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# miR-16 targets Bcl-2 in paclitaxel-resistant lung cancer cells and overexpression of miR-16 along with miR-17 causes unprecedented sensitivity by simultaneously modulating autophagy and apoptosis

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ABSTRACT

Non-small cell lung cancer is one of the most aggressive cancers as per as the mortality and occurrence is concerned. Paclitaxel based chemotherapeutic regimes are now used as an important option for the treatment of lung cancer. However, resistance of lung cancer cells to paclitaxel continues to be a major clinical problem nowadays. Despite impressive initial clinical response, most of the patients eventually develop some degree of paclitaxel resistance in the course of treatment. Previously, utilizing miRNA arrays we reported that downregulation of miR-17 is at least partly involved in the development of paclitaxel resistance in lung cancer cells by modulating Beclin-1 expression [1]. In this study, we showed that miR-16 was also significantly downregulated in paclitaxel resistant lung cancer cells. We demonstrated that anti-apoptotic protein Bcl-2 was directly targeted miR-16 in paclitaxel resistant lung cancer cells. Moreover, in this report we showed that the combined overexpression of miR-16 and miR-17 and subsequent paclitaxel treatment greatly sensitized paclitaxel resistant lung cancer cells to paclitaxel by inducing apoptosis via caspase-3 mediated pathway. Combined overexpression of miR-16 and miR-17 greatly reduced Beclin-1 and Bcl-2 expressions respectively. Our results indicated that though miR-17 and miR-16 had no common target, both miR-16 and miR-17 jointly played roles in the development of paclitaxel resistance in lung cancer. miR-17 overexpression reduced cytoprotective autophagy by targeting Beclin-1, whereas overexpression of miR-16 potentiated paclitaxel induced apoptotic cell death by inhibiting anti-apoptotic protein Bcl-2.



## 1. Introduction

Worldwide, lung cancer appears to be one of the most commonly diagnosed cancers causing almost 1.38 million deaths and 1.61 million new occurrences each year [2]. Taxens, specially paclitaxel has emerged as an important, broad range chemotherapeutic agent since paclitaxel based combination chemotherapies are now being globally prescribed as standard therapies in the treatment of lung cancer as well as other cancers such as breast, ovarian, and prostate [3–5]. However, despite its wide spread usage, resistance of cancer cells to paclitaxel has become frequent and has been recognized as the major reason for reoccurrence, cancer progression and failure of therapy in many cancer types [6]. During paclitaxel resistance, the cancer cells eventually escape the paclitaxel induced apoptotic death and this failure of paclitaxel treatment to induce apoptosis ultimately contributes to relapse and poor prognosis [1,3,4,7–9]. In the last couple of years considerable amount of research is going on to understand the mechanism of paclitaxel resistance to overcome the problem of drug resistance in paclitaxel therapy.

MicroRNAs (miRNAs) are endogenous non-protein coding, single stranded RNA of ~22 nucleotide length which plays important roles in controlling gene expression in humans [10]. miRNAs guide the RNA-induced silencing complex (RISC) to bind to the complementary sequences within the 3′ untranslated region of the cognate mRNA and thereby either translationally repress the mRNA expression or induce destabilization and degradation of the target mRNA bearing fully complementary target sites [10,11]. Previous studies involving NCI-60 panel of cell lines have shown that miRNA expression patterns are better representative of the type and stage in human cancers than classical mRNA profiles [12]. Moreover, dysregulated miRNA expression has already been reported in the case of various human malignancies including non-small cell lung cancer (NSCLC) [1,3,13].

We have been working on the role of miRNA in the development of paclitaxel-resistance in lung cancer cells. In our previous report, we

*Abbreviations:* Tx, paclitaxel; miR/miRNA, micro RNA; PI, propidium iodide; MTT, (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide); AO, acridine orange; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodide; H2-DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; FITC, fluorescein isothiocyanate; MDC, monodansylcadaverine; AVOs, acidic vacuoles; ROS, reactive oxygen species; MMP, mitochondrial membrane potential change

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performed miRNA array analysis to screen differentially expressed miRNAs between paclitaxel resistant and paclitaxel sensitive lung cancer cells and we reported that downregulation of miR-17 contributed to the development of paclitaxel resistance of lung cancer cells by increasing Beclin-1 expression and subsequent autophagy [1]. From the microarray data we found that miR-16 was also significantly downregulated in paclitaxel resistant lung cancer cells. So in this current study, we first investigated the role of downregulation of miR-16 in the development of paclitaxel-resistance in lung cancer cells and then we determined the effect of combined overexpression of miR-16 and miR-17 and subsequent paclitaxel treatment in modulating the sensitivity of resistant cells to paclitaxel. We demonstrated that anti-apoptotic protein Bcl-2 was a direct target of miR-16 in paclitaxel resistant lung cancer cells. Ectopic expression of miR-16 into paclitaxel resistant lung cancer cells significantly sensitized the cells to paclitaxel.

Since it is understood that downregulation of a single miRNA cannot be held solely responsible for the development of paclitaxel resistance. Other miRNAs could be involved in this process. We previously found that miR-17 was involved in the development of paclitaxel resistance in lung cancer cells [1]. So we were interested to compare the effects of combined overexpression of miR-16 and miR-17 and subsequent paclitaxel treatment with the effects of individual miRNA overexpression (miR-16 or miR-17 individually) more clearly, we showed the results for individual miR-16 or miR-17 overexpression and subsequent paclitaxel treatment in parallel with the combined miR-16 and miR-17 overexpression results. Our data clearly demonstrated that both miR-16 and miR-17, without having a common target, jointly played role in the development of paclitaxel resistance in lung cancer cells and combined inhibition of Beclin-1 mediated autophagy by miR-17 overexpression and downregulation of anti-apoptotic protein Bcl-2 by miR-16 overexpression stimulated ROS generation, which was required for paclitaxel mediated apoptotic cell death in paclitaxel resistant lung cancer cells.

## 2. Materials and methods

### 2.1. Reagents and antibodies

Nutrient mixture Dulbecco's Modified Eagle's Medium (supplemented with 1 mM L-glutamine), FBS (fetal bovine serum), penicillin–streptomycin, amphotericin B and 0.25% Trypsin–EDTA were purchased from GIBCO (Invitrogen, USA). Annexin V–FITC apoptosis detection kit was obtained from BD Bioscience, USA. Paclitaxel (Tx), JC-1, H2-DCFDA, zVAD-fmk, MTT, monodansylcadaverine (MDC), acridine orange (A.O) and anti-Bax antibody (mouse monoclonal) were purchased from Sigma, USA. The antibodies to anti-PI3K (p85) (rabbit monoclonal), anti-cleaved caspase 9 (Asp330, rabbit monoclonal), anti-cleaved PARP (Asp214, human specific, mouse monoclonal) (89 kDa), anti-Akt (mouse monoclonal), anti-phospho-Akt (mouse monoclonal) and anti-GAPDH (mouse monoclonal) were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-cleaved caspase 3 (Asp175, rabbit monoclonal) was from Merck Millipore, Germany. Anti-phosphotyrosin (PY20) (mouse monoclonal) and anti-mTOR antibody (N-19, goat polyclonal) were obtained from Santa Cruz Biotechnology, USA. Bradford protein estimation kit was purchased from Genei, India. All other chemicals and reagents were of analytical grade and were purchased from Sisco Research Laboratories, India.

### 2.2. Cell line and cell culture

Human non-small lung epithelial adenocarcinoma cell line Type II, A549, was obtained from the cell repository of the National Centre for Cell Science (NCCS), Pune, India. Human lung adenosquamous carcinoma cell line NCI-H596 and human non-small cell lung adenocarcinoma cell lines NCI-H1734 and NCI-H1299 were obtained from American Type Culture Collection (ATCC). All the cell lines were characterized by mt-rDNA sequencing for species identification, short tandem repeat profiling and isoenzyme analysis for cell line authentication and were confirmed not to be mycoplasma contaminated by the repository. Cells were selected for resistance to paclitaxel in a stepwise manner essentially as described in [1]. All the cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1 mM L-glutamine, 10% FBS, 3.7 g/l $NaHCO_3$, 100 μg/ml each of penicillin and streptomycin and 2.5 μg/ml amphotericin B. Cells were maintained at 37 °C in a humidified atmosphere containing 5% $CO_2$. Cells were grown up to ~80% confluency in tissue culture plates, then trypsinized with 0.25% Trypsin–EDTA and divided into subsequent culture plates as required.

### 2.3. Pre-miRNA transfection

mirVana miRNA 17 mimic precursor (pre-miR-17), mirVana miRNA 16 mimic precursor (pre-miR-16) and mirVana miRNA mimic negative control #1 (pre-miR-negative control) were purchased from Ambion, USA. Pre-miRNAs were transfected into cell lines at ~50% confluency at 100 nM concentration with Lipofectamine RNAiMAX (Invitrogen) transfection reagent. Forty-eight hours after transfection, the expressions of miRNAs were detected by real-time PCR and the expressions of Beclin-1 and Bcl-2 were analysed by qRT-PCR and/or Western blotting.

### 2.4. Quantitative real-time PCR (qRT-PCR)

TaqMan qRT-PCR was performed to evaluate the expression of miRNAs using the TaqMan microRNA reverse transcription kit (Applied Biosystems, USA) and TaqMan microRNA assays kit (Applied Biosystems, USA) following the manufacturer's protocols. Expression of U6 SnRNA was used as the internal reference to normalize the relative miRNA expression data. To analyze the expression of Bax, Beclin-1, Bcl-2, p62, LC3-II and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1) cDNAs were prepared from 2 μg of total RNA using SuperScript VILO cDNA Synthesis kit (Invitrogen, USA). Requisite amount of cDNA was mixed with 2× DyNAmo ColorFlash SYBR Green qPCR Master Mix (Thermo Scientific) and various sets of gene-specific primers (Table 1) and then subjected to qRT-PCR quantification using the StepOne-Plus real time PCR system (Applied Biosystems, USA). Gene expression was normalized with respect to GAPDH (for Beclin-1, Bax, Bcl-2, LC3-II, p62 etc.) or U6 SnRNA (for miR-17 and miR-16) using the comparative cycle time (Ct) method ($2^{-\Delta\Delta Ct}$ method) [14].

### 2.5. Cell proliferation inhibition assay (MTT assay)

A549-T24 and H596-TxR cells, either transfected with 100 nM pre-miR-17 (T24-miR-17 and TxR-miR-17 respectively) or with 100 nM pre-miR-16 (T24-miR-16 and TxR-miR-16) or with 50 nM each of both pre-miR-17 and pre-miR-16 (T24-miR-comb and TxR-miR-comb) or with 100 nM pre-miR-negative control RNA (T24-miR-NC and TxR-miR-NC respectively) were plated in 96-well culture plates (1

**Table 1**
Primer sequences used for real-time PCR.

| Gene name | Forward primer sequence (5′–3′) | Reverse primer sequence (5′–3′) |
|---|---|---|
| *Bcl-2* | TTGGATCAGGGAGTTGGAAG | TGTCCCTACCAACCAGAAGG |
| *LC3-II* | GAGAAGCAGCTTCCTGTTCTGG | GTGTCCGTTCACCAACAGGAAG |
| *Beclin-1* | CAAGATCCTGGACCGTGTCA | TGGCACTTTCTGTGGACATCA |
| *p62* | TGTGGAACATGGAGGGAAGAG | TGTGCCTGTGCTGGAACTTTC |
| *Bax* | GGACGAACTGGACAGTAACATGG | GCAAAGTAGAAAAGGGCGACAAC |
| *P53* | GTTCCGAGAGCTGAATGAGG | TTATGGCGGGAGGTAGACTG |
| *GAPDH* | CACCATGGAGAAGGCTGGGGCTC | GCCCAGGATGCCCTTGAGGG |