

Transmission of Exosomal TPX2 Promotes Metastasis and Resistance of NSCLC Cells to Docetaxel

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Background: Lung cancer, most of which is non-small cell lung cancer (NSCLC), is the most common tumor in the world, and drug resistance, as a major problem in clinical treatment, has attracted extensive attention. However, the role and mechanism of Targeting protein for Xenopus kinesin-like protein 2 (TPX2), which is highly expressed in NSCLC, is still unclear.

Methods: Bioinformatics analysis was used to analyze the relationship between TPX2 and the clinicopathological features of NSCLC. Stable TPX2 overexpression cell lines with were constructed by lentivirus infection, and the effect of TPX2 on proliferation, migration, invasion and chemoresistance to docetaxel was characterized by the CCK8, wound healing, transwell, colony formation assay and FACS. An in vivo lung homing mouse model was used to further confirmed the role of TPX2 on metastasis. Exosomes were extracted by differential centrifugation from the culture supernatant, and their functions were investigated by co-culture with tumor cells. Gene expression was detected via Western blot and real time PCR (RT-qPCR).

Results: Overexpression of TPX2 was related to the poor prognosis of NSCLC. Promoted migration, invasion and metastasis, and reduced the sensitivity of NSCLC cells to docetaxel. The abundance of TPX2 can be packaged in vesicles and transported to other cells. In addition, overexpression of TPX2 induced the accumulation of β -catenin and C-myc.

Conclusion: Our findings indicated that intercellular transfer of exosomal TPX2 triggered metastasis and resistance against to docetaxel in lung cancer cells, through activating downstream WNT/ β -catenin signaling pathway.

Keywords: metastasis, docetaxel, TPX2, exosome, NSCLC

Introduction

Lung cancer is the leading global cause of mortality worldwide,^{1,2} it is mainly divided into small cell lung cancer and Non-small cell lung cancer, which constitutes 85% of lung cancer, and the common treatments are surgical resection, chemotherapy and targeted therapy.^{2,3} Docetaxel, which has a unique mechanism of inhibiting mitosis, has been widely used as a chemotherapeutic drug to treat lung cancer.^{4,5} However, there are still a large number of treatment failure because of drug resistance.^{6,7} Therefore, understanding the molecular determinants of the regulation of chemotherapy resistance in NSCLC will provide the basis for molecular targeted therapy.

TPX2 is a microtubule-related protein that is a key aspect in mobile mitosis and spindle assembly.^{8–10} In many cancers, TPX2 has been identified as a diagnostic and prognostic marker.^{11–14} A large amount of evidence have shown that increased TPX2 can lead to spindle dysfunction, chromosome instability or DNA damage, resulting in a poor prognosis.^{8,9} It has been suggested that TPX2 could be used as a marker for poor prognosis in lung adenocarcinoma (LUAD) by data analysis.^{15–17} TPX2 overexpression also caused resistance to EGFR TKI and radiation in NSCLC.¹⁸ So, understanding the mechanism of TPX2 has become an important issue.

Exosomes are nanosized vesicles (between 30 and 150 nm) that can be ingested. They exist in all biological fluids, mediate cell-to-cell communication, and can also be used as intercellular carriers for various biomolecules, including proteins, mRNA, microRNA and DNA.^{19,20} Studies have shown that tumor cells release exosomes at a significantly higher rate, and exosomes can carry active proteins or RNA, transmit and activate downstream pathways between cells or affect the expression of target cell proteins and play a key function in events related to tumor growth and metastasis.^{21–23}

In this study, we determined the expression of TPX2 and its relationship with the TMN stage and survival of patients in NSCLC. We also investigated its effect on drug resistance, and interestingly, we found that exosomes play a transmission role during this process. Meanwhile, we explored the potential molecular mechanism. Our results may shed light on the role of TPX2 in the progression of NSCLC and help develop potential strategies for clinical treatment.

Materials and Methods

Cell Lines and Cell Culture

The lung cancer cell lines CALU-3, pc-9, H1650, H1299, H1975 and A549 were provided by the China Cell Resource Center. All cell lines were negative for mycoplasma. A549, H1650, PC-9 and H1299 cells were cultured under normal conditions (37 °C, 5% CO₂) with RPMI medium modified (cat. #SH30809.01, Cytiva) +1% (v/v) penicillin–streptomycin (cat. #C0222, Beyotime) and 10% (V/V) heat inactivated fetal bovine serum (Gibco). Calu-3 and H1975 cells were cultured under normal conditions in DMEM (cat. #SH30022.01, cytosol) +1% (v/v) penicillin–streptomycin and 10% (v/v) heat-inactivated fetal bovine serum supplemented with high glucose and glutamine.

Data Collection and Bioinformatics Analysis

The level 3 HTSeq FPKM format RNA-sequencing data of non-small cell lung cancer patients, including 108 normal and 1037 tumor samples, was downloaded from the TCGA data portal (<https://portal.gdc.cancer.gov/>), then was transformed into TPM and log₂-transformed for subsequent analyses. The non-small cell lung cancer dataset from TCGA was used by combining TCGA LUAD and TCGA LUSC datasets. The clinical data of these patients were also obtained from the TCGA. The receiver operating curve (ROC) was used to determine the diagnostic accuracy of TPX2 expression in differentiating normal tissues from NSCLC tissues. The ROC curve was created, and the area under the curve (AUC) value was calculated using the R package “pROC” (v.1.17.0.1). An AUC value greater than 0.9 indicates excellent predictive power. The RNA sequencing data (TPM values) in TCGA-TARGET-GTEX cohort were obtained from UCSC XENA (<https://xenabrowser.net/datapages/>) and used to run pan-cancer analysis of TPX2 expression between normal and tumor tissues.

Gene Set Enrichment Analysis (GSEA) was performed to elucidate differential pathways between high- and low-expression groups according to the median TPX2 expression level. Firstly, differential gene expression analysis between these two groups was performed using DESeq2 (v.1.18.1) to generate a gene list. Secondly, the sorted (by log fold change of expression level) gene list was applied for GSEA by “clusterProfiler” (v.3.14.3) package using the gene-set h.all.v7.2.symbols.gmt. The analysis was based on 1000-times permutations. The pathways were considered to be significantly enriched when adjusted p-value < 0.05 and FDR q-value < 0.25.

All analytics and plots in the bioinformatic analysis were generated using R (v.3.6.3).
2.3 Gene Overexpression Experiment

The lentivirus packaged with TPX2 expression vector or negative control vector (vector as NC) was purchased from Hanheng organism. To infect the target cell line, the cell culture medium diluted to 0.5 mL was spread in a 24-well plate, and the culture medium was removed after adhering to the wall. Then, 0.25 mL of medium diluted with 10 µL lentivirus and polybrene (the final concentration was 8 µg/mL) was replaced for 4 hours and then added to 0.5 mL. Once the cells reached 70% confluence, the selection began: the cells were added to culture medium containing 0.5 µg/mL puromycin (Gibco) and replaced every 2 to 3 days. Once the cells recovered and expanded, the expression of TPX2 was verified by WB and RT-qPCR.

Western Blot

NSCLC cell lines' proteins were extracted with lysis buffer (cat.# P0015A, Beyotime). The protein was separated by SDS-PAGE and transferred to PVDF membranes (0.22 µm microwell). The membrane was blocked in 5% skim milk for 1 hour, incubated overnight with specific antibodies at 4 °C, and then incubated with a horseradish peroxidase-conjugated secondary antibody (Proteintech). The signal band was detected with an ECL kit (Thermo). All the antibodies used in this article are shown in Table 1. The pixel density of the protein bands was analyzed by ImageJ software. The ratio of protein strength to GAPDH protein strength or whole protein strength was calculated and expressed as a percentage.

RT-qPCR

Total RNA was extracted by TRIzol reagent (cat.# R401-01, Vazyme). A HiScrip[®] II First Strand gene synthesis kit (cat.# R211-01, Vazyme) was used to synthesize cDNA. In line with the manufacturer's instructions, SYBR Green (cat.# C0008, TargetMol) was used to quantify RT-qPCR gene expression. GAPDH was used as the endogenous control. Table 2 lists the primer pairs for each target gene. Finally, the relative expression was calculated using $2^{-\Delta\Delta CT}$.²⁴

Wound Healing Assay

The cells (1.5×10^5) used for wound healing analysis were inoculated in a 24-well plate. When the cell confluence reached approximately 80%, the cell layer on each culture plate was scratched with the tip of a 200 µL pipette to form scratches. After injury, the cells were washed with PBS, and the debris was removed. The wound culture was cultured in medium without FBS. An Olympus IX73 inverted microscope was used to image wound healing every 24 hours for 48 hours. Wound healing ability was determined by measuring the average migration distance using Prism 8 statistics. The experiment was carried out three times.

Transwell Migration and Invasion Assays

Cell migration analysis was carried out in a 24-well tissue culture plate using a transwell (cat. #353097, BD Falcon, USA) membrane filter plug-in. The NSCLC cells to be detected were trypsinized and suspended in serum-free medium. Then, the cells were inoculated in the upper transwell chamber, and medium containing 10% FBS was added to the lower chamber. After incubating at 37 °C for 24 hours, the cells were stained with crystal violet. The unigrated cells within

Table 1 Antibodies Used in This Study

Antibody	Supplier	Working Dilutions
Anti-GAPDH	Proteintech (cat.# 60004-I-Ig)	1:5000
Anti-TPX2	Proteintech (cat.# 11741-I-AP)	1:2000
Anti-C-myc	Cell Signaling (cat.# 18583)	1:1000
Anti-β-catenin	Abmart (cat.# M24002)	1:1000
Anti-CD63	Cell Signaling (cat.# 52090)	1:1000
Anti-tsg101	Cell Signaling (cat.# 72312)	1:1000
IgGs, rabbit and mouse	Proteintech	1:5000

Table 2 Primer Sequences Used for RT-qPCR Experiments from Generalbiol

Gene	Forward Primer 5'-3'	Reverse Primer 3'-5'
GAPDH	GGAGCGAGATCCCTCCAAAT	GGCTGTTGTCATACTTCTCATGG
TPX2	ATGGAAGTGGAGGGCTTTTC	TGTTGTCAACTGGTTTCAAAGGT
WNT6	GGCAGCCCCTTGTTATGG	CTCAGCCTGGCACAACCTCG
WNT5B	GCTTCTGACAGACGCCAACT	CACCGATGATAAACATCTCGGG
C-myc	GGCTCCTGGCAAAGGTCA	CTGCGTAGTTGTGCTGATGT

the upper cavity were eliminated, and the migrated cells were imaged via an Olympus IX73 microscope. ImageJ software was used to analyze and count and Prism 8 statistical data. For intrusive analysis, BioCoatMatrigel (cat.#356234, CorningBioCoat) cross-hole inserts are used, and the process is similar to the migration evaluation and conforms to the manufacturer's instructions.

In vivo Metastasis Model

Female BALB/c nude mice (4-week-old) was purchased from Hangzhou Ziyuan Laboratory Animal Technology Co., Ltd. To construct an in vivo lung transfer model, each nude mouse is injected with 100 μ L vector or TPX2 overexpressed A549 cells (1×10^6 cells per mouse, $n=5$ per group) respectively through the tail vein. Four weeks after injection, mice are killed and metastatic lung tumors are analyzed. All animal research procedures are carried out with the approval of the Ethics Committee of the Animal Laboratory Center of the First Affiliated Hospital of University of Science and Technology of China.

Hematoxylin and Eosin (H&E) Staining

First, put the tissue sections into a drying oven and bake the pieces at 66 °C for 20–30min. It is then treated three times sequentially with xylene and ethanol. Slowly rinse and slice under running water until it is clean and transparent, then soak with hematoxylin. Bluing of saturated lithium carbonate solution and redness of eosin dyeing solution. Dehydrated with 95% ethanol several times, and then transparent sections with phenol-xylene, xylene I and II. Finally, a neutral gum seal is used and the results are observed under a microscope.

2.10 Proliferation and Docetaxel Sensitivity Assays. The CCK-8 assay (cat. #C0005, TargetMol, Shanghai, China) and the colony formation assay were both used to monitor cell viability and sensitivity recovery. To quantify the cell growth rate, 2000 cells were inoculated in 96-well plates. At 24, 48, 72 and 96 hours after cell attachment, CCK-8 solution was added to the cells and then cultured for 2 hours. The absorbance value (OD) at 450 nm was measured using a universal microplate spectrophotometer. For the drug sensitivity test, docetaxel sensitivity was evaluated by docetaxel treatment in 96-well plates for 72 hours before adding CCK-8 solution.

For the colony formation test, the cells of different treatments were resuspended and inoculated into each hole of the six-well plate with the same number of cells. The cells were treated with docetaxel for 72 hours and then cultured in new medium without docetaxel. After being cultured for the same time, the clones were fixed with methanol and then stained with crystal violet.

Apoptosis Analysis

To study apoptosis, an Annexin V-Alexa Fluor660/PI (cat.# BB-41037-50T MagneBestbio) kit was used. After washing the tumor cells with PBS twice, 5×10^5 cells were suspended in 100 μ L $1 \times$ binding buffer and incubated in the dark with 5 μ L Annexin V-AF660 for 10 min, and then 10 μ L PI was added for 3 min. Then, 400 μ L PBS was added prior to detection and immediately measured by flow cytometry (LSRFortessa, BD Biosciences, USA), and 10,000 signals were collected from each sample. FlowJo software was used for further analysis.

Isolation of Exosomes by Differential Centrifugation

The cells were cultured in conventional medium until 80% confluence, and then the medium was replaced with RPMI-1640 without FBS. The cell supernatant was collected directly after 48 h. The samples were centrifuged at $3000 \times g$ for 30 min at 4 °C to remove cells and cell debris. The supernatant was transferred into ultracentrifuge tubes and centrifuged at $16,000 \times g$ for 1 h in an L-100xp ultracentrifuge (Beckman, USA) equipped with a Type 70 rotor. The supernatant was centrifuged again at a rate of $100,000 \times g$ for 1 h. The supernatant was discarded, resuspended in PBS, and filtered into a 1.5 mL ultra-detachment tube with a 0.2 μ m membrane. Then, the samples were centrifuged at $100,000 \times g$ for 1 h in an Optima MAX-XP ultracentrifuge (Beckman, USA) equipped with a TLA55 rotor, the supernatant was discarded, and exosome samples were obtained by resuspension with 20 μ L PBS.²⁵ Exosomes samples (20 μ L) were obtained from the initial 20 mL supernatant, and the exosome protein concentration was determined with a two-inch nickel acid (BCA) detection kit (Beyotime). Exosome preparations can be stored at -80 °C for 30 days. The morphological characteristics of

exosomes were observed by transmission electron microscopy (Tecnai G2 F20). The size distribution of the Exosomes body was measured with a nanoparticle tracking analyzer (PMX220, Particle Metrix, Germany). The expression levels of tsg101 and CD63, representative exosome-associated positive markers, were detected by Western blotting.

Statistical Analysis

Student's *t*-test and two-tailed χ^2 test methods were used to determine statistical significance. Data with $p < 0.05$ were considered statistically significant.

Results

TPX2 is Upregulated in NSCLC and is Associated with Poor Prognosis

The pan-cancer analysis showed that compared with the normal tissues, the expression level of TPX2 was significantly higher in most types of cancers, including NSCLC (Figure 1A and C). The results were further validated in paired cancer- corresponding normal tissues (Figure 1B and D). In addition, the ROC curve indicated that TPX2 expression had a good predictive power with an area under the curve (AUC) of 0.981 (95% confidence interval [CI] = 0.973–0.988) to discriminate NSCLC tissues from normal tissues (Figure 1E). Meanwhile, we found that high expression of TPX2 was significantly associated with advanced pathologic stage, T stage, N stage, smoking, shorter overall survival (OS), and disease-specific survival (DSS) events (Figure 1F–K). These results indicated that TPX2 acting as an oncogene, might be participated in the occurrence and development of NSCLC.

TPX2 Promotes the Invasion and Migration Ability of NSCLC Cells

To investigate potential function of TPX2 in the progression of NSCLC, firstly the expression of TPX2 in NSCLC cell lines was determined by Western blot. A549 and H1299, two TPX2 lowly expressing cell lines were selected for further experiments (Figure 2A). Then TPX2 was overexpressed in H1299 and A549 by lentivirus transfection, RT-qPCR and Western blotting analysis showed that the mRNA and protein expression level of TPX2 was significantly increased (Figure 2B and C). Subsequently, we carried out wound healing experiments and transwell assays to determine the effect of TPX2 upregulation on cell invasion and migration. The results showed that compared with the control group, the migration and invasion abilities of A549 OE-TPX2 cells were significantly enhanced (Figure 2D–F), and similar results was confirmed in H1299 cells (Figure 2G–I), which was consistent with previously report.²⁶ To further examine the role of TPX2 in metastasis in vivo, we assessed the metastatic nodules in the lungs in nude mice, which were injected with A549 cells into tail vein. Ectopic overexpression of TXP2 markedly increased the lung homing potential of A549 cells (Figure 2J and K). However, overexpression of TPX2 did not affect the proliferation of both two NSCLC cell lines (Figure 2L and M). All these results demonstrated that upregulation of TXP2 could promote migration, invasion and metastasis in NSCLC.

TPX2 Promotes the Resistance of NSCLC Cells to Docetaxel

Docetaxel is a microtubule depolymerization inhibitor which arrests the cell cycle at G2/M and leads to cell apoptosis, thus killing cancer cells. It has been widely used a second-line chemotherapeutic for treatment of patients with locally advanced and metastatic non-small cell lung cancer.⁴ TPX2, a microtubule-associated protein that mediates spindle assembly during mitosis, is speculated to affect the response of cells to docetaxel. To test this hypothesis, the sensitivity to docetaxel of NSCLC cells after TXP2 overexpression was evaluated by the CCK-8 assay. The results showed that the IC50 value of TPX2-overexpressing cells was significantly higher than that of negative control cells (Figure 3A and B), suggesting that TPX2 desensitized NSCLC cells to the cell death triggered by docetaxel. Besides, colony formation experiments showed that the colony formation ratio was significantly higher in TPX2 overexpressing A549 and H1299 cells after docetaxel treatment than in control cells (Figure 3C and D). Both suggested the promoting role of TPX2 in NSCLC cells against to docetaxel.

In addition, to examine the effect of TXP2 on apoptosis, cells were treated with docetaxel or not for 72 h, then detected by flow cytometry. We found that TPX2 did not inhibit the apoptosis individually but only in combination with

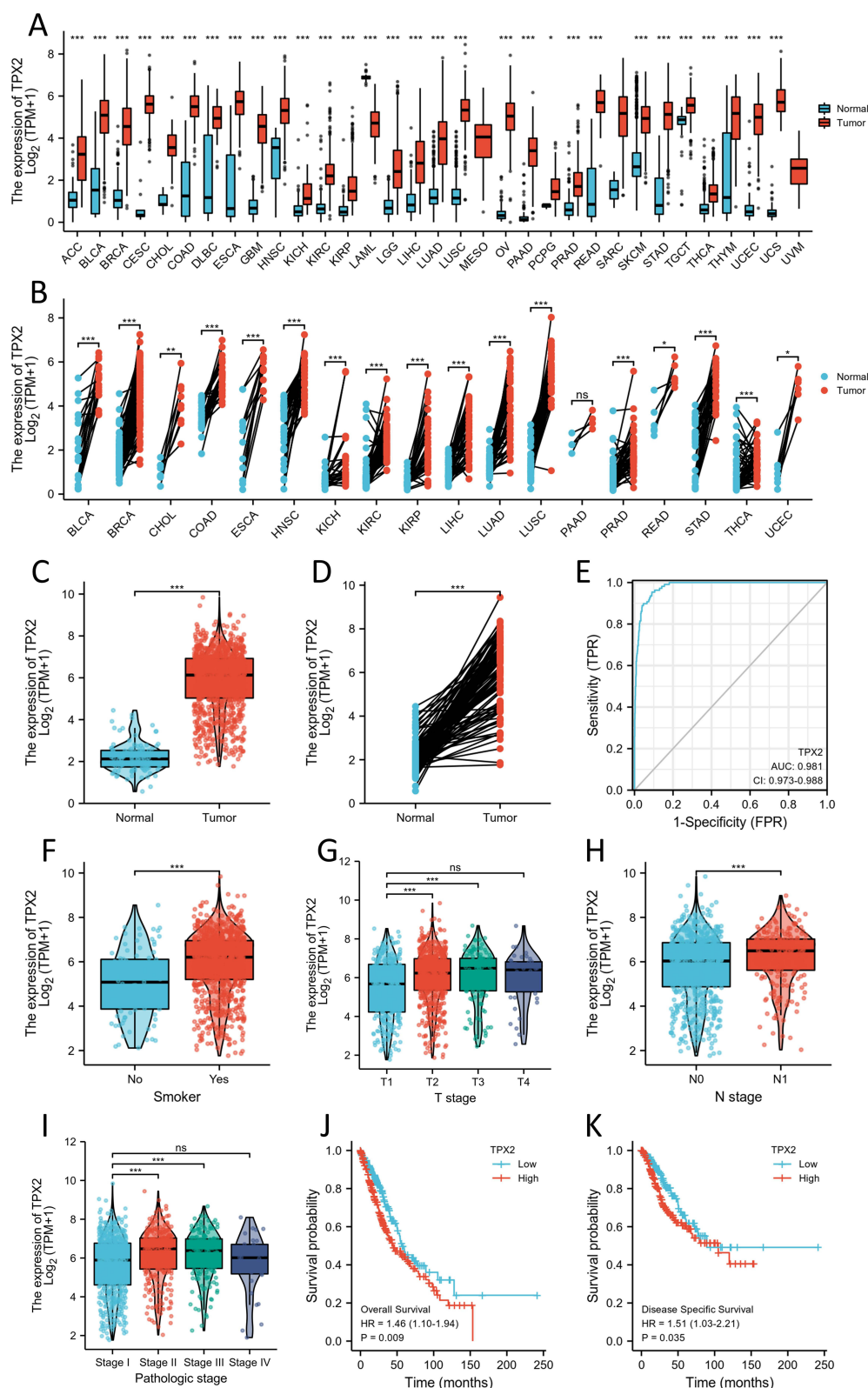


Figure 1 TPX2 expression level in NSCLC and its relationship with clinicopathological features. **(A)** Expression of TPX2 in different tumor types compared to nonmatching normal tissues in TCGA and GTEx databases. **(B)** TPX2 expression in different tumor types compared to matched normal tissues in TCGA and GTEx databases. **(C)** TPX2 expression in NSCLC and nonmatching normal tissues in TCGA databases. **(D)** TPX2 expression in NSCLC in TCGA database with matching normal tissues. **(E)** ROC curves for classifying NSCLC versus normal breast tissues in the TCGA database. **(F–K)** Data are shown for **(F)** smoker; **(G)** T stage; **(H)** N stage; **(I)** Pathologic stage; **(J)** Overall Survival; and **(K)** Disease Specific Survival. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Abbreviations: TCGA, The Cancer Genome Atlas; GTEx, Genotype Tissue Expression Project; ROC, receiver operating characteristic; OS, overall survival; DSS, disease-specific survival.

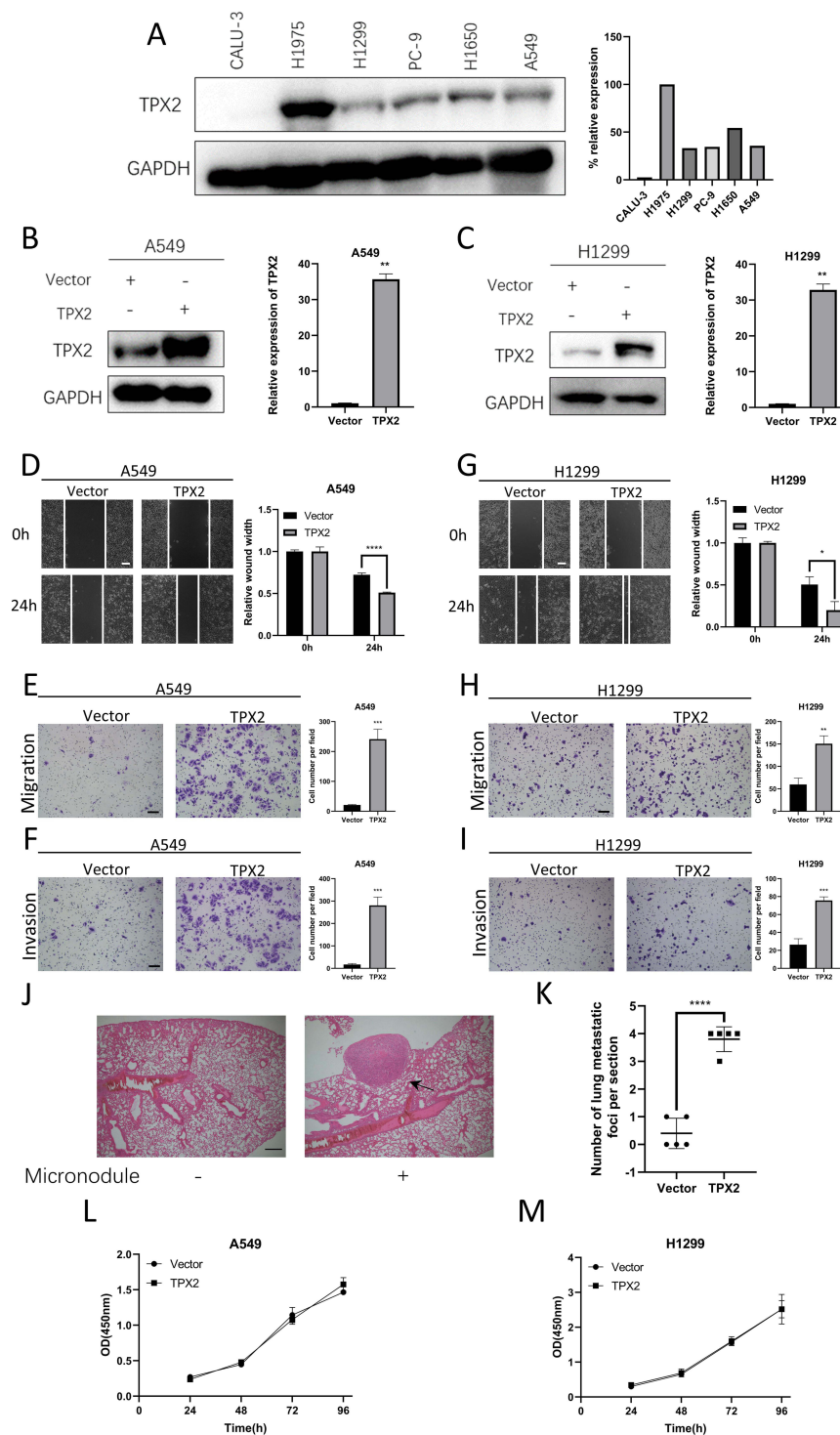


Figure 2 TPX2 overexpression promoted the migration and invasion abilities of NSCLC cells. **(A)** Western blotting analysis of TPX2 expression in six NSCLC cell lines. **(B and C)** The overexpression efficiency of TPX2 in A549 **(B)** and H1299 **(C)** was identified at the protein and RNA levels using Western blotting (left) and RT-qPCR (right). **(D–F)** Effects of TPX2 overexpression on wound healing **(D)**, transwell migration **(E)** and invasion **(F)** abilities of A549 cells. Scale bar: 100 μ m. **(G–I)** Effects of TPX2 overexpression on wound healing **(G)**, transwell migration **(H)** and invasion **(I)** abilities of H1299 cells. Scale bar: 100 μ m. **(J)** H&E staining showing representative photos of positive and negative metastatic nodules in the lung. Scale bar: 200 μ m. **(K)** The scatterplot shows the number of lung metastatic foci per mouse. **(L and M)** Growth curve of A549 **(L)** and H1299 **(M)** cell lines (Vector represents negative control of overexpression, TPX2 represents overexpression of the TPX2 gene). Each bar displays the mean \pm SD of 3 independent experiments as analyzed by paired two-tailed students, t-test. * p <0.05, ** p <0.01, *** p <0.001 and **** p <0.0001.

docetaxel treatment (Figure 3E and F). In summary, these results strongly pointed out that TPX2 triggered drug resistance in NSCLC cells.

TPX2 Can Affect the Expression of TPX2 in Surrounding Cells Through Exosomes

At present, there is a great deal of evidence showed that DNA, mRNA and protein can be transmitted between cells through exosomes, thus affecting the function of tumor cells. It is also commonly used in the diagnosis and treatment of NSCLC.^{27,28} The exosomes in the conditional medium were isolated by differential centrifugation and characterized by transmission electron microscopy (TEM) (Figure 4A and B), nanotracker (NTA) (Figure 4C and D) and Western blot analysis (positive marker: CD63, TSG101) (Figure 4E). At the same time, the RT-qPCR results showed that the level of TPX2 mRNA in exosomes from cells overexpressing TPX2 was significantly higher than that in exosomes from control cells (Figure 4F). This indicates that abundant TPX2 mRNA can be packaged into vesicles and transported outside of the cells.

Next, we studied whether TPX2 in the exosomes can be untaken by the surrounding cells. A549 cells were co-incubated with exosomes derived from different sources, 24 hours later, cells were collected for TPX2 expression examination by Western blot. We found that TPX2 protein level was significantly increased in A549 cells treated with TPX2-overexpressing exosomes than control exosomes (Figure 4G). Consistent results were obtained in H1299 cells (Figure 4H). Taken together, the abundant TPX2 in NSCLC cells could be transferred intracellular via exosomes.

TPX2 Derived from Exosomes Can Affect the Motility and Drug Sensitivity of NSCLC Cells

Next, we wondered whether TPX2 derived from exosomes could induced similar phenotypes as overexpressed by lentivirus transfection. Exosomes isolated from A549-NC and A549 TPX2-overexpression cells were incubate with A549 and H1299 cells. Then the migration, invasion, drug sensitivity to docetaxel and apoptosis of NSCLC cells were

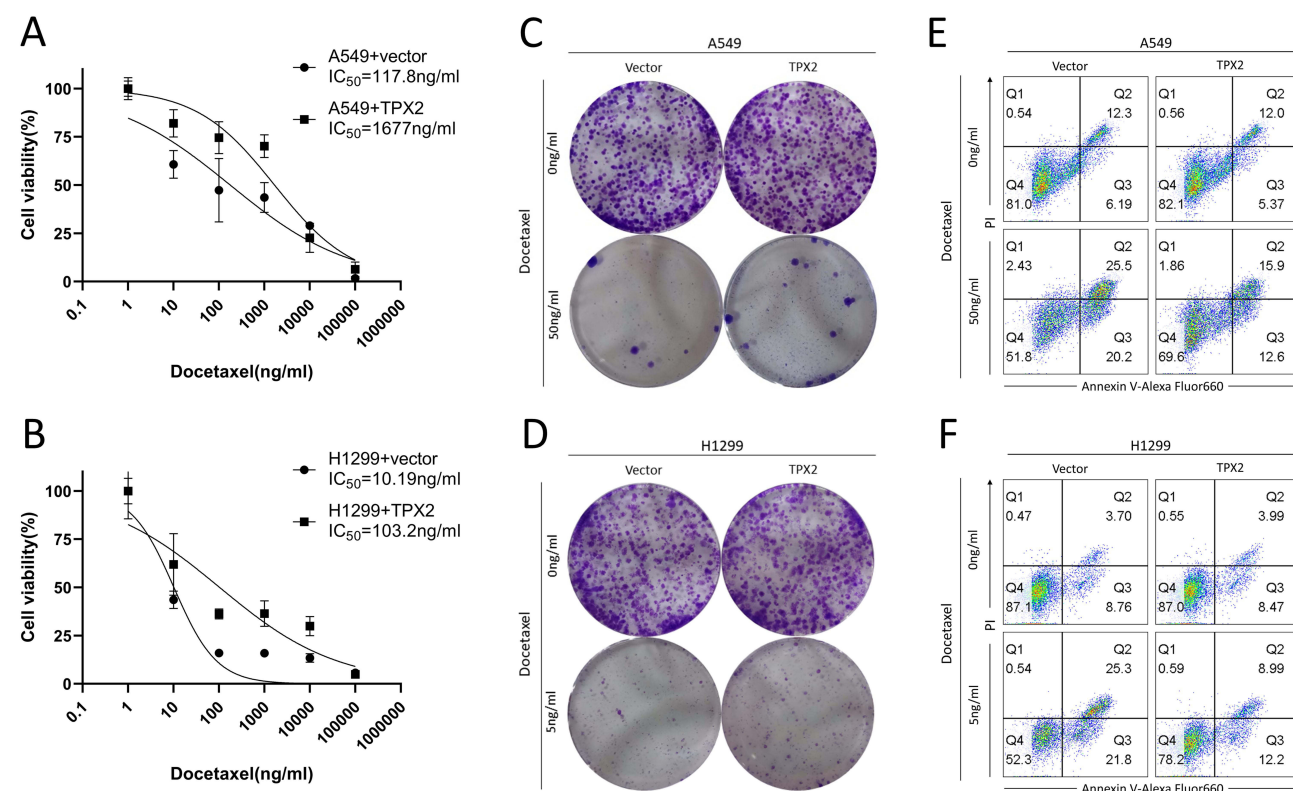


Figure 3 TPX2 overexpression reduced sensitivity to docetaxel. (A and B) Dose–response docetaxel viability curves in A549 (A) and H1299 (B) cell lines. Cell viability was estimated by CCK-8 reagent after 72 h of drug exposure. (C and D) A colony formation assay was used to analyze the clone formation ability of A549 (C) and H1299 (D) cell lines treated with 50 ng/mL docetaxel or not. (E and F) Flow cytometry was used to detect apoptosis in A549 (E) and H1299 (F) cell lines after treated with docetaxel for 72 h.

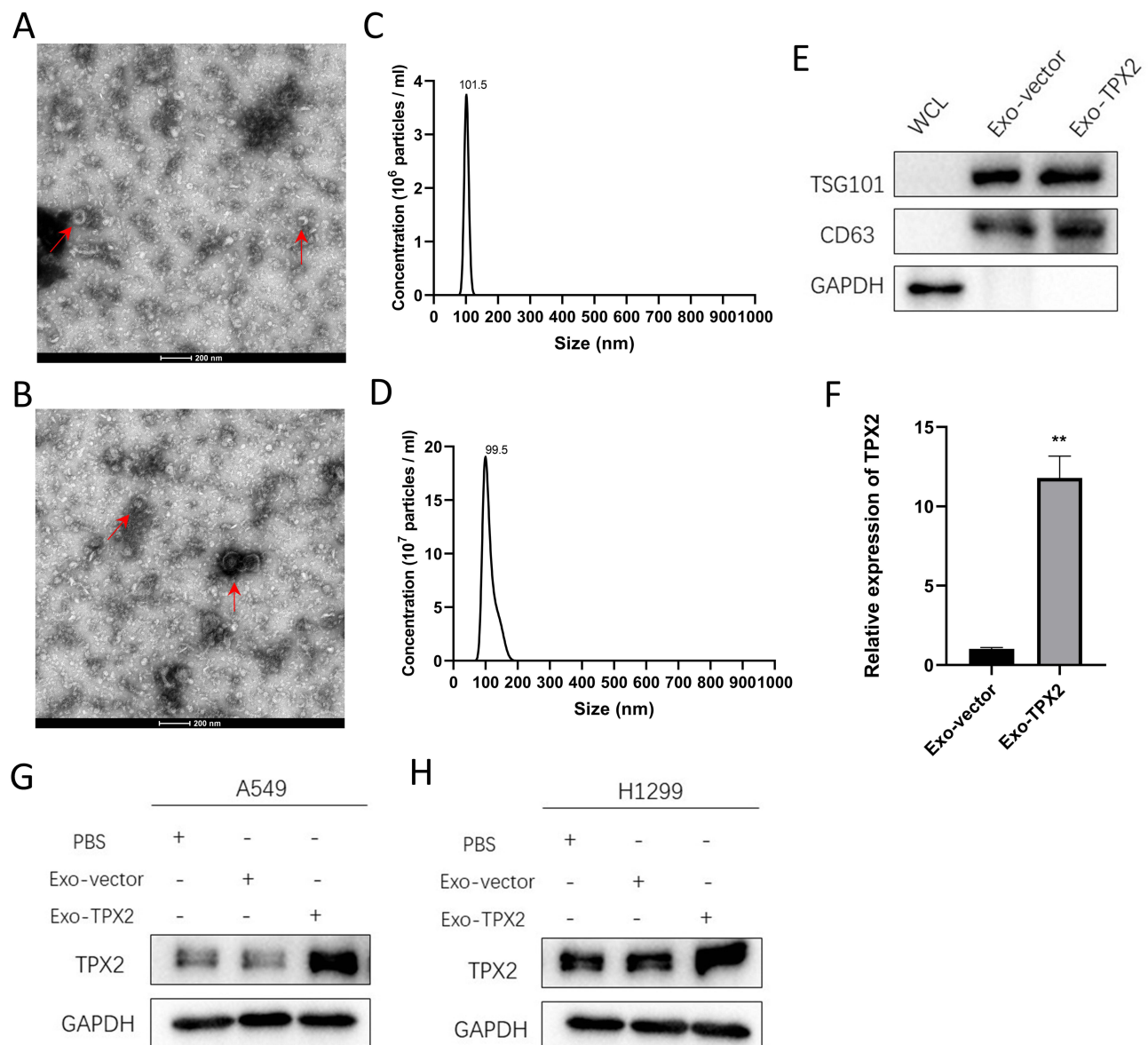


Figure 4 TPX2 can be transferred intercellularly through exosomes. **(A and B)** Transmission electron microscopy (TEM) images of exosomes isolated from A549 cells (Vector: A, TPX2: **(B)**). Red arrows indicate exosomes. Scale bar: 200 μ m. **(C and D)** The size distribution of exosomes determined from A549 cells (Vector: A, TPX2: **(B)**) by nanoparticle tracking analysis (NTA). **(E)** Western blot analysis of exosome markers (tsg101 and CD63) in equivalent amounts of protein from A549-derived exosomes and A549 whole cell lysates (WCL) (as a control). **(F)** The amount of TPX2 mRNA in exosomes isolated from cell supernatant was detected by RT-qPCR. **(G and H)** Western blot analysis of TPX2 expression in A549 **(G)** and H1299 **(H)** cells after incubation with exosomes. Data were statistically analyzed with paired two-tailed students, *t*-test. ***p*<0.01.

determined by wound healing (Figure 5A and B), transwell (Figure 5C–F), CCK-8 assay (Figure 6A and B), colony formation experiments (Figure 6C and D) and flow cytometry (Figure 6E and F). Results showed that exosomal TPX2 could promote the migration, invasion and drug resistance to docetaxel, but inhibited apoptosis in recipient A549 and H1299 cells. Altogether, TPX2 in the NSCLC cells could be secreted into exosomes and ingested by the surrounding cells, and induced higher migratory and invasive capacity, triggered docetaxel resistance, and suppressed apoptosis in NSCLC cells.

TPX2 Functions Through WNT/ β -Catenin/C-Myc Signaling Pathway

We next addressed the molecular mechanism through which TPX2 regulates lung cancer progression. GSEA enrichment analysis of lung cancer data in TCGA showed WNT, MYC, unfolded protein response and spermatogenesis were

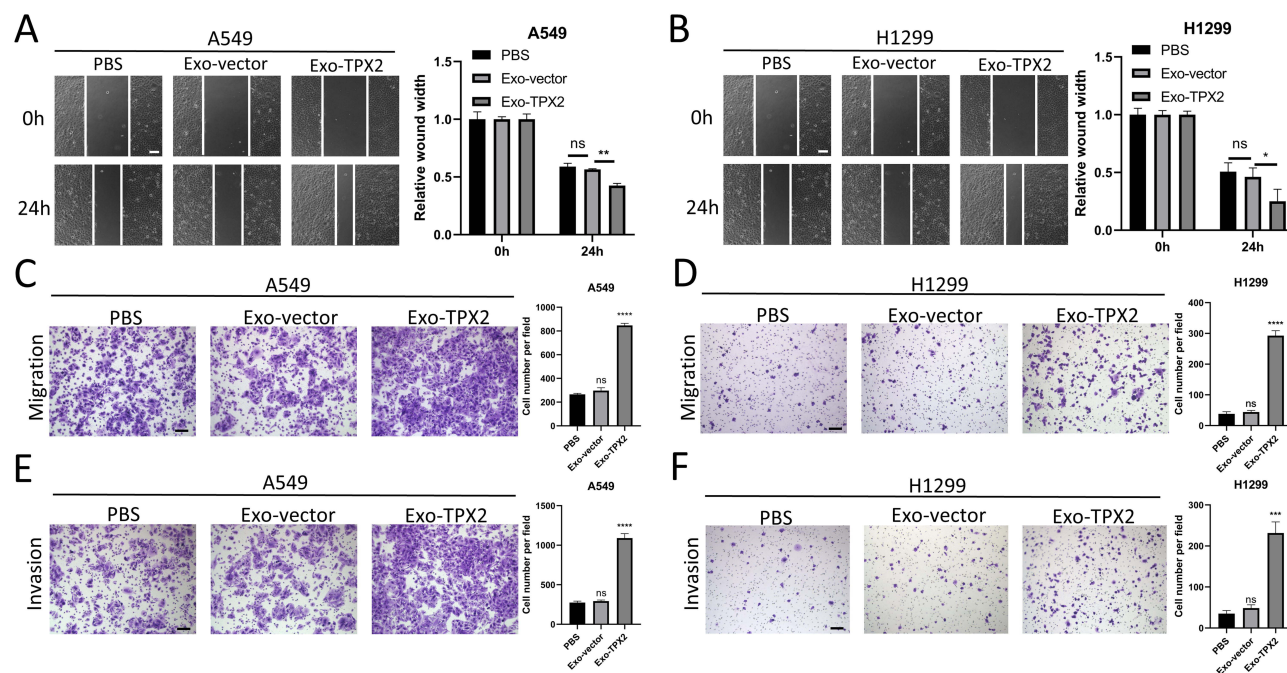


Figure 5 Exosomes from cells with high TPX2 expression promote cell invasion and migration. (A and B) The migration of A549 (A) and H1299 (B) cells after treatment with exosomes from different sources were determined by wound healing. Scale bar: 100 μ m. (C and D) The migration capacity of A549 (C) and H1299 (D) cells after exosomes treatment is determined by transwell assay. Scale bar: 100 μ m. (E and F) The invasive capacity of A549 (E) and H1299 (F) cells after treatment with exosomes is determined by transwell assay. Scale bar: 100 μ m. Each bar shows the mean \pm SD of 3 independent experiments. Data were statistically analyzed with paired two-tailed students, t-test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ and **** $p < 0.0001$.

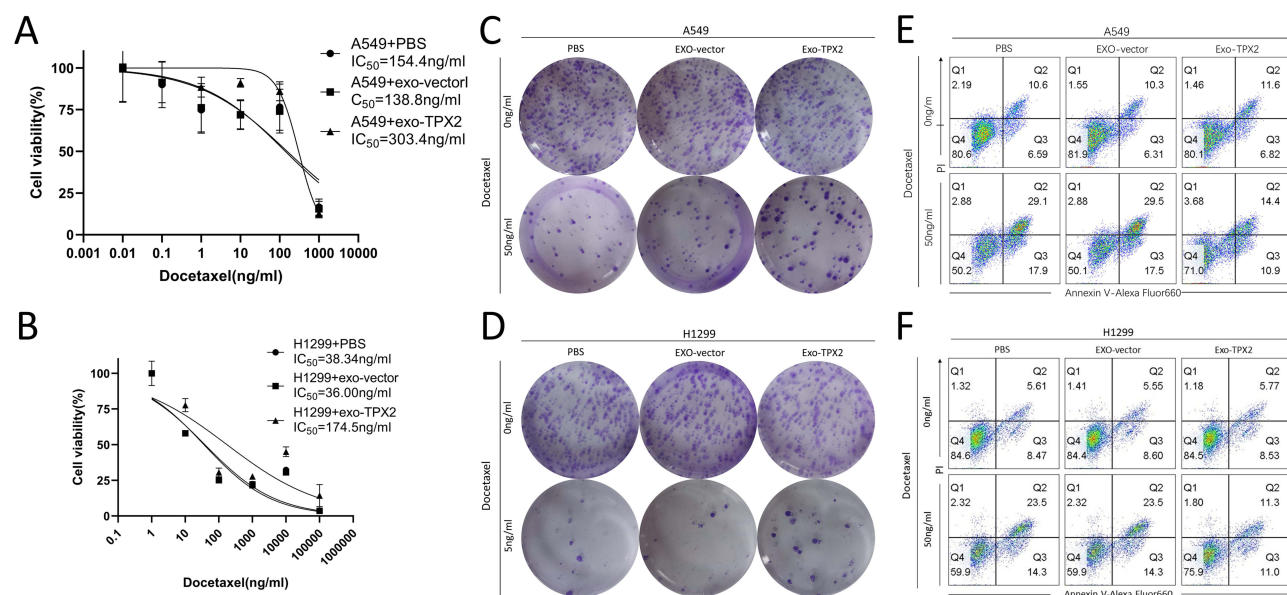


Figure 6 Exosomes from TPX2 high-expressing cells reduce the sensitivity of NSCLC cells to docetaxel. (A and B) Dose-responsive docetaxel activity curves in A549 (A) and H1299 (B) cells treated with exosomes. Cell viability was estimated with CCK-8 reagent after 72 h of drug exposure. (C and D) Effect of exosomes on the clonogenic capacity of A549 (C) and H1299 (D) cells treated with docetaxel or not. (E and F) Flow cytometry was used to detect apoptosis in A549 (E) and H1299 (F) cells treated with docetaxel or not after exosome co-incubation.

positively correlated with TPX2, while coagulation was negatively correlated with TPX2 (Figure 7A and B). Studies have shown that TPX2 and MYC work together in tumors, and c-myc is one of the downstream of WNT pathway.²⁹ Then, we analyzed the relationship between WNT/ β -catenin pathway-related genes and TPX2 expression, and the results

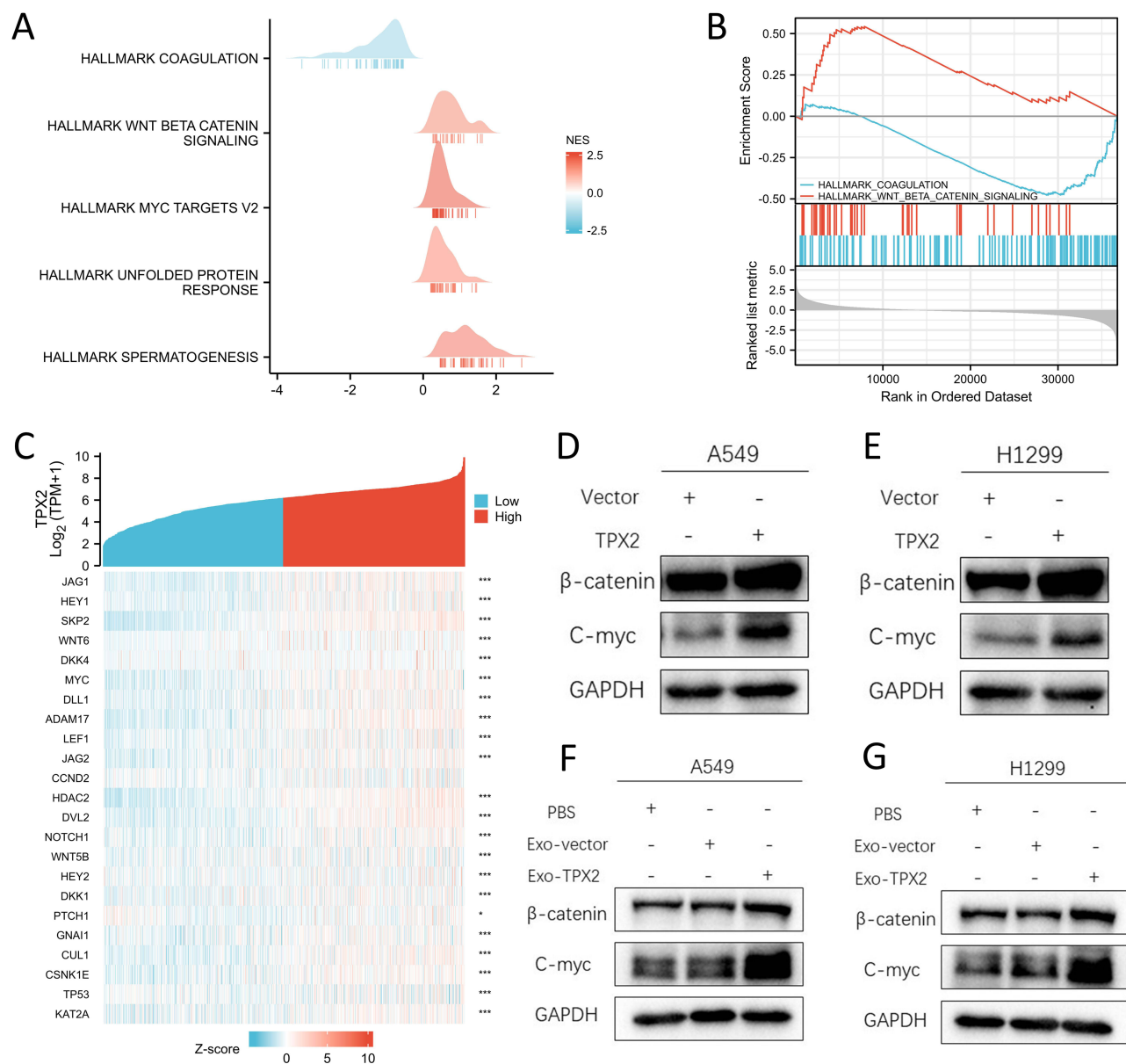


Figure 7 TPX2 activates the downstream WNT/β-catenin signaling pathway. **(A and B)** GSEA of the hallmark gene sets deposited in MSigDB. **(C)** Heatmap of the correlation between TPX2 expression and WNT pathway-associated proteins. **(D and E)** The amount of β-catenin and C-myc proteins in A549 **(D)** and H1299 **(E)** cells with different levels of TPX2 expression was compared by Western blot. **(F and G)** The amount of β-catenin and C-myc proteins in A549 **(F)** and H1299 **(G)** cells treated with different exosomes was compared by Western blot.

showed that TPX2 activated the WNT pathway (Figure 7C). To verify this conjecture, we tested it at the protein level. The Western blot results showed that the levels of β-catenin and C-myc were also higher in the cell lines overexpressing TPX2 (Figure 7D and E), or treated with exosomal TPX2 (Figure 7F and G). These results suggest that TPX2 may play a role through the WNT/β-catenin/C-myc axis.

Discussion

TPX2 is a microtubule-associated protein whose expression is precisely managed in the cell; it appears in the G1/S phase, moves to the mitotic spindle poles during mitosis and disappears after the completion of cytokinesis.^{9,30} A large number of studies have shown that the overexpression of TPX2 is related to the stage, grade and poor prognosis of some malignant tumors,^{31–33} and it has been identified as a driving oncogene. Previous study also pointed out TPX2 could be

used as a poor prognostic marker for NSCLC.^{16,34,35} To verify this, TCGA database analysis showed that the advanced clinical stage and poor prognosis of NSCLC were associated with high TPX2 expression.

Many studies have elucidated the relationship between TPX2 and proliferation, metastasis, and apoptosis in liver cancer, stomach cancer and other cancers,^{13,36,37} including NSCLC.²⁶ In this study, both in vitro and in vivo experiments confirmed that high TPX2 expression can promote NSCLC metastasis. TPX2 has been reported to play an important role as a microtubule-associated protein in the mitosis process and generally promotes cell proliferation.^{13,38,39} A previous bioinformatics analysis predicted that TPX2 is related to the proliferation of LUAD.⁴⁰ However, here we found that although high TPX2 positivity correlated with T stage of NSCLC, which represented tumor size, but it did not affect NSCLC cells proliferation in vitro. This contradiction was probably due to that TPX2 overexpression was the concomitant phenomenon during NSCLC rapid proliferation but not the cause. Or TPX2 promoted larger tumor size not through fast proliferation but other reasons, eg tumor immunity. Indeed, the overexpression of TPX2 was discovered to be associated with reduced immune infiltration in LUAD.¹⁵ Docetaxel is a commonly used chemotherapeutics for NSCLC, which promotes microtubule polymerization, resulting in cell-cycle arrest and apoptosis. We speculated that the microtubule-associated protein, TPX2, could affect the response to docetaxel. Here we demonstrated that overexpression of TPX2 induced resistance against docetaxel in NSCLC cells. Interestingly, we also found that TPX2 could be secreted outside of the cells via exosome which further affect the expression of TPX2 in the surrounding cells and then change their invasion and migration ability and sensitivity to docetaxel. This is a completely new discovery. Bioinformatics data analysis showed that TPX2 may affect the expression of the proto-oncogene MYC through the WNT/ β -catenin signaling pathway, and we further verified this conjecture by protein detection.

Conclusions

This study demonstrates that TPX2 promotes the metastasis of NSCLC cells and induces docetaxel resistance. TPX2 may play a role in NSCLC by further promoting the transcription of C-myc through the accumulation of β -catenin, and it can be intercellularly transferred by exosomal TPX2. In summary, TPX2 may be a marker of NSCLC metastasis and is a key gene involved in the induction of docetaxel resistance, and it may become an important molecular target for the clinical treatment of NSCLC.

Data Sharing Statement

The analyzed datasets for this study can be found in the TCGA (<https://portal.gdc.cancer.gov>).

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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