Am J Cancer Res 2016;6(6):1317-1330
www.ajcr.us /ISSN:2156-6976/ajcr0025646

Original Article

Oncogenic *miR-137* contributes to cisplatin resistance via repressing CASP3 in lung adenocarcinoma

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Received February 5, 2016; Accepted April 3, 2016; Epub June 1, 2016; Published June 15, 2016

Abstract: Although targeted therapy can prolong the survival of non-small cell lung cancer (NSCLC) patients with EGFR mutations, chemotherapy still is the choice for patients with wild-type EGFR or failure in targeted therapy. However, most of the patients will eventually develop chemoresistance. Our previous study showed that *miR-137* is a risky microRNA and is associated with poor prognosis in NSCLC patients. Here we investigated the role of *miR-137* in cisplatin resistance in lung adenocarcinoma patients. Our data indicated that *miR-137* overexpression increases the survival of lung cancer cells exposed to cisplatin and decreases cisplatin-induced apoptosis. Through computational prediction and microarray, we identified caspase-3 (CASP3) as a potential target of *miR-137*. Luciferase reporter and site-directed mutagenesis assays demonstrated that *miR-137* downregulates CASP3 through binding to its 3'-UTR. Moreover, the endogenous CASP3 can be modulated by overexpressing or silencing *miR-137* in lung adenocarcinoma cell lines regardless of EGFR status. Suppression of CASP3 by *miR-137* provides cancer cells with anti-apoptotic ability, leading to cisplatin resistance. Immunohistochemistry results revealed an inverse correlation between *miR-137* and CASP3 expressions in lung adenocarcinoma patients. Together, our data provide a new chemoresistance mechanism in lung adenocarcinoma and a possible target to control chemoresistance in lung adenocarcinoma patients.

Keywords: *miR-137*, lung adenocarcinoma, cisplatin, caspase 3, chemoresistance

Introduction

MicroRNAs (miRNAs) are small non-coding RNAs that regulate their target gene expression by mRNA cleavage or inhibiting translation [1]. miRNAs are important to most of biological processes including differentiation, proliferation, development, metabolism, survival, and apoptosis [2-4]. Many miRNAs serve as oncogenes (onco-miR) or tumor suppressors (ts-miR) in different tissues, and they target important genes involved in the initiation and progression of human cancers [5].

In lung cancer, there are a number of onco-miRs and ts-miRs that have been reported [6, 7]. Several miRNA signatures in lung cancer have also been identified. For example, a 5-miRNA signature composed of miR-25, miR-34c-5p, miR-191, let-7e, and miR-34a can distinguish squamous cell carcinoma from adenocarcinoma and it correlates with poor overall survival among squamous patients [8]; our previously published 5-miRNA signature (let-7a, miR-221, *miR-137*, miR-372, and miR-182*) can predict the outcome of cancer relapse and survival after surgery [9]. In this study, we focus on *miR-137*, a risky miRNA of non-small cell lung cancer (NSCLC), for further investigation.

MiR-137 has been reported to be a ts-miR in several solid tumors including head and neck cancer [10], colorectal cancer [11], glioblastoma [12], and lung cancer [13, 14]. Confirmed

targets are CDK6, CDC42, CSE1L, CTBP1, E2F6, ESRRA, PTGS2, NCOA2, YBX1, KDM1A, PXN, ZNF804A, and MITF [15]. However, a previous report revealed that *miR-137* is significantly up-regulated in the most advanced T-stage after starting chemoradiotherapy in rectal cancer [16]. This suggests the possible oncogenic role of *miR-137*.

Caspase-3 (CASP3) belongs to the cysteine proteases, which plays a critical role in apoptosis by cleaving a number of crucial cellular proteins. CASP3 can be activated by initiator caspases through different death-inducing signals, for instance, the chemotherapy drugs [17]. Acquired chemoresistance to apoptosis-inducing anti-cancer drugs is frequently identified in CASP3 down-regulated cancers [18]. In NSCLC, down-regulated CASP3 has been linked to poor overall prognosis and chemoresistance [19, 20].

It has been suggested that chemotherapy should be provided to NSCLC patients to increase survival, control diseases, and enhance life quality [21]. Cisplatin is the most widely used drug in cancer therapy and the first FDA-approved platinum compound for lung cancer treatments [22, 23]. However, intrinsic and acquired chemoresistance will eventually develop in NSCLC patients [24]. NSCLC patients were treated with higher doses of chemo drugs to overcome the resistance, thus resulting in unfavorable side effects [25].

Here, we present that CASP3 is a novel target gene of *miR-137*. As a result of targeting CASP3, *miR-137* overexpression leads to anti-apoptosis and cisplatin resistance in lung adenocarcinoma cells. The clinical investigations also show an inverse correlation between *miR-137* and CASP3 expressions, thus offering a plausible explanation for the oncogenic feature of *miR-137*.

Materials and methods

Cell lines

The human lung adenocarcinoma cell lines, including CL1-5, CLH9, CLH27, A549, H1299, H1650, H1437, H3255, and H1975, were maintained in RPMI supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Among which, CL1-5 [26], CLH9, and CLH27 cells were derived from the lung adenocarcinoma patients in Taiwan. The HEK-293 cells were maintained in DMEM with 10% fetal bovine serum. All of the cell lines were incubated at 37°C in a humidified atmosphere with 5% CO_2.

Cell proliferation and flow cytometry assays

For cell proliferation assay, the cells were seeded into 96-well plates (10^3 cells/well) and incubated for various durations. At each time point, cell proliferation was evaluated by thiazolyl blue tetrazolium bromide (MTT) assay, according to the manufacturer's protocol (Chemicon, Temecula, CA). For the apoptosis assays, cells were analyzed by flow cytometry using an Annexin V-based apoptosis assay according to the manufacturer's instruction (BD Pharmingen, San Diego, CA).

Microarray and miR-137 target prediction

Human HT12-v4 Illumina Beadchip gene expression array (Illumina, San Diego, CA) was applied according to the manufacturer's protocol. The arrays were scanned and fluorescence signals were obtained using the Illumina Bead Array Reader (Illumina). Array data analysis was performed with GenomeStudio software. Differentially expressed genes were identified with the Mann-Whitney differential expression algorithm ($P < 0.05$) and defined by a fold change of greater than 1.35 between groups. Gene ontology and pathway analysis were done with the Metacore platform (GeneGo Inc. St. Joseph, MI). MicroRNA target prediction was performed by using miRwalk target prediction programs (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk/index.html).

Plasmid construction and transfection

A fragment of CASP3 3'UTR containing the binding site of *miR-137* was amplified from genomic DNA of HEK293 cells using the following primers: forward, 5'-ACCGGTTACTAGTAGAAATGGTTGG-TTGGTGGTTTTTT-3'; and reverse, 5'-AAGCTTAAGTTTGAATGTATATTTTGAAATAA-3'. To generate the mutant CASP3 3'UTR, six nucleotides within the seed region of *miR-137* binding site were mutated by PCR-based mutagenesis method. Both PCR fragments were cloned into pMIR-reporter luciferase vector (Ambion, Austin, TX). The full-length CASP3 cDNA was PCR-amplified and cloned into

pcDNA 3.1 expression vector (Invitrogen) along with V5 tag and CASP3 3'UTR. The precursor sequence of hsa-*miR-137* was synthesized and cloned into the BamHI and HindIII sites of an expression vector pSilencer4.1-CMV puro (Ambion). The pSilencer4.1-CMV puro Negative Control is a negative control plasmid encoding a hairpin siRNA whose sequence is not found in the human genome databases (Ambion). Plasmid transfection was performed by using lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instruction. Mimic endogenous precursor *miR-137*, anti-*miR-137*, siRNA and negative control were purchased from QIAGEN (Valencia, CA). They were transfected into cells using RNAiMAX (Invitrogen) according to the manufacturer's instruction.

Luciferase reporter assay

One day before transfection, HEK293 cells were seeded in 12-well plates at a density of 2.5×10^4 per well. Next, 50, 100, and 200 ng of pSilencer 4.1 vector or *miR-137* plasmid were co-transfected with 50 ng of pMIR-target gene-3'-UTR. The Renilla luciferase plasmid (phRL-TK, Promega, Madison, WI) was co-transfected as a transfection control. Cells were lysed 36 h after transfection, and luciferase activity was measured using a Dual-Luciferase system (Promega) according to the manufacturer's protocol.

Western blot

Immunoblotting was performed as described in a previous study conducted by Ho et al. [27]. Cells were harvested in RIPA lysis buffer, and the protein concentration was measured by the BCA protein assay (BioRad, Hercules, CA). Proteins were resolved by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred onto PVDF membranes, blocked with 5% skimmed milk in Tris-buffered saline (TBS), and reacted with primary antibodies for Tubulin (1:5000; GeneTex, San Antonio, TX), CASP3 (1:5000; Cell Signaling, Danvers, MA), cl-CASP3 (1:1000; Cell Signaling), PARP-1 (1:2000; GeneTex), cl-PARP-1 (1:2000; GeneTex), and V5 tag (1:5000; Invitrogen). Tubulin acts as an internal control.

Real-time quantitative polymerase chain reaction

According to the standard protocol, total RNAs were isolated using TRIZOL reagent (Invitrogen). The mature *miR-137* and endogenous control U6B were analyzed using TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA). miRNA-specific real-time PCR was performed using an ABI 7500 real-time PCR system. For TaqMan quantitative real-time RT-PCR, the primer sets for CASP3 (Hs00234387_m1) and the internal control, TBP (Hs00427621_m1), were purchased from Life Technologies. The relative mRNA expression of target gene was determined by the formula: $-\Delta CT = -[CT_{CASP3} - CT_{TBP}]$. The CASP3/TBP mRNA ratio was calculated as $2^{-\Delta CT} \times K$, in which K is a constant. All experiments were performed in triplicate.

Clinical lung cancer samples and immunohistochemistry

A total of 40 clinical lung adenocarcinoma specimens were collected from Taichung Veterans General Hospital (Taichung, Taiwan), with appropriate institutional review board approval and informed permission and written consent from all participants. miRNA expression level was measured using real-time PCR. The formalin-fixed and paraffin-embedded (FFPE) samples were dissected into 4-μm thick sections and then subjected to immunohistochemistry staining of CASP3 and cleavage of CASP3 using Vantana Medical System (Tucson, AZ). The primary antibodies against CASP3 (3CSP03) and cl-CASP3 (E83-77) were obtained from Abcam Inc. (Cambridge, MA). PBS without primary antibody was used as the negative control. The immunohistochemistry results were scored according to the average staining intensity and area. The immunostaining results were assessed and scored independently by pathologists.

Statistical analysis

Data are presented as the mean ± s.d. The differences between two groups were assessed using the Student's t-test, and the Kaplan-Meier method was used to estimate overall and progression-free survival. Differences in survival between two groups were analyzed using the log-rank test. Multivariate Cox proportional hazard regression analysis with stepwise selection was used to evaluate the independent prognostic factors associated with patient survival, and the expression of *miR-137*, age, gender, and tumor stage were used as covariates. MiRNA-sequencing data of lung adenocarcinoma patients used for validation were from TCGA

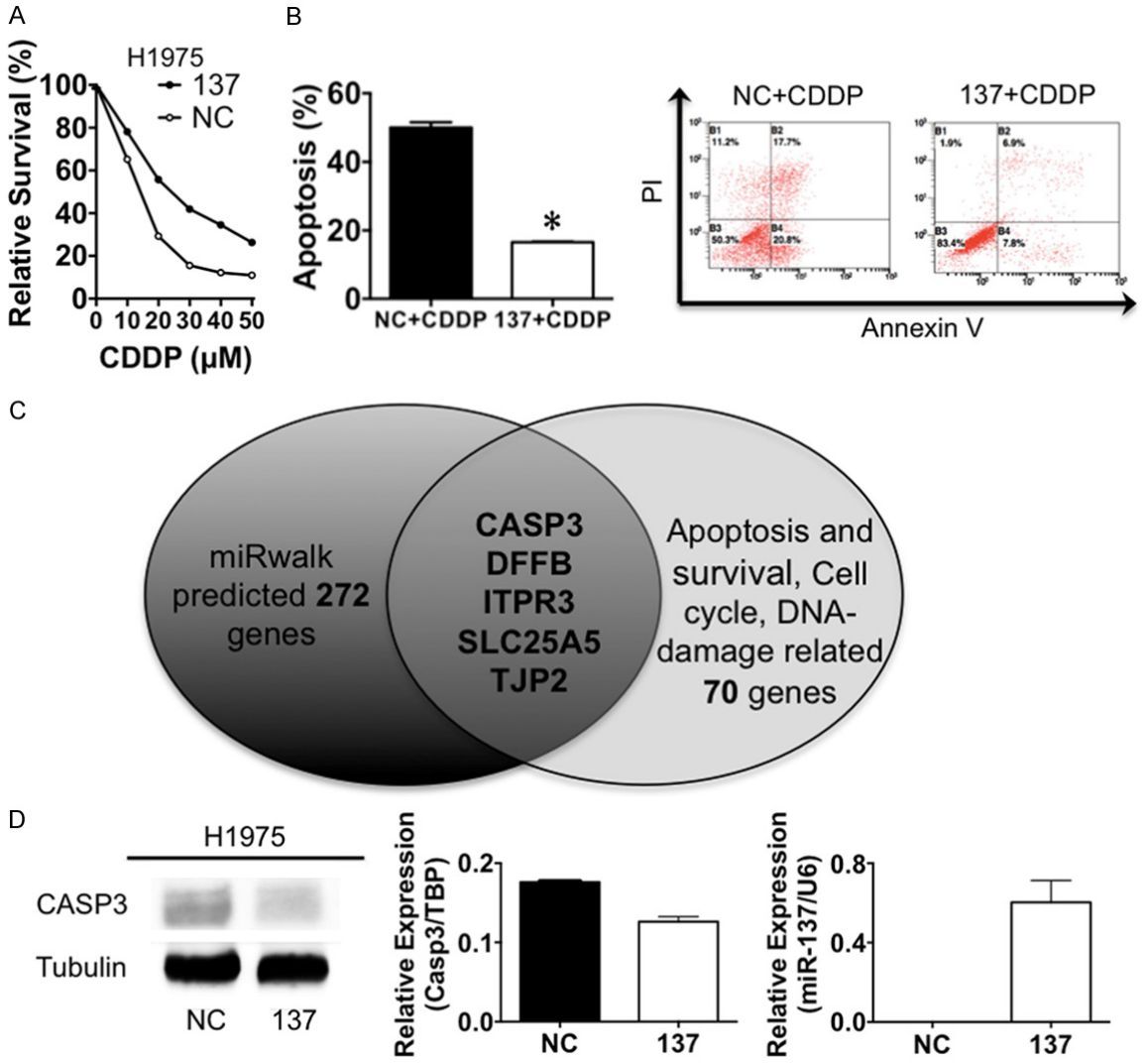

Figure 1. Reduction of cisplatin-induced cell death and caspase-3 expression by *miR-137*. Human lung cancer H1975 cells overexpressing *miR-137* or negative control were treated with the indicated concentrations of cisplatin and then subjected to cell death and microarray analyses. NC: negative control; 137: *miR-137* transfection. A. Cell viability was measured by MTT method after 72 hrs of treatment. B. Cell apoptosis was analyzed by Flow cytometry after 48 hrs. C. Identification of the candidate target of *miR-137* by *in silico* prediction and microarray. Circle with light grey: the down-regulated genes from microarray and involved in apoptosis, survival, cell cycle, and DNA damage pathways. Circle with dark grey: the predicted targets generated by the *in silico* prediction tool miRwalk. D. A decrease of caspase-3 (CASP3) expression by *miR-137*, as determined by Western blot (left panel) and real-time RT-PCR (middle panel). Right panel: *miR-137* expression in NC- and *miR-137*-transfected H1975 cells. *$P < 0.05$, compared with negative control.

datasets [28]. A patient's risk score was calculated using the levels of expression of *miR-137*. With the median of risk scores as the threshold value, patients were classified into the high-risk group or the low-risk group, and then subjected to Kaplan-Meier survival analysis. All analyses were performed with SAS version 9.1 software (SAS Institute Inc., Cary, NC). Two-tailed tests were used, and *P*-values < 0.05 were considered statistically significant.

Results

miR-137 expression decreases cisplatin-induced cell death and caspase-3 expression

Because drug resistance is a serious issue in chemotherapy of NSCLC patients, we further investigated whether *miR-137* expression is associated with chemoresistance. Overexpression of *miR-137* made H1975 cells more resis-

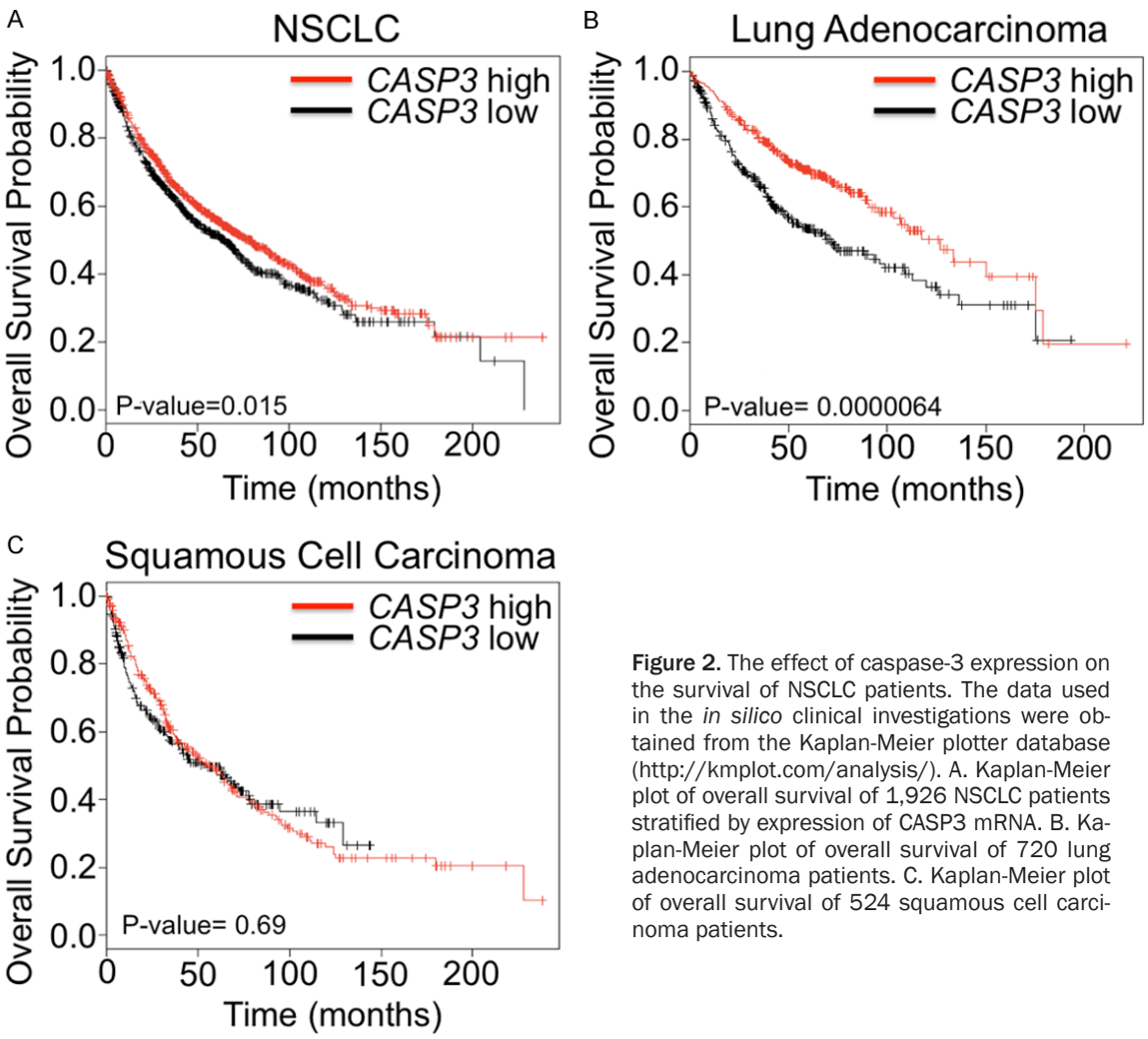

Figure 2. The effect of caspase-3 expression on the survival of NSCLC patients. The data used in the *in silico* clinical investigations were obtained from the Kaplan-Meier plotter database (http://kmplot.com/analysis/). A. Kaplan-Meier plot of overall survival of 1,926 NSCLC patients stratified by expression of CASP3 mRNA. B. Kaplan-Meier plot of overall survival of 720 lung adenocarcinoma patients. C. Kaplan-Meier plot of overall survival of 524 squamous cell carcinoma patients.

tant to cisplatin after 72 hours (**Figure 1A**). The IC50 of *miR-137* in transfected H1975 is 36.6 μM, which is 1.5-fold higher than negative control (NC) (IC50 is 24.2 μM). Furthermore, the ectopic expression of *miR-137* decreased the apoptotic cell population in H1975 treated with 30 μM of cisplatin for 48 hours (**Figure 1B**), from 50±0.8% down to 16.5±0.17%.

To identify the potential target genes of *miR-137*, microarray and computer-based prediction algorithm (miRwalk) [29] were applied in this study. Cisplatin resistance has been believed to be related to apoptosis, cell-cycle, and DNA-damage pathways [30]. Therefore, the differentially expressed and down-regulated genes involved in these pathways (70 genes; data not shown) were employed to intersect with the predicted targets by miRwalk (262 genes; data not shown). There are caspase-3 (CASP3), DFFB, IP3 receptor (ITPR3), ANT2 (SLC25A5), and ZO-2 (TJP2) in the intersection (**Figure 1C**), and amongst these 5 genes, CASP3 appears in many of the genetic pathways. Next, we examined the effects of *miR-137* on *CASP3* at the mRNA and protein level in H1975 (**Figure 1D**). Overexpressing *miR-137* precursor could remarkably inhibit the protein and the mRNA expressions of CASP3.

Expression of CASP3 benefits lung adenocarcinoma patients

The expression status of *CASP3* in NSCLC has been verified to correlate with survival [31, 32]. We then evaluated the role of *CASP3* in the prognosis of lung cancer by using a large public clinical microarray database [33] and found that a higher expression of *CASP3* is associated with better overall survival in lung cancer

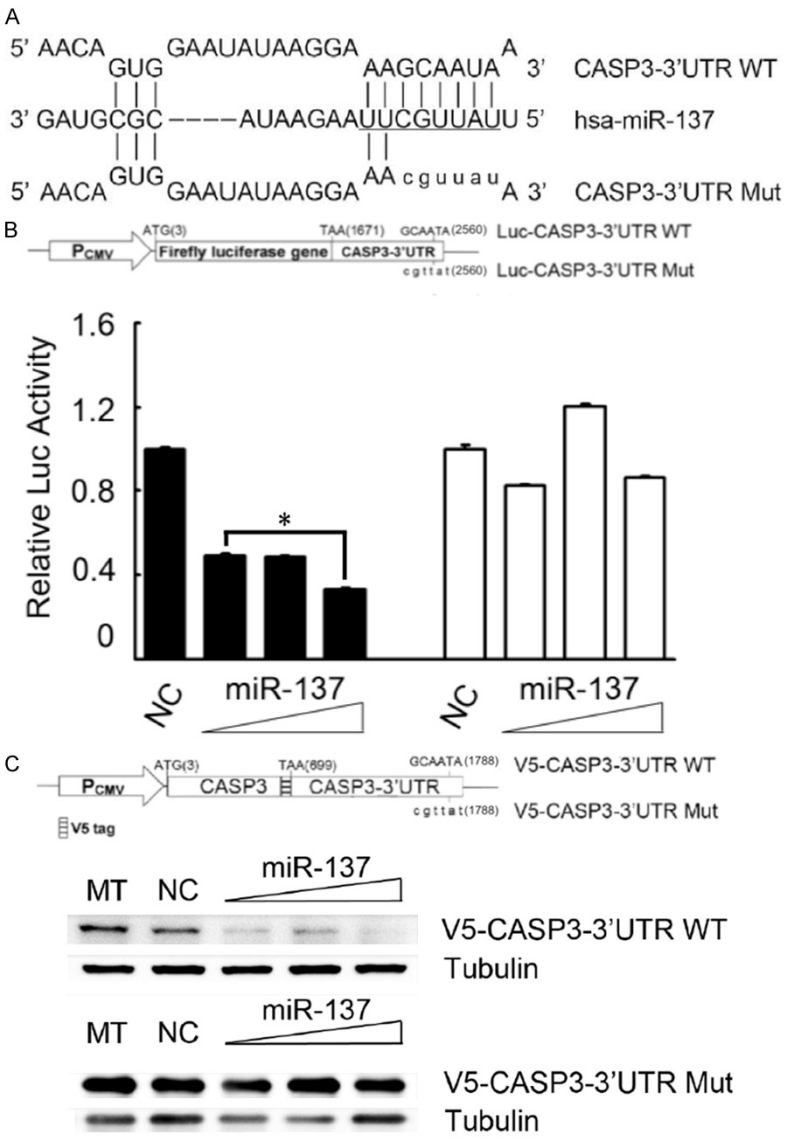

Figure 3. CASP3 is a direct target of *miR-137*. A. The predicted binding site of *miR-137* in the 3'UTR region of CASP3 mRNA. The seed region of *miR-137* is underlined. The mutated binding site used for the luciferase assay and Western blot is shown in lowercase. B. Reduction of CASP3 expression by *miR-137*, as determined by luciferase reporter assay. *miR-137* dose-dependently decreased the activity of luciferase with wild-type CASP3-3'UTR (left panel) but not the mutant one (right panel). C. A decrease in CASP3 protein expression by *miR-137*. Also, *miR-137* dose-dependently reduced V5-tagged CASP3 with wild-type 3'UTR but not the mutant. *$P < 0.05$, compared with negative control (NC) and mock transfection (MT).

patients ($P = 0.013$, log rank test; **Figure 2A**). Interestingly, when we further look into this correlation in adenocarcinoma and squamous cell carcinoma cohorts, only adenocarcinoma group showed significance ($P = 0.0000064$, log rank test; **Figure 2B**) but not squamous cell carcinoma group ($P = 0.69$, log rank test; **Figure 2C**). Therefore, we focused on the adenocarcinoma in the following experiments.

CASP3 is the direct target of miR-137

In accordance with the prediction, the seed region of *miR-137* was complementary to the 1053-1060 nucleotides of the 3'UTR of CASP3 (**Figure 3A**). Co-transfection of *miR-137* expression vector and reporter construct with wild-type CASP3 3'UTR (Luc-CASP3-3'UTR WT) into HEK293 cells significantly and dose-dependently reduced luciferase activity compared to the control vector (**Figure 3B**, left panel). To validate target specificity, we mutated the binding site of *miR-137* in 3'UTR. Co-transfection of *miR-137* and reporter construct with mutant 3'UTR (Luc-CASP3-3'UTR Mut) significantly diminished the reduction capability of *miR-137* on the luciferase activity of corresponding wild-type construction (**Figure 3B**, right panel). Furthermore, we generated the expression vector of V5-tagged CASP3 with wild-type or mutant 3'UTR (V5-CASP3-3'UTR WT and V5-CASP3-3'UTR Mut). The expression of CASP3 with wild-type 3' UTR could be dose-dependently inhibited by *miR-137* (**Figure 3C**). However, CASP3 with mutant 3'UTR could not be suppressed by mi*R-137*, suggesting that *miR-137* negatively regulates CASP3 expression by directly interacting with its 3'UTR.

The effect of miR-137 on CASP3 expression is independent of EGFR genotype

Because the expression of *CASP3* could predict the survival of lung adenocarcinoma patients

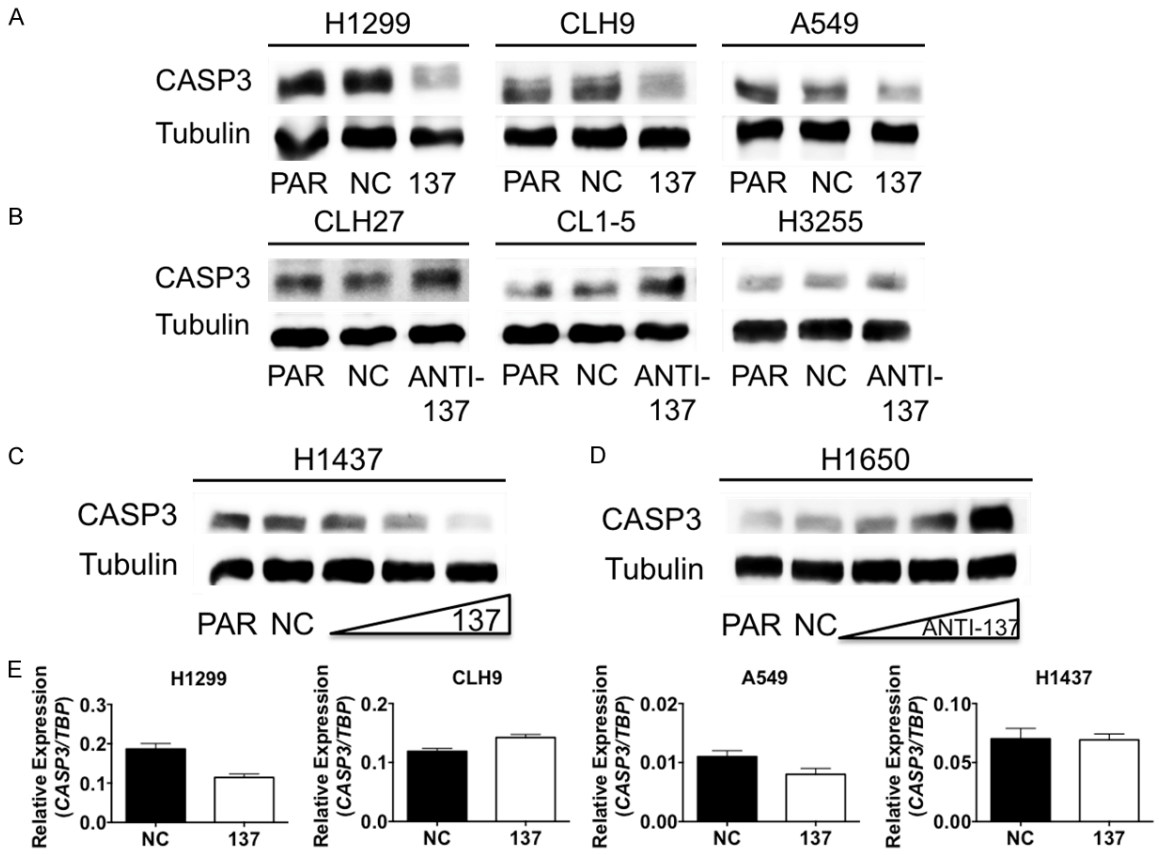

Figure 4. Downregulation of endogenous CASP3 expression by *miR-137* in lung adenocarcinoma cells. *miR-137* was overexpressed or silenced in lung adenocarcinoma cell lines with different EGFR status and then subjected to Western blotting. A. Reduction of CASP3 protein level in *miR-137*-overexpressed H1299, CLH9, and A549 cell lines. B. An increase of CASP3 protein level in anti-*miR-137* overexpressing CLH27, CL1-5, and H3255 cell lines. C. Dose-dependent decrease of CASP3 by increasing the amount of *miR-137* transfection in H1437 cells. D. Dose-dependent elevation of CASP3 by increasing amount of anti-*miR-137* in H1650 cells. PAR: parental cell; NC: negative control; 137: *miR-137* transfection; Anti-137: anti-*miR-137* transfection. E. The expression levels of CASP3 mRNA in various lung adenocarcinoma cell lines with *miR-137* overexpression, as determined by real-time RT-PCR. Left panel: H1299; middle left panel: CLH9; middle right panel: A549; right panel: H1437 lung cancer cells.

(**Figure 2B**) and lung adenocarcinoma is closely associated with *EGFR* genotype, we then examined the inhibitory effect of *miR-137* on CASP3 in lung adenocarcinoma cell lines bearing different *EGFR* status. Overexpression of *miR-137* reduced the protein expression levels of CASP3 in H1299 (WT-*EGFR*), CLH9 (Del19-*EGFR*), and A549 (WT-*EGFR*) cells (**Figure 4A**). Conversely, knockdown of *miR-137* increased the expressions of CASP3 in CLH27 (Del19-*EGFR*), CL1-5 (WT-*EGFR*), and H3255 (L858R-*EGFR*) cells (**Figure 4B**). In addition, the data illustrated in **Figure 1D** showed that *miR-137* overexpression could decrease CASP3 expression in H1975 cells with L858R/T790M mutation of *EGFR*. Moreover, we found that manipulating *miR-137* expression could dose-dependently reduce or promote CASP3 expression in H1437 (WT-*EGFR*) and H1650 (Del19-*EGFR*) cells (**Figure 4C** and **4D**), suggesting the sensitivity and specificity of *miR-137* targeting CASP3 in lung adenocarcinoma cells. Interestingly, we also observed the different changes on CASP3 mRNA expression level in *miR-137*-overexpressed lung cancer cells (**Figure 4E**), implying that the inhibitory machineries are cell line specific.

miR-137 modulates the cisplatin sensitivity in lung adenocarcinoma cells

Overexpression of *miR-137* in H1437 cells resulted in more resistance to cisplatin after treatment for 72 hours. The IC50 of H1437 cells with *miR-137* overexpression is 49.0 μM, which is almost a 2-fold change compared to

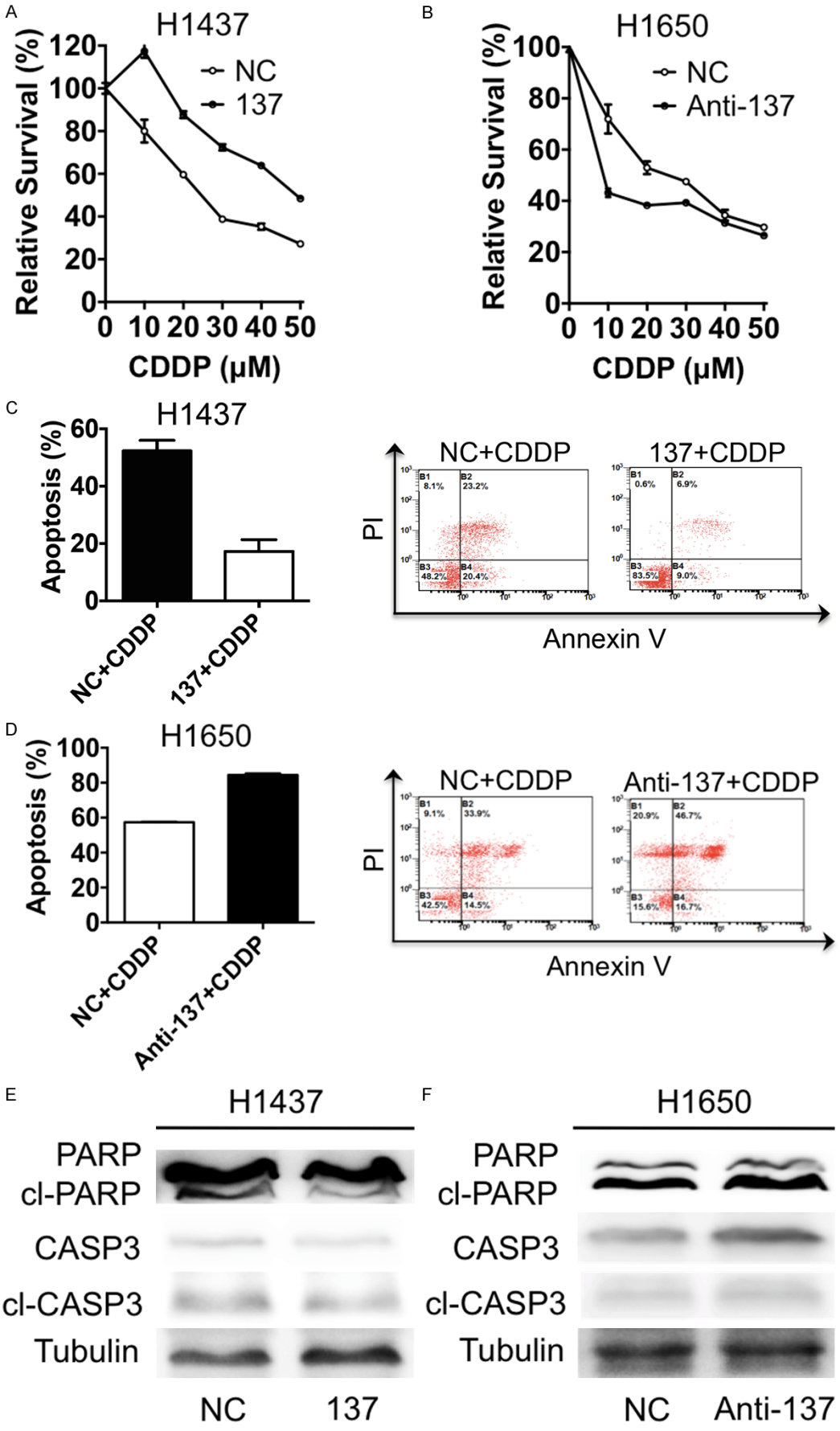
A
H1437
Relative Survival (%)
NC
137
CDDP (μM)
B
H1650
Relative Survival (%)
NC
Anti-137
CDDP (μM)
C
H1437
Apoptosis (%)
NC+CDDP
137+CDDP
NC+CDDP
137+CDDP
PI
Annexin V
D
H1650
Apoptosis (%)
NC+CDDP
Anti-137+CDDP
NC+CDDP
Anti-137+CDDP
PI
Annexin V
E
H1437
PARP
cl-PARP
CASP3
cl-CASP3
Tubulin
NC
137
F
H1650
PARP
cl-PARP
CASP3
cl-CASP3
Tubulin
NC
Anti-137

Figure 5. The change in cisplatin sensitivity and induced apoptosis in lung adenocarcinoma cells by altering *miR-137* expression. (A) Human lung cancer H1437 cells overexpressing *miR-137* and negative control were treated with the different concentrations of cisplatin for 72 hours and then subjected to MTT cell viability assays. (B) Cell viability of human lung cancer H1650 cells overexpressing anti-*miR-137* under the same condition of treatment mentioned above. Flow cytometry analyses of H1437 cells overexpressing *miR-137* (C) and H1650 cells overexpressing anti-*miR-137* (D) in the treatment of cisplatin for 48 hrs. The protein levels of CASP3, cl-CASP3, PARP, and cl-PARP were then detected in H1437 cells with *miR-137* overexpression (E) and in H1650 cells with anti-*miR-137* overexpression (F). NC: negative control; 137: *miR-137* transfection; Anti-137: anti-*miR-137* transfection.

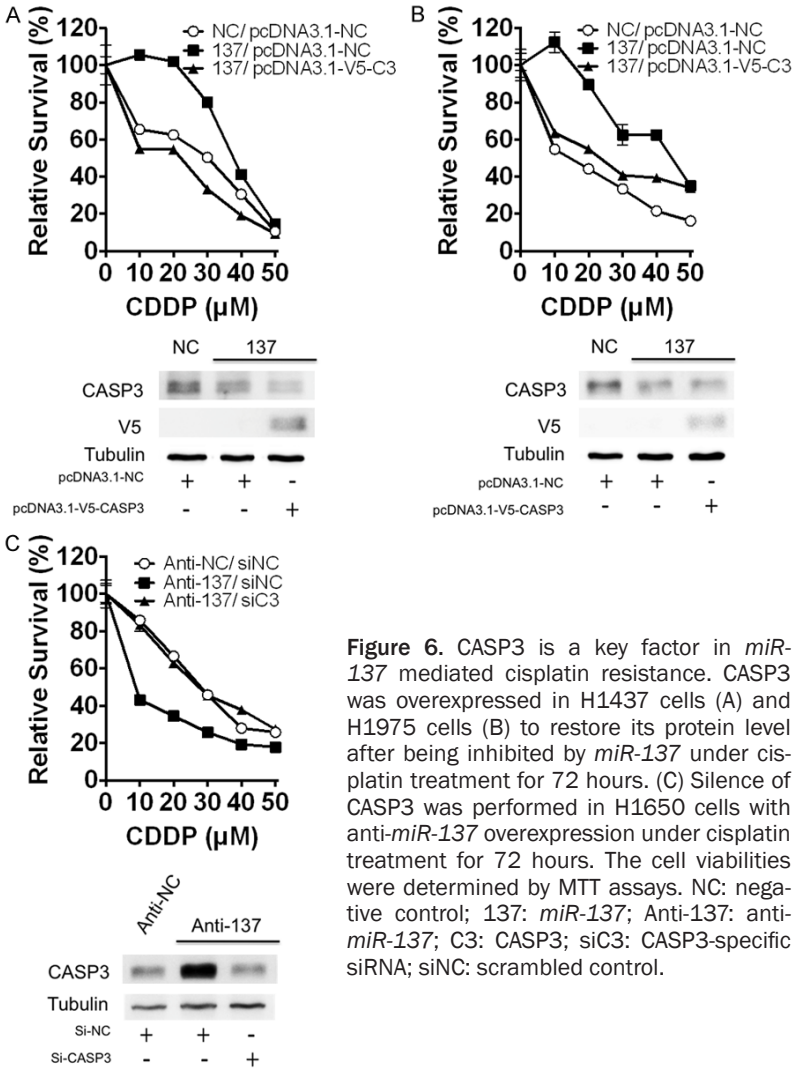

Figure 6. CASP3 is a key factor in *miR-137* mediated cisplatin resistance. CASP3 was overexpressed in H1437 cells (A) and H1975 cells (B) to restore its protein level after being inhibited by *miR-137* under cisplatin treatment for 72 hours. (C) Silence of CASP3 was performed in H1650 cells with anti-*miR-137* overexpression under cisplatin treatment for 72 hours. The cell viabilities were determined by MTT assays. NC: negative control; 137: *miR-137*; Anti-137: anti-*miR-137*; C3: CASP3; siC3: CASP3-specific siRNA; siNC: scrambled control.

negative control (IC50 is 24.6 μM) (**Figure 5A**). On the contrary, knockdown of *miR-137* in H1650 cells led to more sensitivity to cisplatin. The IC50 of H1650 transfected with negative control (25.4 μM) is around 2.5 folds higher than that of H1650 with anti-*miR-137* transfection (9.86 μM) (**Figure 5B**). Furthermore, *miR-137* overexpression decreased the apoptotic cell population in H1437, from 52.2±2.1% down to 17.3±2.3% (**Figure 5C**), while *miR-137* silencing increased the apoptotic cell population in H1650, from 57.4±0.15% up to 84.3±0.54% (**Figure 5D**). To further confirm that the attenuation of cisplatin-induced apoptosis by *miR-137* is through CASP3, the expression of the molecular markers of apoptosis was investigated by Western blot. Under cisplatin treatment, the H1437 cells overexpressing *miR-137* showed less apoptosis and reduced PARP cleavage and cl-CASP3 expression (**Figure 5E**). On the contrary, silencing *miR-137* rendered H1650 cells more apoptosis and expressing more PARP cleavage and cl-CASP3 compared to negative control (**Figure 5F**). CASP3 is the most active effector caspase, which plays a key role in the execution step of apoptosis. To determine the possibility of a functional interaction between miR-137 and CASP3, revealed in cisplatin resistance, we overexpressed V5-CASP3 in H1437 and H1975 cells after endogenous CASP3 was inhibited by *miR-137* and knocked down the endogenous CASP3 in H1650 cells after CASP3 has been restored by anti-*miR-137*. The enforced expression of CASP3 decreased cisplatin resistance of *miR-137*-overexpressing H1437 cells (IC50: from 37.7 μM down to 22.2 μM, **Figure 6A**) and H1975 cells (IC50: from 44.5 μM down to 23.4 μM, **Figure 6B**). Knockdown of CASP3 significantly increased cisplatin resistance of H1650 cells with anti-*miR-137* overexpression (IC50: from 8.8 μM to 27.4 μM, **Figure 6C**). Taken together, *miR-137*

Table 1. Clinical features of lung adenocarcinoma patients

Characteristic	All (N = 40)	*miR-137* low expression (N = 18)	*miR-137* high expression (N = 22)
Age (mean ± SD)	67.38±11.44	67.91±12.32	66.94±10.94
Sex			
Female	16	8	8
Male	24	10	14
Stage			
Unknown	1	0	1
I	25	13	12
II	4	2	2
III	9	3	6
IV	1	0	1

confers cisplatin resistance through inhibiting cell apoptosis by targeting CASP3.

miR-137 inversely correlates to CASP3 in lung adenocarcinoma patients

To further understand the potential biological significance of *miR-137* expression in lung cancer progression, we evaluated the correlation between the *miR-137* expression in tumor specimens and the clinical outcomes of lung adenocarcinoma patients (**Table 1**). Kaplan-Meier survival analyses showed that high levels of *miR-137* expression were significantly associated with decreased overall survival (P = 0.028, log rank test; **Figure 7A**) and progression-free survival (P = 0.014, log rank test; **Figure 7B**). Furthermore, a multivariate Cox proportional hazards regression showed that *miR-137* is an independent prognostic factor, without respect to overall (hazard ratio [HR] = 3.45, P = 0.049) or progression-free survival (HR = 2.79, P = 0.041). We next examined the expression of CASP3 and active CASP3 in the same cohort by immunohistochemistry (IHC) staining. Excluding the failed staining, there were 36 tissues applied for IHC scoring. We identified an inverse correlation between CASP3 and *miR-137* (R = -0.47, P = 0.004) (**Figure 7C**), as well as between active CASP3 and *miR-137* (R = -0.35, P = 0.038) (**Figure 7D**). In addition to Taiwanese patients, the TCGA lung adenocarcinoma dataset consisted of 458 patients was employed to investigate the relationship between *miR-137* expression and survival. The data showed that the patients with a higher *miR-137* expression experience significantly worse outcome (P = 0.0009; **Figure 7E**), which is consistent with the Taiwanese cohort.

Discussion

MiRNAs play critical roles in tumor progression. *miR-137* is a highly conserved miRNA among different species. It is frequently silenced by promoter methylation in several cancers [34-36], and potentially functions as a tumor suppressor miRNA due to the lower expression in various cancers including breast cancer [37] and colorectal cancer [38]. In lung cancer, there are few targets that have been verified, for example, BMP-7 [14], Kit [39], PXN [40], CDK6, and CDC42 [13]. By targeting these genes, *miR-137* inhibits cancer cell proliferation, invasion, and migration *in vitro*. However, the role of *miR-137* in tumor biology is still controversial. In the clinical aspect, the previous report has demonstrated that *miR-137* is a risk gene in NSCLC patients and can promote invasiveness [9]. In addition, the data from TCGA showed that higher expression of *miR-137* leads to poorer survival in 458 lung adenocarcinoma patients as well. Further investigations are needed to clarify the inconsistent biological functions of *miR-137* in cancer progression.

To better understand if miR-137 plays the oncogenic role in lung cancer is to find other potential targets of miR-137. In this study, we use microarray and in silico tool to identify CASP3 as a candidate target of miR-137. Moreover, our molecular and cellular biology approaches demonstrated that CASP3 is the direct and novel target gene of miR-137. Cisplatin-induced apoptosis can be reduced via the inhibition of CASP3 by miR-137, suggesting the oncogenic role of miR-137 in chemoresistance. Most importantly, the clinical evidence revealed that those patients with lower miR-137 expression have better prognosis and that there is a negative correlation between miR-137 and CASP3 expression in lung adenocarcinoma patients.

Cisplatin could induce apoptosis through the extrinsic pathway or intrinsic pathway. Abnormalities of apoptotic factors are related to platinum resistance [41]. As an important intrinsic and extrinsic apoptotic factor, CASP3 has been associated with chemoresistance of certain types of malignancies because of its

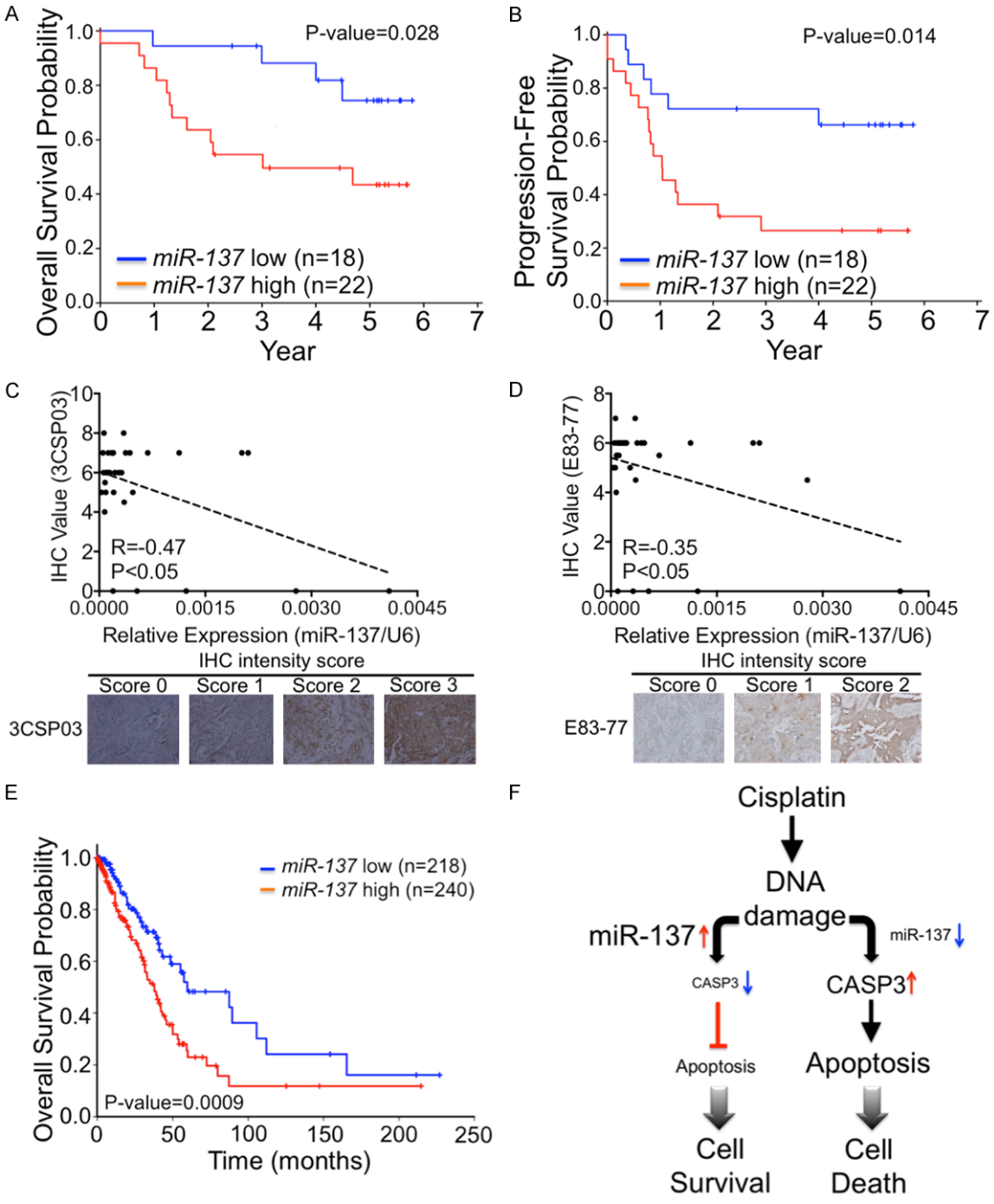

Figure 7. *miR-137* expression is negatively correlated with CASP3 and positively correlated with poor survival in lung adenocarcinoma patients. Kaplan-Meier plots of overall survival (A) and progression-free survival (B) of 40 lung adenocarcinoma patients, stratified by expression of *miR-137*. (C) An inverse correlation between CASP3 and *miR-137* expression; (D) An inverse correlation between cleaved CASP3 and *miR-137* expression, as determined by XY scatter plots using the IHC staining data of 36 tumor FFPEs. (E) Kaplan-Meier survival estimate of 458 lung adenocarcinoma patients obtained from the TCGA database according to *miR-137* expression. (F) An illustration of the potential mechanism of *miR-137* regulating cisplatin sensitivity in lung adenocarcinoma.

executioner role [42]. Loss of CASP3 is frequently observed in various solid tumors and is correlated with poor survival of patients with stomach and prostate cancer [43, 44]. In addition, CASP3-negative lung cancer has also been linked to the worst overall survival [45].

However, the low expression and malfunction of CASP3 caused by coding region mutation are rarely proposed, implying that non-genetic alterations might lead to the downregulation of CASP3 in lung cancer [46]. According to the public microarray data [33], we found that higher expression of CASP3 is associated with better survival in NSCLC (HR = 0.85), especially in lung adenocarcinoma (HR = 0.59). Our gene transfection experiments indicated that a change in *miR-137* level would alter CASP3 expression in lung adenocarcinoma cell lines, suggesting that the reduced expression of CASP3 might be caused by the increased *miR-137* level in lung adenocarcinoma patients. This speculation could be supported by the clinical results of a significantly negative correlation between *miR-137* and CASP3 in lung adenocarcinoma tissues using IHC staining (R = -0.47, *P* =0.004). Moreover, the *in vitro* studies also showed that the manipulation of *miR-137* level could change the sensitivity of lung adenocarcinoma cells to cisplatin. Overexpressing *miR-137* makes cells more resistant to cisplatin by inhibiting apoptosis; on the contrary, silencing *miR-137* induces more apoptotic cells under cisplatin treatment. Taken together, our findings suggest that the oncogenic *miR-137* confers lung adenocarcinoma the chemoresistance to cisplatin by functionally targeting CASP3.

Lung cancer is the major cause of cancer-related deaths worldwide. The most diagnosed histological subtype of non-small-cell lung cancer is lung adenocarcinoma, followed by squamous cell carcinoma. Although patients undergoing targeted therapy and immunotherapy tend to have better outcome, most patients with advanced adenocarcinoma are treated with chemotherapy due to cost issues. Chemotherapy is still widely used in the treatment for lung adenocarcinoma and remains slightly effective. Acquired chemoresistance is one of the most important problems in the treatment of lung cancer [30]. There are several studies indicating that miRNAs may act as regulators of chemosensitivity in various types of human cancer [47, 48]. More specifically in lung cancer, there are few miRNAs that have been linked to the chemosensitivity regulation, including let-7, miR-29, miR-34, miR-200, and miR-141 [49]. Although EGFR-TKI is much more effective, around 50% of the patients bearing wild-type EGFR, as well as those patients with developed TKI resistance, can only receive chemotherapy or radiotherapy in Taiwan. In our study, lung adenocarcinoma cell lines with different EGFR status (T790M, L858R, and Del19) were applied to manipulate *miR-137* expression. Unsurprisingly, CASP3 can be downregulated in these cell lines, suggesting that *miR-137* could serve as a chemoresistance marker for lung adenocarcinoma patients. Furthermore, *miR-137* and CASP3 could also be the targets for cancer treatment.

In summary, our studies show that CASP3 is a previously unidentified target of *miR-137*, and plays an essential role in the *miR-137*-mediated lung cancer progression. Furthermore, we demonstrate that the suppression of CASP3 via *miR-137* is involved in the apoptotic regulation and cisplatin resistance in lung adenocarcinoma (**Figure 7F**). Thus, *miR-137* might be a good diagnostic marker and an ideal therapeutic target for chemotherapy in lung adenocarcinoma patients.

Acknowledgements

This study was supported by grants from the Ministry of Science and Technology, Taiwan, R.O.C. (NSC 100-3112-B-005-001) and the Ministry of Health and Welfare, Taiwan, R.O.C. (DOH97-TD-G-111-017), as well as in part by the Ministry of Education, Taiwan, R.O.C. under the ATU plan.

Disclosure of conflict of interest

None.

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