest that the MS2-GFP-tagged KRT17P3 was specifically enriched for miR-497-5p (Fig. 4C). Then we further conducted anti-Ago2 RIP in A549 cells transiently overexpressing miR-497-5p. Endogenous KRT17P3 pulldown by AGO2 antibody was specifically enriched in miR-497-5p-transfected cells, but not in empty vector -transfected cells (Fig. 4D). These data suggested that miR-497-5p is a miRNA binding partner of KRT17P3.

To confirm that miR-497-5p is involved in KRT17P3-mediated cisplatin resistance of NSCLC cells, we suppressed the function of miR-497-5p with miRNA inhibitors in shRNA-KRT17P3 cells. The miR-497-5p inhibitor attenuated the cisplatin sensitivity with increases in the cell viability (Fig. 5A) and IC50 (Fig. 5B) for shKRT17P3 cells as compared to the control cells transfected with inhibitor-NC. Meanwhile, miR-497-5p inhibitor alone enhanced the cell viability and IC50. Consistently, miR-497-5p inhibitor also decreased cisplatin-induced apoptosis and cleaved PARP-1 in sh-KRT17P3 cells (Fig. 5C-D). Conversely, increased miR-497-5p expression via the use of miRNA mimic reversed the cisplatin-resistance in Lv-KRT17P3 cells as compared to cells transfected with mimic-NC, with decreased cell viability and IC50 (Fig. 6A-B). The miR-497-5p mimic also increased cisplatin-induced apoptosis and expression level of cleaved PARP-1 in Lv-KRT17P3 cells (Fig. 6C-D). These data suggest that miR-497-5p plays an important role in KRT17P3-dependent cisplatin-resistance.

**KRT17P3 contributes to cisplatin resistance in part through regulation of the miRNA-497-5p/mTOR axis**

Based on the above results, we sought to explore potential targets of miR-497-5p by MIRANDA (http://www.microrna.org) and TARGETSCAN (http://www.targetscan.org) algorithms. Among them, mTOR was predicted to be targeted by miR-497-5p through a recognition sequence in its 3’ UTR (Fig. 7A). To verify this prediction, we performed luciferase reporter assays with plasmid containing the predicted wild type or mutant miR-497-5p binding site. The miR-497-5p mimic significantly weakened the luciferase activity of the mTOR 3’UTR-WT but not in its mutant plasmid (Fig. 7B). Furthermore, modulation of miR-497-5p expression changed the protein level of mTOR, as detected by Western blotting (Fig. 7C). These data suggest that mTOR is a direct target of miRNA-497-5p.

Next, to test whether KRT17P3 expression regulates the miRNA-497-5p/mTOR axis, we performed Western blotting assays in KRT17P3 overexpression and knockdown cells. The results verify that
KRT17P3 induces expression of mTOR, whereas KRT17P3 shRNA represses mTOR expression (Fig. 7D). Furthermore, miRNA-497-5p inhibitor rescued the expression of mTOR in KRT17P3 knockdown NSCLC cells, while miRNA-497-5p mimic decreased mTOR expression in KRT17P3-overexpressing cells (Fig. 7E). Collectively, these data suggest that KRT17P3 enhances cisplatin resistance of NSCLC cells by regulating the miRNA-497-5p/mTOR axis.

**KRT17P3 expression is increased in the plasma of chemoresistant NSCLC patients**

To determine whether the expression of KRT17P3 in plasma is associated with cisplatin chemoresponse, we collected sera from 30 chemoresistant patients and 32 chemosensitive patients and measured the expression of KRT17P3 by RT-qPCR. As shown as Fig. 8, plasma KRT17P3 was significant up-regulated in the chemoresistant patient group, which is consistent with the results for 60 LUSC tissue samples in our previous study [7]. The association between chemoresponse and KRT17P3 expression was also confirmed by analysis of 62 samples ($P=0.031$, Table 2). These results suggest that increased expression of plasma KRT17P3 is associated with poor chemotherapy response and may be useful as a chemoresistance marker for NSCLC.
Table 2  
Association of relative expression of KRT17P3 with clinicopathological parameters.

| Feature                  | Cases (n) | KRT17P3 expression | P value |
|--------------------------|-----------|--------------------|---------|
|                          |           | Log2 (KRT17P3/β-actin) Mean±SD |         |
| Gender                   |           |                    | 0.029*  |
| Female                   | 14        | -12.501±1.117      |         |
| Male                     | 48        | -8.522±0.903       |         |
| Age (yr)                 |           |                    | 0.54    |
| <65                      | 30        | -9.912±1.026       |         |
| ≥65                      | 32        | -8.959±1.148       |         |
| Smoking                  |           |                    | 0.447   |
| No                       | 26        | -10.114±1.123      |         |
| Yes                      | 40        | -8.919±1.052       |         |
| Histological type        |           |                    | 0.151   |
| Adenocarcinoma           | 45        | -10.186±1.523      |         |
| Squamous cell carcinoma  | 21        | -7.813±1.523       |         |
| Chemotherapy sensitivity |           |                    | 0.031*  |
| Resistant                | 33        | -7.715±1.254       |         |
| Sensitive                | 33        | -11.019±0.840      |         |
| Tumor size(cm)           |           |                    | 0.309   |
| <4                       | 38        | -10.068±0.939      |         |
| ≥4                       | 28        | -8.461±1.306       |         |
| TNM stage                |           |                    | 0.600   |
| I+II                     | 3         | -11.655±3.965      |         |
| III+IV                   | 63        | -9.346±0.787       |         |

*For analysis of association of KRT17P3 levels and clinicopathological features, Student’s t-test was used. *P* < .05
Discussion

Non-coding RNAs, including IncRNAs, cir-RNAs, and pseudogenes, have been widely verified to participate in tumorigenesis, tumor progression, and chemoresistance [14]. Several lines of evidence have demonstrated roles for pseudogenes, providing new insights into cisplatin chemoresistance [4, 9, 10]. In a previous study, we determined that the pseudogene KRT17P3 is highly expressed in cisplatin-resistant lung cancer [10]; however, the effect of KRT17P3 on drug resistance in NSCLC cells and the precise molecular mechanism was not evaluated.

Initially, we explored the role and possible mechanism of KRT17P3 in cisplatin resistance of NSCLC in vitro and in vivo. In vitro results demonstrate that KRT17P3 overexpression significantly reduces cell apoptosis and enhances cell viability, whereas KRT17P3 knockdown increases cell apoptosis and decreases cell viability upon cisplatin treatment. In a xenografted mouse model, we observed that cisplatin, in combination with sh-KRT17P3, inhibits tumor growth, thus verifying that KRT17P3 promotes resistance of NSCLC cells to cisplatin both in vitro and in vivo.

Recent studies suggest that pseudogenes may exert functions via targeting of miRNAs [15]. To determine how KRT17P3 mediates cisplatin resistance in NSCLC cells, we used online algorithms to search for miRNAs with complementary base pairing to KRT17P3 and identified 9 miRNA families. Because of its function as a potential "suppressor-miR", we focused on miR-497-5p. miR-497-5p is downregulated in multiple tumors and is involved in malignancy-associated processes, including cancer initiation, progression, metastasis, and chemosensitivity [12, 16-19]. In this study, we found that miR-497-5p inhibition was sufficient to increase cisplatin sensitivity in NSCLC cells, while miR-497-5p mimic decreased cisplatin sensitivity. Moreover, miR-497-5p mimic reversed the cisplatin resistance induced by KRT17P3 overexpression. Conversely, miR-497-5p inhibitor attenuated the cisplatin sensitivity induced by KRT17P3 knockdown. These findings suggest that KRT17P3-dependent cisplatin resistance is partly mediated via miR-497-5p suppression.

We also explored the underlying mechanism by which KRT17P3 negatively regulates miR-497-5p. Luciferase assays validated the direct binding ability of the miR-497-5p binding site to KRT17P3, as predicted by bioinformatics analysis. In addition, by performing RIP assays, we determined that KRT17P3 and miR-497-5p are in the same RISC complex. These results suggest that KRT17P3 and miR-497-5p directly interact, and that KRT17P3 promotes cisplatin resistance through 'sponging' miR-497-5p. These data also imply that miR-497-5p may act as a potential target for therapy for patients who are resistant to cisplatin.

Notably, miR-497-5p has been shown to regulate cisplatin chemosensitivity in many types of cancer through multiple targets [12, 19]. In cervical cancer, miR-497-5p improves cisplatin chemosensitivity by regulating the expression of transketolase [19]; while in ovarian cancer, miR-497-5p increases cisplatin sensitivity by targeting the mTOR/P70S6K signaling pathway [12]. In the current study, we demonstrate that mTOR is a downstream target of miR-497-5p. Treatment with miR-497-5p mimic markedly reduced mTOR expression and improved cisplatin chemosensitivity. Furthermore, luciferase assays confirmed
bioinformatics predictions that the mTOR 3'-UTR is a putative target of miR-497-5p. In addition, Western blotting assays demonstrated that miR-497-5p expression suppresses protein levels of mTOR. Finally, KRT17P3 was found to modulate expression of mTOR, which was reversed by miRNA-497-5p mimic in NSCLC cells.

mTOR has been reported as a serine-threonine kinase that is frequently dysregulated in cancer. Overexpression of mTOR has been found in various types of cancer and correlates with tumor progression and poor prognosis. Accumulating evidence demonstrates the mTOR signaling pathway is a critical regulator of drug resistance, and a multitude of mTOR inhibitors are in development to overcome it [13, 20-22]. The mTOR inhibitor rapamycin can modulate P-gp1 or MDR1 mediated drug resistance [23, 24]. Furthermore, the mTOR inhibitor CCI-779 is able to restore cisplatin sensitivity in small cell lung cancer cell lines that are selected for cisplatin resistance, as well as cell lines derived from patients who failed cisplatin [20]. Our present study indicates that KRT17P3 repression is a potential method for restoring cisplatin sensitivity by inhibiting mTOR, though it is likely that other mechanisms contribute to KRT17P3-mediated chemoresistance. Moreover, the mechanism of KRT17P3 in mediating cisplatin chemoresistance in other types of cancers remains to be explored.

Furthermore, the expression of KRT17P3 in peripheral blood was significantly higher in the chemoresistant patient group compared to the chemosensitive patient group, which is consistent with the results for 60 LUSC tissue samples in our previous study [10]. The consistent result further enhanced the reliability in the plasma examination. Recent studies have demonstrated that IncRNAs, including pseudogene, were present in the blood of cancer patients and showed great potential as powerful and non-invasive tumor markers [25-27]. Our results indicate KRT17P3 maybe a potential non-invasive tumor markers for discriminating against cisplatin resistant patients and sensitive patients. However, the quantity of samples used in this study is still not enough. Therefore, the future research will expand the sample sizes to verify its value.

In conclusion, we show for the first time that KRT17P3 is involved in cisplatin resistance in NSCLC cells. Furthermore, miR-497-5p mediates the cisplatin resistance exerted by KRT17P3, which involves modulation of mTOR. Understanding the key role of ‘pseudogene-miRNA’ modules in NSCLC may lead to the identification of new therapeutic targets for reversing cisplatin resistance and therefore warrants further investigation.

**Abbreviations**

KRT17P3, Keratin 17 Pseudogene 3; NSCLC, non-small-cell lung cancer; LUAD, Lung Adenocarcinoma; LUSC, Lung squamous cell carcinoma; RT-qPCR, real-time polymerase chain reaction; PR, partial response; PD, progressive disease; TCGA: The Cancer Genome Atlas

**Declarations**

**Ethics approval and consent to participate**
Written informed consent for the biological studies was obtained from each patient involved in the study, and the study was approved by the Ethics Committee of Jinling Hospital, Nanjing University School of Medicine. All animal studies were approved by the Animal Experimental Committee of Nanjing Medical University.

**Consent for publication**

The content of this manuscript has not been previously published and is not under consideration for publication elsewhere.

**Availability of data and materials**

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

**Competing interests**

The authors declare no conflict of interest.

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**Authors’ contributions**

ZH and QZ: performed experiments, analyzed data and drafted the manuscript; NX and TL: collected clinical samples and clinical information; XY and YS: design the study and revised the manuscript. All authors read and approved the final version of the manuscript.

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**Supplementary Data**

Supplementary file 1: Figure S1: miRNAs that have complementary base pairing with KRT17P3 predicted by miRcode.

Supplementary file 2: Figure S2: miRNAs that have complementary base pairing with KRT17P3 displayed by the UCSC browser.
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Figures

| shRNA-Ctrl/ IC50 (μg/ml) | A549+Cisplatin | SK-MES-1+Cisplatin |
|--------------------------|----------------|-------------------|
| 3.194±0.336              | 9.936±0.232    |
| shRNA-KRT17P3/ IC50 (μg/ml) | 1.518±0.187 | 6.92±0.203 |

*P value* <0.05 <0.05

Figure 1

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