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ORIGINAL RESEARCH

Integrative gene expression profiling reveals that dysregulated triple microRNAs confer paclitaxel resistance in non-small cell lung cancer via co-targeting MAPT

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Background: Paclitaxel has shown significant anti-tumor activity against non-small cell lung cancer (NSCLC); however, resistance to paclitaxel frequently occurs and represents a significant clinical problem and its underlying molecular mechanism remains elusive.

Methods: Long-term treatment of culture cell with paclitaxel was carried out to mimic the development of acquired drug resistance in NSCLC. Cell proliferation and clonogenic assay and apoptosis evaluation were carried out to determine the efficacy of paclitaxel on NSCLC cells. Western blot analyses were performed to determine the expression and activation of proteins. Apoptosis enzyme-linked immunosorbent assay was used to quantify cytoplasmic histone-associated DNA fragments. Microarray analyses were applied to explore both mRNA and miRNA expression profiles in NSCLC cells followed by integrative analysis. qRT-PCR was carried out to verify the differentially expressed mRNAs and miRNAs.

Results: The expression of 652 genes was shown to be changed at least 2-fold in paclitaxel-resistant NSCLC (H460_TaxR) cells with 511 upregulated and 141 downregulated as compared with that in parental H460 cells. The differentially expressed genes were functionally enriched in regulating the cell proliferation, cell death, and response to endogenous stimulus, and clustered in pathways such as cancer and signaling by the G protein-coupled receptor (GPCR). Moreover, 43 miRNAs were shown to be differentially expressed in H460_TaxR cells with 15 upregulated and 28 downregulated as compared with parental H460 cells. A total of 289 pairs of miRNA-potential target gene were revealed in H460_TaxR cells by bioinformatics analysis. Furthermore, integrative analysis of miRNAs and gene expression profiles revealed that dysregulated miR-362-3p, miR-766-3p, and miR-6507-3p might confer paclitaxel resistance in NSCLC via targeting MAPT simultaneously.

Conclusion: Our findings suggested that specific manipulation of MAPT-targeting miRNAs may be a novel strategy to overcome paclitaxel resistance in patients with NSCLC especially large-cell lung carcinoma.

Keywords: integrative analysis, non-small cell lung cancer, paclitaxel-resistance, gene expression profile, miRNAs

Introduction

Lung cancer remains the most commonly diagnosed cancer and the leading cause of cancer-related deaths, with 2.1 million new cases and 1.8 million deaths in 2018 worldwide.[1,2] Of all pathological types, non-small cell lung cancer (NSCLC)

accounts for approximate 85% of all lung cancers and more than 65% of the patients with NSCLC present with locally advanced or metastatic disease.[3,4]

The major treatment options for patients with NSCLC are determined on the basis of histologic features and staging according to the eighth edition of the TNM (T, N, and M represent tumor, lymph node, and metastasis, respectively) classification for lung cancer.[5] Generally, the current treatment for lung cancer patients who have been diagnosed at an early stage is surgical resection followed by chemotherapy; however, majority of the patients will eventually experience disease progression and require further treatment.[6,7] Paclitaxel, either as single agent or combined with other therapeutics, has shown significant anti-NSCLC activity.[8–10] In advanced stages of NSCLC, the efficacy of paclitaxel in combination with a platinum compound has also been confirmed by a meta-analysis of 16 randomized trials.[11] However, either intrinsic or acquired resistance to paclitaxel frequently occurs and represents a significant clinical problem.[12] We had previously shown that microRNA-mediated epigenetic targeting of survivin significantly enhances the antitumor activity of paclitaxel against NSCLC.[13] In the current report, we investigated both the gene and miRNAs expression profiles in paclitaxel-resistant large-cell lung carcinoma cells, a pathological type of relative small population of NSCLC. An integrative analysis of miRNAs and gene expression profiles was also carried out.

Materials and methods

Reagents and antibodies

Paclitaxel was obtained from the 900th Hospital of the Joint Logistics Team pharmacy (the Former Fuzhou General Hospital). The CellTiter96 AQ cell proliferation kit (Cat.#G3582) was a product of Promega (Madison, WI, USA). Oligonucleotides were synthesized in Sangon (Shanghai, China). Antibodies against PARP (Cat.#9542) and Caspase-3 (Cat.#9665) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA); antibody against β-actin (Cat.#A5441) was the product of Sigma (St. Louis, MO, USA).

Cells and cell culture

Human large-cell lung carcinoma cell line H460 was obtained from American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS). All cells were cultured in a 37ºC humidified atmosphere containing 95% air and 5% CO_2 and were split twice a week.

To mimic the development of acquired paclitaxel resistance in vitro, parental H460 cells were maintained in culture medium with low dose of paclitaxel started from 1 nmol/L initially. Cells were then split twice a week and maintained in culture medium with gradually increased dose of paclitaxel every 2 weeks. After 6 months, paclitaxel-resistance NSCLC cells developed from H460 were maintained in culture medium with 40 nmol/L of paclitaxel for normal culture.

Cell viability assay

The CellTiter96 AQ cell proliferation kit (Cat.#G3582, Promega) was used to determine cell viability as previously described.[13] Briefly, cells were plated onto 96-well plates for 24 hrs, and then grown in either RPMI1640 medium with 0.5% FBS as control or the same medium containing different concentrations of paclitaxel, and then incubated for another 72 hrs. During this period, the medium was refreshed daily maintaining the same treatment. After reading all wells at 490 nm with a microplate reader, the percentages of surviving cells from each group relative to controls, defined as 100% survival, were determined by reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS).

Clonogenic assay

The parental and paclitaxel-resistant human SCLC cancer cells H460 were plated onto 6-well plates and incubated at 37°C with 5% CO_2. After 24 hrs, the culture medium was replaced daily with 2 mL fresh medium containing 0.5% FBS or the same medium containing indicated concentrations of paclitaxel for long-term incubation. After 2 weeks, cells were stained with 0.5% crystal violet (dissolved in 25% methanol) and clone number was quantified with QuantiOne software of Fluor-S^{TM} Multimager (Bio-Rad Laboratories, Inc., Hercules, CA) at the end of the experiments.

Western blotting analysis and quantification of apoptosis

Protein expression and activation were determined by Western blotting analysis as previously described.[14] In brief, equal amounts of cell lysates in a buffer (containing 50 mmol/L Tris (pH 7.4), 50 mmol/L NaCl, 0.5% NP40, 50 mmol/L

NaF, 1 mmol/L Na_3VO_4, 1 mmol/L phenylmethylsulfonyl fluoride, 25 μg/mL leupeptin, and 25 μg/mL aprotinin) were boiled in sodium dodecyl sulfate (SDS) sample buffer (0.0625 mol/L Tris (pH 6.8), 2% SDS, 10% Glycerol, 5% 2-mercaptoethanol, 0.002% Bromophenol-B), resolved by SDS-polyacrylamide gel electrophoresis and Western blotted with specific antibodies directed against PARP (1:1000), Caspase-3 (1:1000), or β-actin (1:10000), as described in the figure legends. For quantification of apoptosis, an apoptosis enzyme-linked immunosorbent assay kit (Cat.#11774425001, Roche Diagnostics Corp., Indianapolis, IN, USA) was used to quantitatively measure cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) as previously reported.[14]

Microarray analysis of both mRNA and miRNA followed by integrative analysis

Total RNAs were prepared from parental and paclitaxel-resistant H460 cells (H460_Parental and H460_TaxR, respectively) with miRNeasy Mini Kit (QIAGEN, GmbH, Germany). For mRNA and miRNA profiling, Agilent G3 Human (8*60 K) Chip and Agilent Human

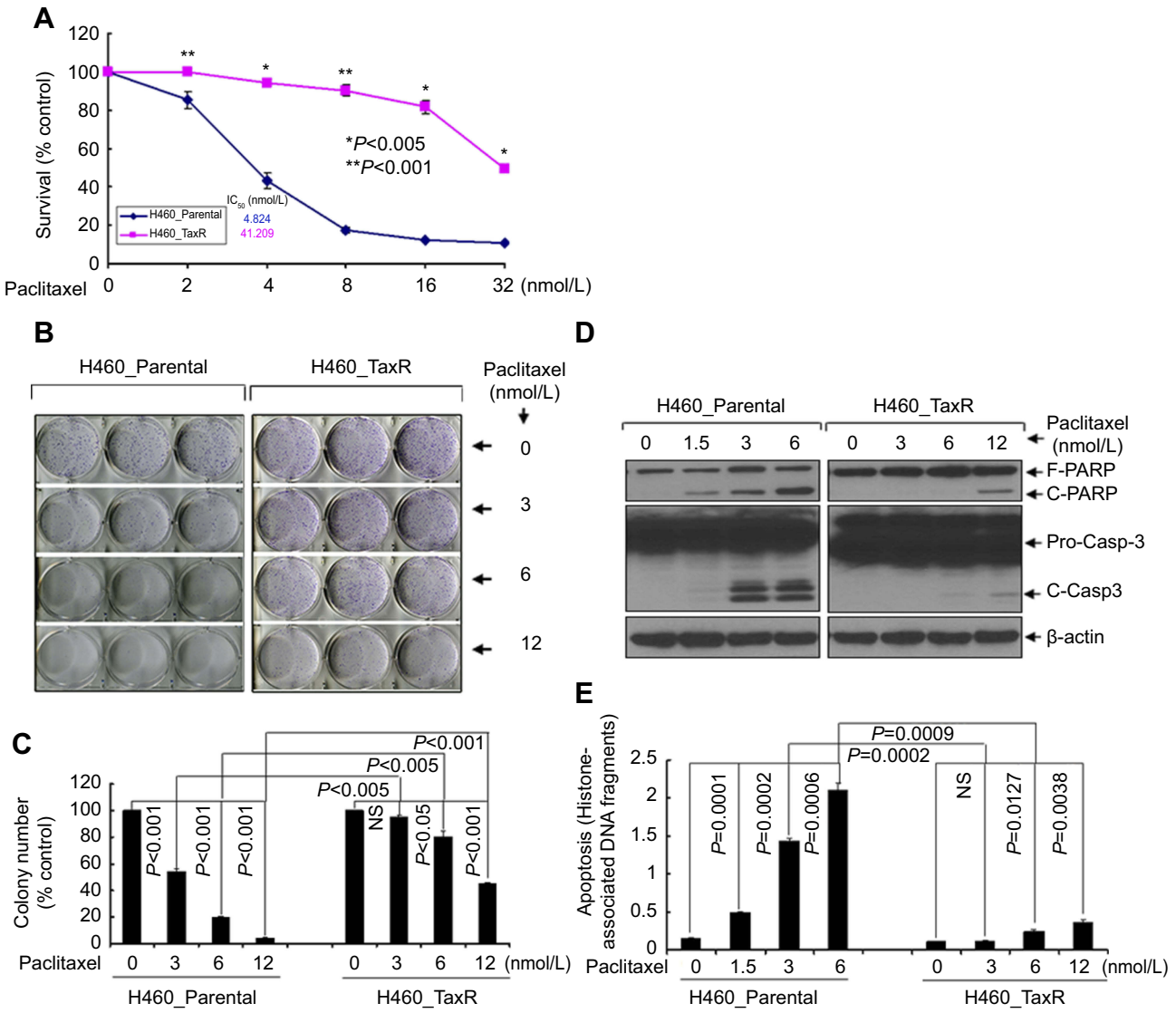

Figure 1 Identification of paclitaxel-resistant NSCLC cells.

Notes: (**A**) Human NSCLC cells (H460_Parental and H460_TaxR) treated with indicated concentrations of paclitaxel for 72 h were subjected to cell viability assay. (**B, C**) H460_Parental and H460_TaxR cells were grown in triplicates in the absence or presence of indicated concentrations of paclitaxel for 2–3 weeks. The pictures and numbers of the cell colonies were obtained by the QuantiOne software of Fluor-S™ Multimager. (**D, E**) H460_Parental and H460_TaxR cells were treated with indicated concentrations of paclitaxel for 24 hrs. Cells were collected and subjected to Western blot analyses of PARP, Casp-3 or β-actin (**D**), or apoptotic-ELISA (**E**).

Abbreviations: F-PARP, full length of poly(ADP-ribose) polymerase; C-PARP, cleaved PARP; Pro-Casp-3, Caspase-3; C-Casp-3, cleaved caspase-3; ELISA, enzyme-linked immunosorbent assay.

miRNA Chip (8*60 K) V19.0 (Agilent technologies, Santa Clara, CA, USA) were used, respectively. Microarray analysis was performed in triplicate in Shanghai Biochip Co., Ltd (Shanghai, China). Either mRNA or miRNA with two-fold change and a $P<0.05$ between parental and paclitaxel-resistant H460 cells was taken as significantly differentially expressed. The mRNA and miRNA expression profiles were then subjected to integrative analysis in Shanghai Center for Bioinformation Technology (Shanghai, China).

Analysis of differentially expressed mRNAs and miRNAs with real-time quantitative reverse transcriptase PCR (qRT-PCR)

Total RNA was prepared using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was generated using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) following the manufacturer's instructions. To quantify the mRNA level of *MAPT* (forward primer:

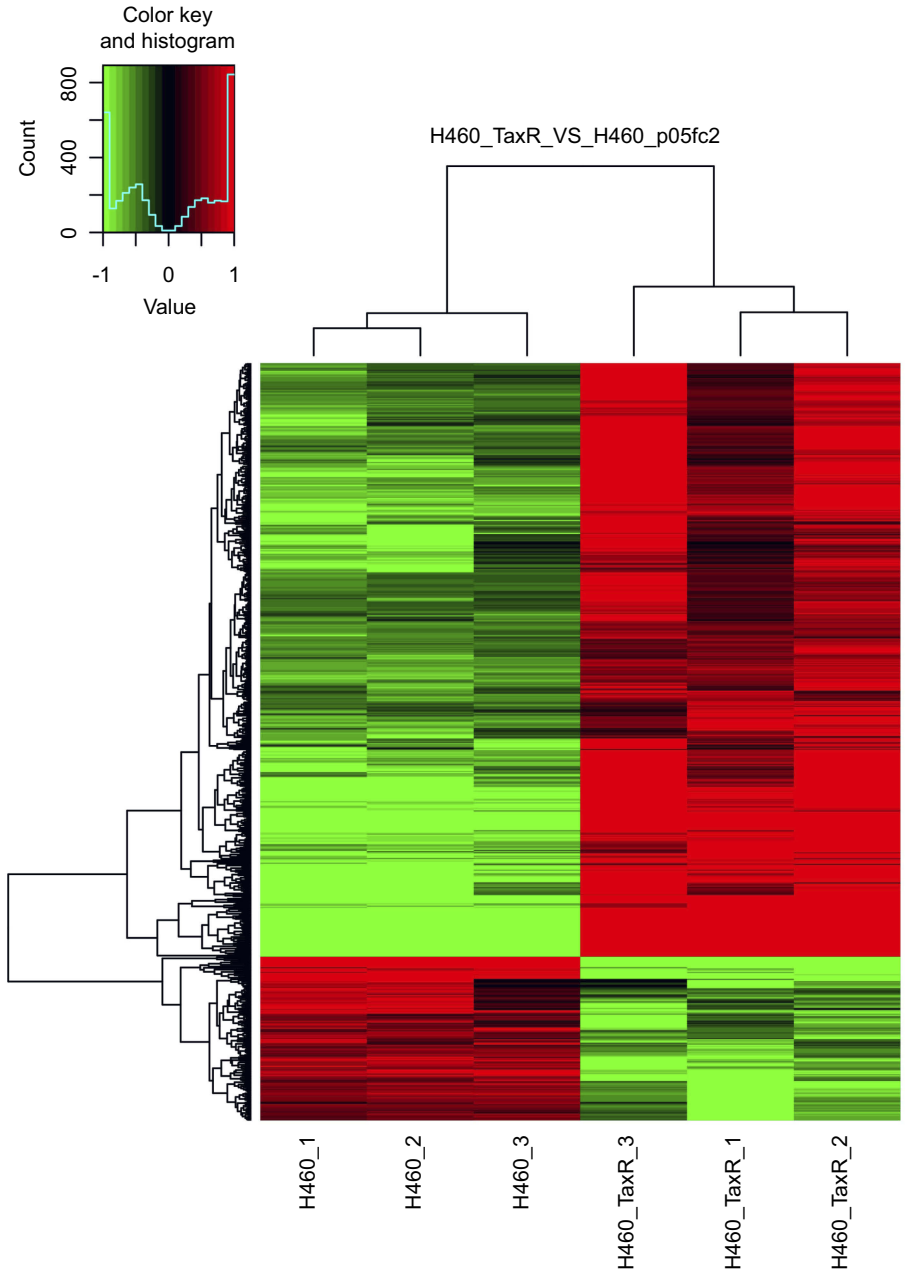

Figure 2 mRNA expression profiles in parental and paclitaxel-resistant NSCLC cells.
Note: The heatmap from unsupervised hierarchical clustering showed mRNAs with high expression in red and mRNAs with low expression in green.

5′-AAGATCGGCTCCACTGAGAA-3; reverse primer: 5′-ATGAGCCACACTTGGAGGTC-3′), qRT-PCR was performed using the FastStart Universal SYBR Green Master Mixes (Roche) by a 7900HT Fast Real-Time PCR system (Applied Biosystems) as we had described previously.[15] The expression of β-actin was used as an internal control (forward primer: 5′-AGAGCTACGA GCTGCCTGAC-3; reverse primer: 5′-AGCACTGTG TTGGCGTACAG-3′). The expression of mature miR-362-3p, miR-766-3p, miR-6507-3p, and RNU6B was measured by qRT-PCR using TaqMan Assays (Applied Biosystems) as described previously.[15] The relative mRNA and miRNA levels were calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$).

Statistical analysis

All results were confirmed by at least three independent experiments. Data are presented as mean±SD. Student's *t*-test or one-way ANOVA was carried out to analyze the differences between two groups or multiple groups. Values of $P<0.05$ were considered significant.

Results

Establishment of paclitaxel-resistant NSCLC cells

To establish a stable NSCLC cell subline with significant resistance to paclitaxel, parental H460 cells were subjected to growth in culture medium with gradually increased dose of paclitaxel persistently to mimic the development of acquired drug resistance in vitro. Six months later, NSCLC cell subline with significant resistance to paclitaxel was developed from parental H460 without obvious morphology change (data not shown). The subline was nominated as H460_TaxR. As shown in Figure 1A, the IC_{50} values of paclitaxel were 4.824 nmol/L and 41.209 nmol/L for parental and paclitaxel-resistant H460 cells, respectively. The IC_{50} value for paclitaxel-resistant H460 cells increased 8.542-fold as compared with that of parental H460 cells. The significant resistance to anti-proliferative/anti-survival effects of paclitaxel in H460_TaxR cells was further confirmed by clonogenic assay (Figure 1B and C). Moreover, results from both Western blotting analysis and quantification of apoptosis showed that while treatment with as low as a dose of 1.5 nmol/L of paclitaxel induced

Table 1 Representative differentially expressed genes in paclitaxel-resistant NSCLC cells

Gene symbol	Gene name	Genbank accession no.	Fold change	*P*-values
Top ten upregulated genes in H460_TaxR as compared with H460_Parental				
TIMP3	TIMP metallopeptidase inhibitor 3	NM_000362	84.39827628	4.48E-05
LINGO2	Leucine rich repeat and Ig domain containing 2	NM_152570	46.80088471	0.002291
MYRIP	Myosin VIIA and Rab interacting protein	NM_015460	34.75002707	0.01062
GPR65	G protein-coupled receptor 65	NM_003608	29.05555243	0.008347
MARCKS	Myristoylated alanine-rich protein kinase C substrate	NM_002356	28.25107774	0.00012
ASTN1	Astrotactin 1	NM_207108	27.44982007	0.004438
ANXA3	Annexin A3	NM_005139	27.28251861	0.014142
ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	NM_000927	24.97292343	0.001563
WNT4	Wingless-type MMTV integration site family, member 4	NM_030761	24.6898816	0.001894
INSM1	insulinoma-associated 1	NM_002196	23.84303987	0.002524
Top ten downregulated genes in H460_TaxR as compared with H460_Parental				
SLFN11	Schlafen family member 11	NM_001104587	0.059533855	0.026053
NTS	Neurotensin	NM_006183	0.067125478	0.001581
PAEP	Progestagen-associated endometrial protein	NM_002571	0.06737948	0.005147
DRD2	Dopamine receptor D2	NM_000795	0.069612941	0.002336
KIAA1324L	KIAA1324-like	NM_152748	0.070988816	0.004444
LOC100507127	Uncharacterized LOC100507127	NR_038291	0.089365066	0.004397
CHRNA9	Cholinergic receptor, nicotinic, alpha 9	NM_017581	0.106667335	0.005473
GABBR2	Gamma-aminobutyric acid (GABA) B receptor, 2	NM_005458	0.111175139	0.018236
PTX3	Pentraxin 3, long	NM_002852	0.114626788	0.006955
BAIAP2L2	BAI1-associated protein 2-like 2	NM_025045	0.13930831	0.000128

Table 2 Function enrichment analysis of differentially expressed genes in paclitaxel-resistant NSCLC cells

Gene set name	Genes in overlap	*P*-value	FDR q-value
GO_REGULATION_OF_CELL_PROLIFERATION	78	6.10E-30	3.75E-26
GO_REGULATION_OF_MULTICELLULAR_ORGANISMAL_DEVELOPMENT	80	3.16E-28	9.70E-25
GO_TISSUE_DEVELOPMENT	75	2.53E-27	5.18E-24
GO_RESPONSE_TO_LIPID	56	1.45E-25	2.23E-22
GO_RESPONSE_TO_OXYGEN_CONTAINING_COMPOUND	69	1.98E-25	2.43E-22
GO_REGULATION_OF_CELL_DIFFERENTIATION	71	6.39E-25	6.54E-22
GO_RESPONSE_TO_ORGANIC_CYCLIC_COMPOUND	55	4.45E-24	3.91E-21
GO_REGULATION_OF_CELL_DEATH	69	7.39E-24	5.67E-21
GO_RESPONSE_TO_ENDOGENOUS_STIMULUS	68	1.58E-23	1.08E-20
GO_POSITIVE_REGULATION_OF_DEVELOPMENTAL_PROCESS	60	2.88E-23	1.77E-20
GO_POSITIVE_REGULATION_OF_MULTICELLULAR_ORGANISMAL_PROCESS	66	4.69E-23	2.62E-20
GO_REGULATION_OF_CELLULAR_COMPONENT_MOVEMENT	49	1.30E-22	6.65E-20
GO_POSITIVE_REGULATION_OF_CELL_DIFFERENTIATION	49	2.09E-21	9.87E-19
GO_RESPONSE_TO_EXTERNAL_STIMULUS	73	2.82E-21	1.24E-18
GO_REGULATION_OF_HYDROLASE_ACTIVITY	61	9.90E-21	4.05E-18
GO_RESPONSE_TO_HORMONE	50	1.10E-20	4.23E-18
GO_EPITHELIUM_DEVELOPMENT	51	2.17E-20	7.39E-18
GO_NEUROGENESIS	62	3.19E-20	9.78E-18
GO_RESPONSE_TO_STEROID_HORMONE	37	1.37E-19	4.00E-17
GO_POSITIVE_REGULATION_OF_CELLULAR_COMPONENT_ORGANIZATION	55	1.69E-19	4.73E-17
GO_POSITIVE_REGULATION_OF_MOLECULAR_FUNCTION	68	1.29E-18	3.44E-16
GO_POSITIVE_REGULATION_OF_CELL_DEVELOPMENT	35	1.53E-18	3.92E-16
GO_REGULATION_OF_NERVOUS_SYSTEM_DEVELOPMENT	43	2.65E-18	6.50E-16
GO_POSITIVE_REGULATION_OF_RESPONSE_TO_STIMULUS	70	4.10E-18	9.69E-16
GO_REGULATION_OF_ANATOMICAL_STRUCTURE_MORPHOGENESIS	49	1.47E-17	3.35E-15
GO_NEGATIVE_REGULATION_OF_CELL_PROLIFERATION	39	1.60E-17	3.51E-15
GO_REGULATION_OF_CELL_DEVELOPMENT	44	2.52E-17	5.33E-15
GO_EMBRYO_DEVELOPMENT	45	5.68E-17	1.16E-14
GO_POSITIVE_REGULATION_OF_NERVOUS_SYSTEM_DEVELOPMENT	32	6.71E-17	1.33E-14
GO_REGULATION_OF_CELLULAR_LOCALIZATION	54	6.98E-17	1.34E-14
GO_NEGATIVE_REGULATION_OF_CELL_COMMUNICATION	52	7.61E-17	1.42E-14
GO_RESPONSE_TO_ABIOTIC_STIMULUS	48	8.02E-17	1.45E-14
GO_CELLULAR_RESPONSE_TO_ORGANIC_SUBSTANCE	66	9.02E-17	1.58E-14
GO_BIOLOGICAL_ADHESION	48	1.08E-16	1.80E-14
GO_CELL_DEVELOPMENT	57	1.09E-16	1.80E-14
GO_REGULATION_OF_PHOSPHORUS_METABOLIC_PROCESS	61	1.26E-16	2.04E-14
GO_NEGATIVE_REGULATION_OF_RESPONSE_TO_STIMULUS	55	2.38E-16	3.75E-14
GO_POSITIVE_REGULATION_OF_CELL_PROLIFERATION	42	2.76E-16	4.20E-14
GO_MOVEMENT_OF_CELL_OR_SUBCELLULAR_COMPONENT	53	2.80E-16	4.20E-14
GO_POSITIVE_REGULATION_OF_CATALYTIC_ACTIVITY	58	4.23E-16	6.18E-14
GO_POSITIVE_REGULATION_OF_HYDROLASE_ACTIVITY	44	4.49E-16	6.36E-14
GO_LOCOMOTION	49	4.56E-16	6.36E-14
GO_POSITIVE_REGULATION_OF_CELL_COMMUNICATION	58	6.32E-16	8.44E-14
GO_CARDIOVASCULAR_SYSTEM_DEVELOPMENT	40	2.49E-15	3.19E-13
GO_CIRCULATORY_SYSTEM_DEVELOPMENT	40	2.49E-15	3.19E-13
GO_CELL_MOTILITY	41	3.38E-15	4.15E-13
GO_LOCALIZATION_OF_CELL	41	3.38E-15	4.15E-13

significant apoptosis in parental H460 cells; however, for H460_TaxR cells, even treatment with 12 nmol/L of paclitaxel could only induce a few apoptosis (Figure 1D and E). Collectively, our results demonstrated that an NSCLC cell subline H460_TaxR with significant resistance to paclitaxel was established successfully.

Table 3 Clustered pathways analysis of differentially expressed genes in paclitaxel-resistant NSCLC cells

Gene set name	Genes in overlap	*P*-value	FDR q-value
KEGG_PATHWAYS_IN_CANCER	17	2.13E-07	1.18E-04
REACTOME_SIGNALING_BY_GPCR	30	2.19E-07	1.18E-04
KEGG_LYSOSOME	10	1.11E-06	4.00E-04
REACTOME_HEMOSTASIS	19	1.59E-06	4.28E-04
REACTOME_GPCR_LIGAND_BINDING	17	4.20E-06	7.82E-04
REACTOME_PLATELET_ACTIVATION_SIGNALING_AND_AGGREGATION	12	4.36E-06	7.82E-04
REACTOME_CELL_SURFACE_INTERACTIONS_AT_THE_VASCULAR_WALL	8	8.38E-06	1.29E-03
KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION	13	1.11E-05	1.49E-03
REACTOME_GPCR_DOWNSTREAM_SIGNALING	24	1.57E-05	1.88E-03
REACTOME_GPVI_MEDIATED_ACTIVATION_CASCADE	5	2.23E-05	2.40E-03
REACTOME_METABOLISM_OF_LIPIDS_AND_LIPOPROTEINS	17	3.21E-05	2.99E-03
KEGG_BASAL_CELL_CARCINOMA	6	3.35E-05	2.99E-03
REACTOME_SIGNALLING_BY_NGF	11	3.60E-05	2.99E-03
REACTOME_COLLAGEN_FORMATION	6	4.55E-05	3.28E-03
REACTOME_DEVELOPMENTAL_BIOLOGY	15	4.57E-05	3.28E-03
BIOCARTA_ALK_PATHWAY	5	5.42E-05	3.47E-03
REACTOME_EXTRACELLULAR_MATRIX_ORGANIZATION	7	5.47E-05	3.47E-03
REACTOME_IMMUNE_SYSTEM	25	5.98E-05	3.58E-03
REACTOME_G_ALPHA_I_SIGNALLING_EVENTS	10	7.35E-05	4.17E-03
BIOCARTA_CYTOKINE_PATHWAY	4	9.43E-05	4.85E-03
KEGG_FOCAL_ADHESION	10	9.45E-05	4.85E-03
KEGG_AXON_GUIDANCE	8	1.04E-04	5.04E-03
KEGG_EPITHELIAL_CELL_SIGNALING_IN_HELICOBACTER_PYLORI_INFECTION	6	1.12E-04	5.04E-03
KEGG_COMPLEMENT_AND_COAGULATION_CASCADES	6	1.22E-04	5.04E-03
KEGG_P53_SIGNALING_PATHWAY	6	1.22E-04	5.04E-03
KEGG_PPAR_SIGNALING_PATHWAY	6	1.22E-04	5.04E-03
REACTOME_KERATAN_SULFATE_BIOSYNTHESIS	4	1.86E-04	7.42E-03
REACTOME_SIGNALING_BY_ILS	7	2.02E-04	7.77E-03
REACTOME_BILE_ACID_AND_BILE_SALT_METABOLISM	4	2.16E-04	8.04E-03
KEGG_MAPK_SIGNALING_PATHWAY	11	2.26E-04	8.11E-03
REACTOME_CYTOKINE_SIGNALING_IN_IMMUNE_SYSTEM	11	2.49E-04	8.38E-03
REACTOME_GLYCOSAMINOGLYCAN_METABOLISM	7	2.53E-04	8.38E-03
REACTOME_INTEGRIN_CELL_SURFACE_INTERACTIONS	6	2.57E-04	8.38E-03
REACTOME_SIGNALING_BY_RHO_GTPASES	7	2.83E-04	8.86E-03
BIOCARTA_INFLAM_PATHWAY	4	2.88E-04	8.86E-03
KEGG_CHEMOKINE_SIGNALING_PATHWAY	9	3.02E-04	9.02E-03
REACTOME_KERATAN_SULFATE_KERATIN_METABOLISM	4	3.29E-04	9.58E-03
KEGG_HEDGEHOG_SIGNALING_PATHWAY	5	3.99E-04	1.13E-02
KEGG_ERBB_SIGNALING_PATHWAY	6	4.33E-04	1.20E-02
BIOCARTA_CLASSIC_PATHWAY	3	4.54E-04	1.22E-02

(*Continued*)

Table 3 (Continued).

Gene set name	Genes in overlap	*P*-value	FDR q-value
REACTOME_GASTRIN_CREB_SIGNALLING_PATHWAY_VIA_PKC_AND_MAPK	9	5.24E-04	1.32E-02
REACTOME_ION_TRANSPORT_BY_P_TYPE_ATPASES	4	5.38E-04	1.32E-02
KEGG_NEUROTROPHIN_SIGNALING_PATHWAY	7	5.46E-04	1.32E-02
REACTOME_AXON_GUIDANCE	10	5.59E-04	1.32E-02
BIOCARTA_ERYTH_PATHWAY	3	5.63E-04	1.32E-02
BIOCARTA_NUCLEARRS_PATHWAY	3	5.63E-04	1.32E-02
REACTOME_CLASS_A1_RHODOPSIN_LIKE_RECEPTORS	11	6.90E-04	1.58E-02

Gene expression profile in paclitaxel-resistant NSCLC cells

Next, to gain a more comprehensive knowledge of the underlying molecular mechanism of paclitaxel-resistance in NSCLC, microarray analysis was carried out to investigate the gene expression profile in H460_TaxR cells with significant resistance to paclitaxel. The expression of 652 genes was found to be changed at least 2-fold in H460_TaxR cells with 511 upregulated and 141 downregulated as compared with parental H460 cells (Figure 2, $P<0.05$). Of these genes, the top 10 genes that were most significantly up- or downregulated in H460_TaxR cells are shown in Table 1. Gene Ontology (GO, http://www.geneontology.org) function enrichment analysis based on our own microarray data showed that the differentially expressed genes in H460_TaxR cells mainly involved in regulating the cell proliferation, cell death, and response to endogenous stimulus. (Table 2). Meanwhile, Gene Set Enrichment Analysis (GSEA) revealed that those differentially expressed genes were clustered in pathways such as cancer,[16] signaling by GPCR, cytokine and cytokine receptor interaction, and so on (Table 3).

MiRNAs expression profile in paclitaxel-resistant NSCLC cells

MiRNA has emerged as an important regulator in chemoresistance.[17] Thus, microarray analysis was also carried out to investigate the miRNAs expression profile in H460_TaxR cells with significant resistance to paclitaxel in our study. As shown in Figure 3 and Table 4, the expression of 43 miRNAs was found to be changed at least 2-fold in H460_TaxR cells with 15 upregulated and 28 downregulated as compared with parental H460 cells ($P<0.05$). A total of 289 pairs of miRNA-potential target gene were revealed in paclitaxel-resistant NSCLC cells by bioinformatics analysis.

Integrative analysis of miRNAs and gene expression profiles revealed that dysregulated MAPT-targeting microRNAs confer paclitaxel resistance in NSCLC

Cumulative studies had indicated that microtubule-associated protein tau (MAPT) play an important role in mediating the sensitivity of paclitaxel in several types of tumor including breast cancer.[18,19] Since our current gene expression profiling also showed that the *MAPT* mRNA was significantly upregulated in H460_TaxR cells as compared with parental H460 (Fold-change=6.31, $P=3.46E-03$; Figure 4A), we then focused our integrative analysis on candidate miRNAs targeting *MAPT* mRNA. Of all 28 downregulated miRNAs in H460_TaxR cells, 3 of them including miR-362-3p, miR-766-3p, and miR-6507-3p whose expression was verified by qRT-PCR were predicted to target *MAPT* mRNA via searching in miRDB (http://www.mirdb.org) with at least one binding site in its 3′-UTR (Figure 4B and C). Meanwhile, miR-362-3p and miR-766-3p were also predicted to target *MAPT* mRNA by TargetScan (http://www.targetscan.org). Furthermore, a MAPT-subnetwork was constructed via searching in STRING database (http://string-db.org) in combination with analysis of potential targets of miR-362-3p, miR-766-3p, and miR-6507-3p. As shown in Figure 4D, 13 of 14 potential targets of miR-362-3p, miR-766-3p, and miR-6507-3p were upregulated in H460_TaxR cells (Table 5) and could form a MAPT-subnetwork with protein–protein interactions. Taken together, our integrative analysis of

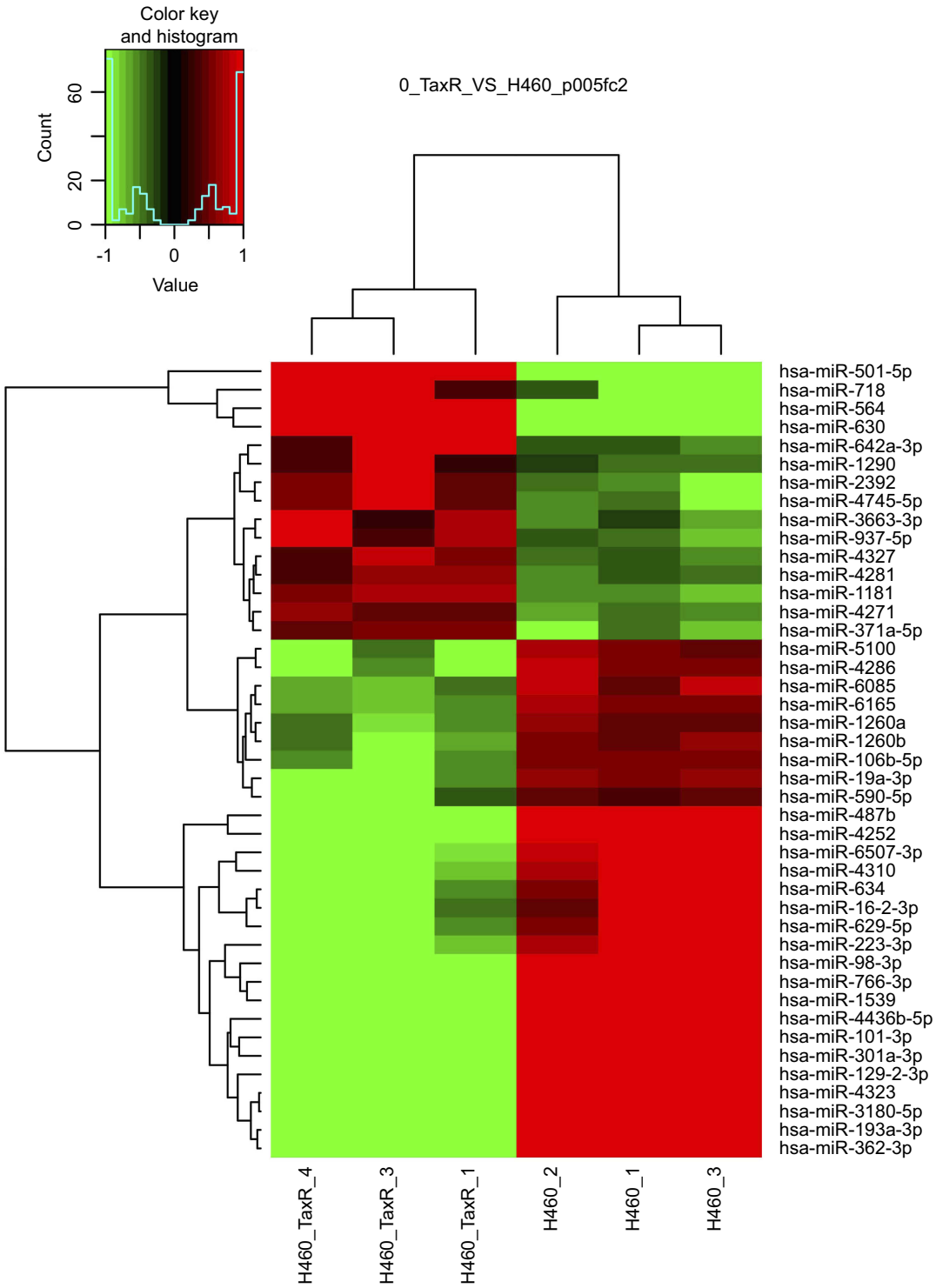

Figure 3 miRNA expression profiles in parental and paclitaxel-resistant NSCLC cells.
Note: The heatmap from unsupervised hierarchical clustering showed miRNAs with high expression in red and miRNAs with low expression in green.

miRNAs and gene expression profiles revealed that dysregulated a couple of microRNAs might confer paclitaxel resistance in NSCLC especially large-cell lung carcinoma via targeting MAPT simultaneously.

Discussion

Paclitaxel, initially derived from the bark of the Pacific yew tree, has long been used to treat NSCLC either as single-agent or combined with other therapeutics.[8,9,19]

Table 4 Differentially expressed miRNAs in paclitaxel-resistant NSCLC cells

Systematic name	Active_sequence	mirbase Accession No	Fold change	Standard deviation (SD)	*P*-values
Upregulated miRNAs in H460_TaxR as compared with H460_Parental					
hsa-miR-501-5p	TCTCACCCAGGGACAAAG	MIMAT0002872	30.43403	1.335591866	0.02809
hsa-miR-630	ACCTTCCCTGGTACAGA	MIMAT0003299	28.5287	0.364971021	0.0346
hsa-miR-564	GCCTGCTGACACCGT	MIMAT0003228	27.43658	0.65322048	0.015002
hsa-miR-718	CGACGCCCGGC	MIMAT0012735	11.46438	1.074424332	0.044477
hsa-miR-4745-5p	CGCCGTCCCGG	MIMAT0019878	3.028384	0.504524739	0.025545
hsa-miR-2392	CACCTCTCACCCCC	MIMAT0019043	2.828347	0.408171554	0.01474
hsa-miR-642a-3p	GGTTCCCTCTCCAAAT	MIMAT0020924	2.471685	0.487946192	0.037868
hsa-miR-1181	CGGCTCGGGTGG	MIMAT0005826	2.447857	0.126743628	0.000237
hsa-miR-3663-3p	GCGCCCGGCCT	MIMAT0018085	2.371832	0.391359374	0.017977
hsa-miR-937-5p	CCAGCCCCACCC	MIMAT0022938	2.323616	0.293576568	0.006291
hsa-miR-371a-5p	AGTGCCCCCACAG	MIMAT0004687	2.31623	0.039261838	0.009611
hsa-miR-4327	CCAGTCCCCCATGC	MIMAT0016889	2.076601	0.276185652	0.011899
hsa-miR-4281	CCCCCCTCCCCG	MIMAT0016907	2.064659	0.147628703	0.001511
hsa-miR-4271	CCCCACCTTTTCTTCC	MIMAT0016901	2.023521	0.111864933	0.000326
hsa-miR-1290	TCCCTGATCCAAAAATCC	MIMAT0005880	2.002767	0.40383008	0.048135
Downregulated miRNAs in H460_TaxR as compared with H460_Parental					
hsa-miR-1260a	TGGTGGCAGAGGTGG	MIMAT0005911	0.468833	0.227683765	0.003831
hsa-miR-1260b	ATGGTGGCAGTGGTG	MIMAT0015041	0.437184	0.267583797	0.008619
hsa-miR-6165	CTCCCCTCACCTCC	MIMAT0024782	0.419638	0.094514223	0.000272
hsa-miR-590-5p	CTGCACTTTTATGAATAAGCTC	MIMAT0003258	0.409267	0.4610021	0.036474
hsa-miR-106b-5p	ATCTGCACTGTCAGCAC	MIMAT0000680	0.402297	0.532130403	0.046242
hsa-miR-6085	TGTGCTCCCCCAGC	MIMAT0023710	0.397764	0.124920683	0.002828
hsa-miR-5100	AGAGGCACCGCTGG	MIMAT0022259	0.386885	0.360047705	0.010945
hsa-miR-4286	GGTACCAGGAGTGGG	MIMAT0016916	0.335645	0.369893481	0.007076
hsa-miR-19a-3p	TCAGTTTTGCATAGATTTGCA	MIMAT0000073	0.330737	0.405804204	0.018967
hsa-miR-16-2-3p	TAAAGCAGCACAGTAATATTGG	MIMAT0004518	0.161867	0.804218051	0.015342
hsa-miR-634	GTCCAAAGTTGGGGTGCT	MIMAT0003304	0.161148	0.764124037	0.014464
hsa-miR-629-5p	AGTTCTCCCAACGTAAAC	MIMAT0004810	0.145186	0.936137741	0.015935
hsa-miR-193a-3p	ACTGGGACTTTGTAGGC	MIMAT0000459	0.105037	0.882816038	0.020123
hsa-miR-362-3p	TGAATCCTTGAATAGGTGTG	MIMAT0004683	0.097067	0.877197155	0.018939
hsa-miR-3180-5p	CGACGTGGGGCG	MIMAT0015057	0.093896	0.872493751	0.013877
hsa-miR-223-3p	TGGGGTATTTGACAAACTGAC	MIMAT0000280	0.082392	1.534040276	0.02651
hsa-miR-4436b-5p	GGCAGGGCAGGC	MIMAT0019940	0.067577	0.812083164	0.00396
hsa-miR-129-2-3p	ATGCTTTTTGGGGTAAGGG	MIMAT0004605	0.065478	0.849298793	0.003717
hsa-miR-301a-3p	GCTTTGACAATACTATTGCAC	MIMAT0000688	0.062341	1.298015049	0.028585
hsa-miR-4310	GGGACATGAATGCTGC	MIMAT0016862	0.053797	1.294332779	0.012988
hsa-miR-6507-3p	GGGAAAAATAGGAAGGAC	MIMAT0025471	0.04823	1.046598716	0.020597

(*Continued*)

Table 4 (Continued).

Systematic name	Active_sequence	mirbase Accession No	Fold change	Standard deviation (SD)	*P*-values
hsa-miR-1539	GGGCATCTGGGACG	MIMAT0007401	0.04443	1.256147046	0.018077
hsa-miR-101-3p	TTCAGTTATCACAGTACTGT	MIMAT0000099	0.04416	0.996798994	0.007266
hsa-miR-766-3p	GCTGAGGCTGTGGGGCT	MIMAT0003888	0.039739	0.928303117	0.008182
hsa-miR-98-3p	GGGAAAGTAGTAAGTTGTA	MIMAT0022842	0.039122	1.176827277	0.013799
hsa-miR-487b	AAGTGGATGACCCTGTAC	MIMAT0003180	0.033905	0.378464844	0.002894
hsa-miR-4252	TGGTGCTGACTCAGTG	MIMAT0016886	0.017371	0.506691708	0.000647

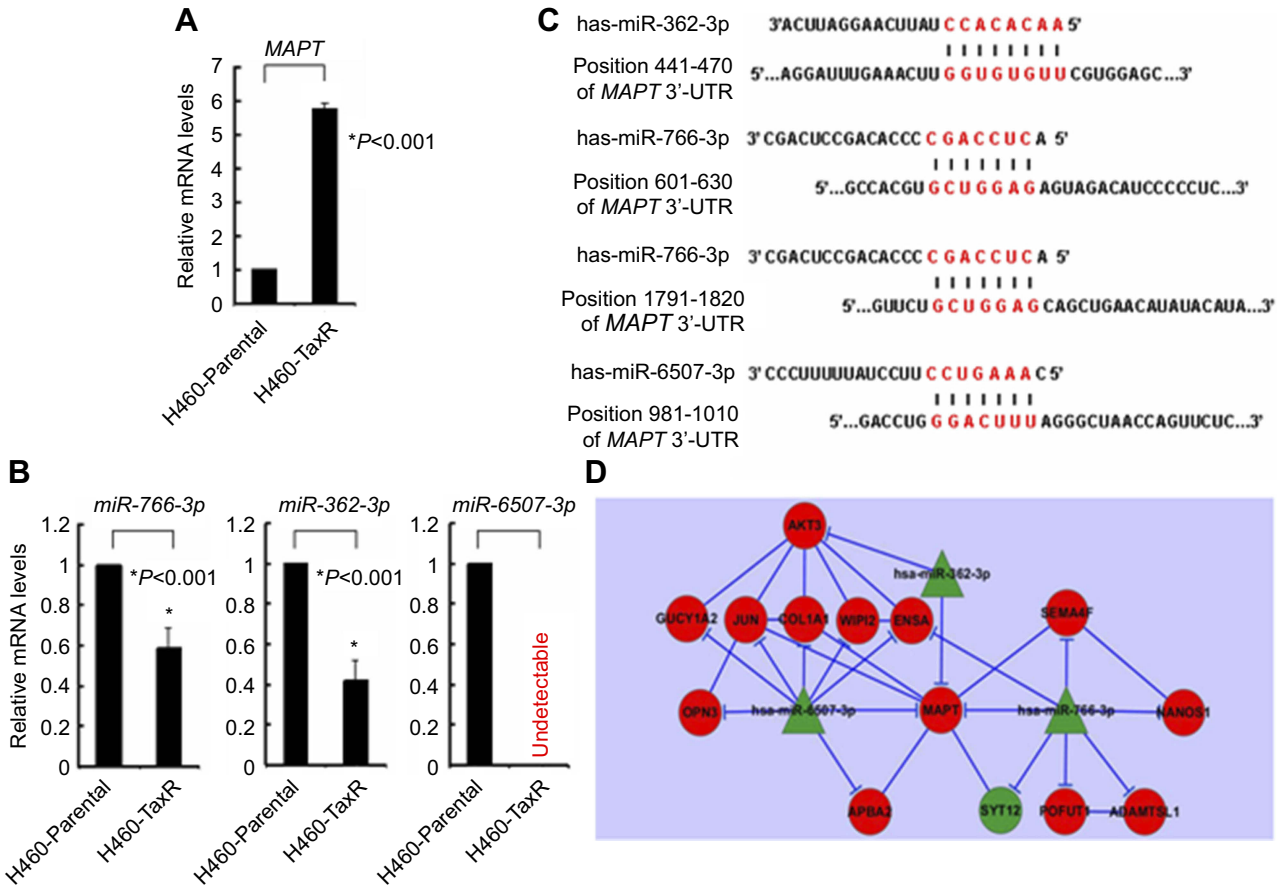

Figure 4 MAPT-subnetwork in paclitaxel-resistant NSCLC cells.
Notes: (**A**) qRT-PCR analysis of mRNA level of *MAPT* in H460_Parental and H460_TaxR. (**B**) qRT-PCR analysis of miRNAs levels of miR-766-3p, miR-362-3p, and miR-6507-3p in H460_Parental and H460_TaxR. (**C**) Diagram of targeting sequences of miR-766-3p, miR-362-3p, and miR-6507-3p in 3′-UTR of *MAPT* mRNA. (**D**) Diagram of constructed MAPT-subnetwork in paclitaxel-resistant non-small cell lung cancer cells. Green, downregulated; Red, upregulated.
Abbreviations: MAPT, microtubule-associated protein tau; qRT-PCR, quantitative reverse transcriptase PCR; 3′-UTR, 3′-untranslated region.

Treatment with paclitaxel disables spindle division and causes cell cycle arrest in phase G1/G2 of mitosis as well as induces apoptosis.[20] However, resistance to paclitaxel remains one of the main causes of treatment failure in NSCLC.[12] It is of particular significance to elucidate the underlying mechanism of paclitaxel resistance and identify new strategy to abrogate it.

Mechanisms of the resistance to paclitaxel are complex as indicated by cumulative studies, which include alterations in microtubule dynamics, altered expression of β-tubulin

Table 5 Differentially expressed genes in MAPT-subnetwork in paclitaxel-resistant NSCLC cells

Gene symbol	Gene name	Genbank accession	Fold change	*P*-values
Upregulated genes in H460_TaxR as compared with H460_Parental				
MAPT	Microtubule-associated protein tau	NM_016835	6.315843305	0.007459131
AKT3	V-akt murine thymoma viral oncogene homolog 3	NM_181690	4.530119529	0.007494871
GUCY1A2	Vuanylate cyclase 1, soluble, alpha 2	NM_000855	10.44950117	0.000226547
JUN	Jun proto-oncogene	NM_002228	4.464571876	0.000278815
COL1A1	Collagen, type I, alpha 1	NM_000088	4.129272402	0.019058353
WIPI2	WD repeat domain, phosphoinositide interacting 2	NM_015610	2.935071819	0.027975988
ENSA	Endosulfine alpha	NM_207168	2.08112235	0.019189465
SEMA4F	Sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4F	NM_004263	2.554550998	0.038460426
OPN3	Opsin 3	NM_014322	2.167589681	0.002040084
NANOS1	Nanos homolog 1	NM_199461	2.021603195	0.02355948
APBA2	Amyloid beta (A4) precursor protein-binding, family A, member 2	NM_005503	2.337305219	0.010474828
POFUT1	Protein O-fucosyltransferase 1	NM_172236	2.38753214	0.018840513
ADAMTSL1	ADAMTS-like 1	NM_001040272	6.100066811	0.011256879
Downregulated gene in H460_TaxR as compared with H460_Parental				
SYT12	Synaptotagmin XII	NM_177963	0.216113965	0.038129845

isotypes, and also deregulated signaling pathways.[21–24] In our own serial studies, we have demonstrated that activation of PI-3K/Akt signaling plays a critical role in ErbB2/ErbB3-mediated therapeutic resistance to paclitaxel mainly via upregulation of survivin.[14,25] In addition, we have also found that microRNA-mediated epigenetic targeting of survivin significantly enhances the antitumor activity of paclitaxel against NSCLC.[12] Unexpectedly, as we did not observe the change of survivin in our established paclitaxel-resistant NSCLC cells (data not shown), an integrative gene expression profiling was applied to explore the possible novel underlying molecular mechanism of paclitaxel-resistance in NSCLC in our current study. We showed that a total of 43 miRNAs and 652 protein-encoding genes, functionally enriched in regulating the fundamental biological processes including cell proliferation and clustered in pathways such as cancer, are differentially expressed in paclitaxel-resistant NSCLC cells, which suggest that the mechanism of resistance to paclitaxel is more complicated than we had ever recognized.

Upon binding to a pocket in beta-tubulin, paclitaxel inhibits the microtubule depolymerization process and interferes the progression of normal cell cycle.[19] MAPT, being a microtubule-associated protein which promotes the assembly of tubulin into microtubules to stabilize microtubule structure,[26] has been shown to compete with paclitaxel via sharing the same site.[27] As anticipated, a large body of studies had indicated that MAPT plays an important role in mediating the sensitivity of paclitaxel in several types of tumor.[18,19,28–30] Consistently, we also found that the mRNA expression of MAPT was significantly upregulated in paclitaxel-resistant NSCLC cells. However, the underlying mechanism of deregulated expression of MAPT remains largely unknown currently. A study by Wu and colleagues showed that miR-34c-5p determines the chemosensitivity of gastric cancer to paclitaxel via regulating MAPT.[31] Recently, miR-186 was also showed to regulate paclitaxel sensitivity of NSCLC via targeting MAPT.[32] Interestingly, although both aforementioned two miRNAs remain unchanged in our established paclitaxel-resistant NSCLC cells, our integrative analysis of miRNAs and gene expression profiles revealed that deregulated miR-362-3p, miR-766-3p, and miR-6507-3p and their 13 potential targets could form a MAPT-subnetwork with protein–protein interactions and confer paclitaxel resistance in NSCLC (Figure 4D). Currently, since the

molecular mechanism underlying deregulated miR-362-3p, miR-766-3p, and miR-6507-3p remains largely unclear, it would be of great interest to unveil it. Besides, whether there exists a causal relationship between specific silence of triple microRNAs (miR-362-3p, miR-766-3p, and miR-6507-3p) and upregulation of MAPT awaits further investigation. Taken together, our findings shed new light on the mechanism of paclitaxel resistance in NSCLC especially large-cell lung carcinoma and suggested that specific manipulation of MAPT-targeting miRNAs may be a novel strategy to overcome paclitaxel resistance in patients with NSCLC in the future.

Abbreviations

NSCLC, non-small cell lung cancer; MAPT, microtubule-associated protein tau; qRT-PCR, quantitative reverse transcriptase PCR; 3′-UTR, 3′-untranslated region; PARP, poly (ADP-ribose) polymerase; C-PARP, cleaved PARP; C-Casp-3, cleaved Caspase-3; ELISA, enzyme-linked immunosorbent assay.

Data sharing statement

Supplementary data is available on request.

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Disclosure

The authors report no conflicts of interest in this work.

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