MicroRNA-17 downregulation contributes to paclitaxel based chemo-resistance of lung cancer cells through altering Beclin 1 expression.
Graphical abstract showing ectopic overexpression of miR-17 into paclitaxel resistant lung cancer cells resulted in reduction of cellular autophagy. It also demonstrated that forced overexpression of miR-17 into paclitaxel resistant lung cancer cells and subsequent paclitaxel treatment for 24 h resulted in induction of apoptosis in paclitaxel resistant lung cancer cells.
5.1. Introduction

Lung cancer is one of the most common malignancies and one of the leading causes of cancer related deaths in this world. Paclitaxel based combination chemotherapies are now been considered as standard therapies for nearly all patients diagnosed with NSCLC \[210\]. Unfortunately, the clinical affectivity of paclitaxel is limited because some tumours show resistance or become resistant to it after repeated cycles of paclitaxel based chemotherapy which ultimately leads to relapse and poor prognosis. The most reported mechanisms of paclitaxel resistance involves upregulation of P-glycoprotein and related drug efflux pumps \[207,211\], inadequate interaction with spindle microtubules due to posttranslational modification or altered expression of tubulin isotypes and microtubule-associated proteins \[212-214\] or functional change in cell signalling and cell survival pathways \[153,229-231\]. Recent studies show that autophagic induction by paclitaxel plays a major role in the development of paclitaxel resistance in tumor cells \[102-104\].

MicroRNAs, a highly conserved family of small, non- coding RNAs which recently emerged as novel class of gene expression modulators at posttranscriptional level \[127,220,221\]. Aberrant miRNA expression has been frequently observed in various human cancers including NSCLC \[222,223\]. In recent years, attempts have been made to correlate dysregulation of particular miRNA expression with tumor responsiveness to chemotherapies, including paclitaxel \[103,156,162,177,216\].

In this study, we were interested to examine the role of miRNAs in the development of paclitaxel resistance in lung cancer cells related to autophagy. We performed miRNA arrays to screen differentially expressed miRNAs between paclitaxel- sensitive (A549) and paclitaxel- resistant lung cancer cells (A549-T24). We identified that miR-17 was downregulated in paclitaxel resistant lung cancer cells (A549-T24 and H596-TxR) and its overexpression promoting paclitaxel induced cytotoxicity and apoptosis. Moreover, our data demonstrated that beclin1, one of the most important regulators of cellular autophagy, was a direct target of miR-17 in lung cancer cells.

Taken together all the findings we concluded that miR-17 played a critical role in the development of paclitaxel resistance by regulating cellular autophagy. Suppression of expression of miR-17 was associated with the upregulation of beclin1 expression and concordant autophagy which played a cyto-protective role and protected the cells from paclitaxel induced apoptosis and cell death.
5.2. Methods:

5.2.1. Cell line and cell culture

Both A549 and NCI-H596 cells were selected for resistance to paclitaxel (Sigma, USA) in a stepwise manner essentially as previously described. All the cell lines were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 1mM L-glutamine, 10% fatal bovine serum, 3.7 gm/L NaHCO₃, 100 µg/mL each of penicillin and streptomycin and 2.5 µg/mL amphotericin B.

5.2.2. miRNA micro array expression analysis

miRNA profiling of A549 and A549-T24 cells were done from Exiqon (Vedbaek, Denmark) and the procedure was essentially described in the precious section. Only those miRNAs which were dysregulated by at least two fold (ΔLMR ≥ 2) were taken into consideration.

5.2.3. Pre- miRNA transfection

mirVana miRNA 17 mimic precursors (pre-miR-17) and mirVana miRNA mimic negative control #1 (pre-miR-negative control) were transfected into cell lines at ~50% confluency at 100 nM concentration with Lipofectamine RNAiMAX (Invitrogen) transfection reagent as previously described. Forty-eight hours after transfection, the expression of miR-17 was detected by real-time PCR and the expression of BECN1 was tested by qRT-PCR and/or Western blotting.

5.2.4. Quantitative real-time PCR (qRT–PCR)

For miRNA expression analysis, qRT-PCR was done by using the TaqMan microRNA reverse transcription kit (Applied Biosystems) and TaqMan microRNA assays kit (Applied Biosystems) following the manufacturer’s protocols. U6 SnRNA served as the internal control.

To analyse the expression of BECN1, Bax, Bcl-2, LC3-II and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and caspase-3 (Table 1), cDNAs were synthesized from 1µg of total RNA using SuperScript VILO cDNA Synthesis kit (Invitrogen). The cDNA was mixed with 2x DyNAmo ColorFlash SYBR Green qPCR Master Mix (Thermo Scientific) and various sets of gene-specific primers and then subjected to qRT-PCR quantification using the StepOne- Plus real time PCR system (Applied Biosystems). Gene expression was calculated relative to GAPDH (for BECN1, Bcl-2, Bax, LC3-II, caspase-3 etc) or U6 SnRNA (for miR-17) using the comparative cycle time (Ct) method (2⁻ΔΔCt method) [232,233].
5.2.5. 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-negative control RNA (T24-miR-NC and TxR-miR-NC respectively) were plated in 96-well culture plates (1×10⁴ cells per well). After 24 h incubation, cells were treated with different concentrations of paclitaxel for another 24 h and the cell viability was measured by MTT assay, as previously described.

5.2.6. Construction of luciferase reporter constructs and luciferase activity assay

The 3’UTR-luciferase reporter constructs containing the 3’UTR of BECN1 with or without miR-17 binding site were PCR amplified from total cDNAs prepared from total RNA obtained from A549-T24 cells. The PCR products were cloned into pCI-neo-RL-luc reporter vector (A generous gift from Dr. SN Bhattacharyya, IICB, Kolkata, India.) between XbaI and NotI restriction sites, immediately downstream of the renilla luciferase gene. All the luciferase constructs were sequence verified. Cells were transiently co-transfected with renilla luciferase reporter plasmids (pCI-neo-RL-Bec-3’UTR-wt or pCI-neo-RL-Bec-3’UTR-mut), firefly luciferase plasmid (pGL3-FF) and pre-miR-17 precursor and/ or anti-miR-17 or pre-miR-negative control precursor RNA using lipofectamine2000 transfection reagent following manufacturer’s protocol. After 48 h of transfection, luciferase activities were measured by using Promega dual luciferase reporter assay kit on a VICTOR X3 Plate Reader system (PerkinElmer). The relative luciferase activities were calculated by the ratio of Renilla luc/Firefly luc activity and normalized to that of the control cells and fold repression was calculated. pGL3-FF vector was used as the internal control.
5.2.7. Detection of acidic vesicular organelles (AVOs)

To observe the reduction in AVOs formation, T24-miR-17 and T24-miR-NC cells were seeded on cover slips. After 48 h of transfection, cells were stained with acridine orange (AO) (1 µg/ml) at 37°C in the dark for 15 min, then visualized immediately using fluorescence microscope (OLYMPUS IX70, Japan). The cytoplasm and nucleus of stained cells fluoresced bright green, whereas the acidic autophagic vacuoles fluoresced bright red. Similar experiments were also performed with A549 cells.

To quantify the change in number of acidic vesicles (AVOs) in A549, T24-miR-NC and T24-miR-17 cells, they were stained with AO (1 µg/ml) in PBS at 37°C for 15 min, the cells were harvested, washed twice in PBS and resuspended in 500 µl PBS and then analysed immediately by flow cytometry assay. The flow cytometric data was analysed with CellQuest analysis software (Becton Dickinson) [105].

5.2.8. Labelling of autophagic vacuoles with monodansylcadaverine

A549-T24 cells were transfected either with pre-miR-17 (100 nM) or with pre-miR-negative control RNA (100 nM), seeded on coverslips and kept for another 48 h. Cells were incubated with 50 µM monodansylcadaverine for 10 min at 37°C in PBS and images were taken by fluorescence microscope (OLYMPUS IX70, Japan). Moreover, for quantitative analysis of autophagosome formation same samples were trypsinized and analysed by flow cytometry assay [234].

5.2.9. Flow cytometric analysis for apoptotic cells

T24-miR-NC and T24-miR-17 cells were treated with 24 nM and 50 nM paclitaxel for 24 h. After 24 h of paclitaxel treatment cells were stained with FITC-conjugated annexinV (1 µg/ml) and propidium iodide (PI) (0.5 µg/ml) in a Ca²⁺-enriched binding buffer and analyzed by a two color flow cytometric assay as previously described. AnnexinV and PI emissions were detected in the FL1 and FL2 channels of a FACSCalibur flow cytometer (Becton-Dickinson, USA) respectively [235]. The data was analysed by CellQuest program from Becton-Dickinson.

5.2.10. Determination of Mitochondrial Membrane Potential (ΔΨ)

T24-miR-NC and T24-miR-17 cells were treated with 24 nM and 50 nM paclitaxel for 24 h. Cells were then harvested, washed twice with PBS, stained with 5 µM JC-1 for 30 min at 37°C in the dark [236]. Cells were rinsed with PBS twice, resuspended in 500µl PBS...
and instantly assessed for red fluorescence (JC-1) with FACSCalibur flow cytometer (Becton-Dickinson, USA).

5.2.11. Measurement of Reactive Oxygen Species (ROS)

ROS levels were determined using the fluorescent marker (DCFH-DA) [237]. Briefly, T24-miR-NC and T24-miR-17 cells were treated with 24 nM and 50 nM paclitaxel for 24h. Cells were trypsinized, washed with PBS and incubated with 10 mM DCFH-DA for 30 min in the dark at room temperature and the shift in the green fluorescence intensity, as detected in FL1 channel, was followed by FACSCalibur flow cytometer (Becton-Dickinson, USA) and the data was analysed with CellQuest analysis software (Becton-Dickinson, USA).

5.2.12. Statistical analysis

qRT-PCR reactions were run in triplicate for each sample and repeated at least 3 times and the data were statistically analyzed with Student’s ‘t-test’ or Wilcoxon rank sum test. IC\textsubscript{50} data from the MTT assay were analyzed with Wilcoxon rank sum test. All data were shown as the means ± S.E. (Standard error). Two measurements were statistically significant if the corresponding p value was <0.05.
5.3. Results

5.3.1. Profiles of miRNAs in paclitaxel-sensitive and resistant lung cancer cells

To search for the critical miRNAs involved in the development of paclitaxel resistance we performed differential miRNA profiling between paclitaxel sensitive and resistant A549 cells. From the array results it was identified that 23 miRNAs were differentially expressed in A549-T24 cells than A549 cells (Fig. 1).

To identify the target genes of these differentially expressed miRNAs, we searched miRNA target prediction databases miRBase, microRNA.org and TargetScanHuman 6.2 and we identified miR-17 could probably have a role in autophagy by targeting autophagy related protein beclin 1 (BECN1) by binding directly to its 3’ UTR region between position 135 to 141. It is already reported that induction of autophagy by paclitaxel treatment plays a major role in the development of paclitaxel resistance in tumor cells with upregulation of BECN1 [102-104]. We observed increased level of autophagy as indicated by upregulation of beclin 1 and increased LC3-I to LC3-II conversion and downregulation of p62 expression in A549-T24 cells compared to A549 cells (Fig. 2A).

Figure 1. Micro-RNA profiles of paclitaxel resistant and paclitaxel sensitive A549 cells.

Figure 2. (A) Expression status of autophagic marker proteins BECN1, MAP-LC3, p62 and GAPDH (loading control) were measured by Western blotting. (B-C) Relative BECN1 and LC3-II mRNA expression levels were quantified by qRT-PCR analysis in A549 and A549-T24 cells, bars represent mean ± S.E.
We also measured relative mRNA levels of BECN1 and LC3-II (Fig. 2B and 2C) and found both these mRNAs were upregulated in A549-T24 cells compared to A549 cells.

Figure 3. (A) Expression status of certain autophagic marker proteins BECN1, MAP-LC3 and GAPDH (loading control) were measured by Western blotting. (B-C) Relative BECN1 and LC3-II mRNA expression levels were quantified by qRT-PCR analysis in TxR-miR-NC and TxR-miR-17 cells, bars represent mean ± S.E.

To extend our finding we examined the status of BECN1 and LC3 in this resistant lung cancer cell line (H596-TxR). We found similar upregulation of BECN1 and increased LC3-I to LC3-II conversion in paclitaxel resistant H596 cells (H596-TxR) compared to parental H596 cells both in mRNA and protein expression level (Fig. 3).

5.3.2. miRNA-17 is Downregulated in Paclitaxel- Resistant Lung Cancer Cells

Using Taqman probe- based qRT- PCR assay, we compared the expression level of miR-17 in paclitaxel sensitive and resistant A549 cells.

Figure 4. Downregulation of miR-17 expression in paclitaxel resistant A549-T24 and H596-TxR cells compared to A549 and H596 cells, respectively. Taqman qRT-PCR was performed to detect the relative levels of miR-17 in A549 and A549-T24 cells (A) and also in H596 and H596-TxR cells (B). Results were normalized to snU6 expression level and represented as mean ± S.E. from three independent replicates.
Fig. 4A shows a ~7.2 fold downregulation in the relative miR-17 expression level in A549-T24 cells compared to A549 cells, validating the micro-array results. Moreover, estimation of expression level of miR-17 in H596-TxR and H596 cells revealed that H596-TxR cells exhibited almost ~2.62 fold downregulation of relative miR-17 expression compared to H596 cells (Fig. 4B) indicating association between miR-17 and paclitaxel resistance was not cell line specific.

5.3.3. Sensitivity to Paclitaxel is modulated by over-expression of miR-17 in vitro.

To investigate whether miR-17 overexpression sensitized A549-T24 cells to paclitaxel, we transfected A549-T24 cells with 100 nM pre-miR-17 (T24-miR-17) or 100 nM pre-miR- negative control RNA (T24-miR-NC) and 24 h following transfection cells were treated with different doses of paclitaxel (0- 200 nM).

![Figure 5. miR-17 overexpression modulates paclitaxel response to paclitaxel resistant lung cancer cells. T24-miR-NC and T24-miR-17 cells (A) and TxR-miR-NC and TxR-miR-17 cells were seeded into 96 well plates at a density of 1x 10^4 cells per well. After 24 h, cells were treated with 0- 200 nM Paclitaxel for another 24 h. The cell viability was assessed by MTT assay. Data are presented as % of cell viability measured in cells treated with Paclitaxel. Columns, mean of three independent experiments; bars, mean ±S.E. (*P < 0.03 vs control, where n=3).](image)

It was observed that compared with the negative control (T24-miR-NC), overexpression of miR-17 significantly sensitized the A549-T24 cells to paclitaxel (Fig. 5A). Similarly with H596-TxR cells, it was observed that compared to the negative control (TxR-miR-NC), TxR-miR-17 cells exhibited much lower cell viability when treated with increasing concentrations of paclitaxel (Fig. 5B).

5.3.4. Beclin 1 is a direct target of miR-17 in lung cancer cells

To determine whether miR-17 directly binds to the 3’ UTR region of beclin 1 mRNA, we constructed 3’ UTR reporters of beclin 1 containing putative miR-17 binding site and
corresponding mutant construct, lacking miR-17 binding site, downstream of the luciferase reporter gene were also constructed (Fig 6A).

Figure 6. miR-17 directly binds to the 3’ UTR of beclin 1 gene in A549-T24 cells. A549-T24 cells were co- transfected with renilla luciferase reporter plasmids (pCI-neo-RL-Bec-3’UTR-wt or pCI-neo-RL- Bec-3’UTR-mut), firefly luciferase plasmids (pGL3-FF) and pre-miR-17 precursor or anti-miR-17 or pre-miR-negative control precursor RNA using lipofectamine2000 transfection reagent. After 48 h, cells were harvested and lysed with passive lysis buffer. Luciferase activity was measured by using Promega dual luciferase reporter assay kit. The results were represented as relative fold repression (Renilla luc/ Firefly luc activity) compared to control cells.

Co-transfection of pre-miR-17 with BECN1 wild-type reporter construct in A549-T24 cells greatly repressed renilla luciferase activity (Fig. 6B), while co-transfection with mutant reporter construct showed no significant change in relative luciferase activity with that of control (Fig. 6B). Moreover, when A549-T24 cells were co-transfected with anti-miR-17 (100 nM) and 3’ UTR reporters of BECN1, with or without putative miR-17 binding site, no significant decrease in relative luciferase activity was observed (Fig. 6B). These results collectively confirmed that BECN1 was a direct target of miR-17 in lung cancer cells.

5.3.5. Over-expression of miR-17 in paclitaxel resistant lung cancer cells leads to Beclin 1 downregulation

To experimentally validate target prediction, we assessed the protein and mRNA expression levels of BECN1 following miR-17 overexpression into both A549-T24 and H596-TxR cells. We observed that irrespective of the cell type, overexpression of miR-17 significantly decreased Beclin 1 expression as compared to the negative control (T24-miR-NC and TxR-
miR-NC) (Fig. 7A-B and Fig. 7C-D). Moreover, overexpression of miR-17 into A549-T24 and H596-TxR cells reduced conversion of LC3-I to LC3-II (Fig. 7A and 7C and Fig. 7D and 7E). All these data collectively proved that overexpression of miR-17 into paclitaxel resistant lung cancer cells caused reduction in cellular autophagy by directly targeting autophagy related protein BECN1.

Figure 7. miR-17 modulates BECN1 expression in paclitaxel resistant lung cancer cells. (A-C) A549-T24 cells were transfected either with 100nM pre-miR-negative (T24-miR-NC) or pre-miR-17 (T24-miR-17) precursor RNA. 24 h following transfection, the status of BECN1 and LC3II were determined either by Western blotting or qRT-PCR. (D-E) Relative BECN1 and LC3-II expression levels were quantified either by Western blotting or by qRT-PCR analysis in TxR-miR-NC and TxR-miR-17 cells.

5.3.6. Overexpression of miR-17 inhibits autophagy in paclitaxel resistant A549-T24 cells

Paclitaxel induces autophagy in cancer cells [102-104]. Autophagy induction under stressed conditions is generally considered to have a prosurvival role [103,105,238]. During autophagy, autophagosomes fuse with lysosomes to form autophagolysosomes which are acidic vacuoles (AVO) that bind acridine-orange giving red fluorescence. Fig. 8A-B showed compared to A549 cells (Fig. 8A) T24-miR-NC cells exhibited large number of red fluorescent vesicles were observed in the cytoplasm (Fig. 8B) which reduced significantly in T24-miR-17 cells (Fig. 8C). Upon flow cytometric analysis (Fig. 8D-G), we observed compared to A549 cells, T24-miR-NC cells showed higher levels of autophagic flux (Fig. 8D)
vs. Fig. 8E) which significantly diminished in T24-miR-17 cells (Fig. 8F vs. Fig. 8E and 8G).

Figure 8. T24-miR-NC and T24-miR-17 cells were stained with AO for AVO observation under fluorescence microscope (B and C). AO staining of A549 served as the control (A). (D-F) Flow cytometric quantitation of AVOs following miR-17 overexpression into A549-T24 cells. (H- I) MDC staining of T24-miR-NC and T24-miR-17 cells under fluorescence microscope. (J) Quantitation of change in autophagic vacuole formation in T24-miR-17 compared to T24-miR-NC cells by MDC staining and Flow-cytometry.

Moreover, onset of autophagy the formation of autophagosome that can be indicated by MDC staining which specifically binds to the autophagosome [103]. We found that overexpression of miR-17 in A549-T24 cells resulted in the decrease in mean MDC fluorescence as compared to negative control (T24-miR-NC) (Fig. 8H vs. Fig. 8I and Fig. 8J) indicating reduction in autophagosome count.

5.3.7. miR-17 overexpression induced apoptosis in paclitaxel-resistant lung cancer cells

Paclitaxel exerts its cytotoxic effect by inducing apoptosis [104]. However, in drug resistant cancer, tumor cells overcome this cytotoxic effect of paclitaxel and become resistant to apoptosis. So, we examined whether overexpression of miR-17 and subsequent treatment with paclitaxel was capable of inducing apoptotic death in drug resistant lung cancer cells. T24-miR-17 or T24-miR-NC cells were treated either with 24 nM or with 50 nM paclitaxel for 24 h and subjected to apoptosis assay. Fig. 9A and 9B revealed that significantly more apoptotic
cells were detected upon paclitaxel treatment of T24-miR-17 cells compared to T24-miR-NC cells. Moreover, consistent with the above results, when paclitaxel induced apoptosis following miR-17 overexpression in H596-TxR cells was determined; it was observed that miR-17 overexpression increased apoptosis in H596-TxR cells (Fig. 9C and 9D). Taken together it appears that overexpression of miR-17 and subsequent paclitaxel treatment induces apoptotic cell death in lung cancer cells.

Figure 9. miR-17 overexpression and subsequent paclitaxel treatment induced apoptosis in paclitaxel resistant lung cancer cells. (A) T24-miR-NC or T24-miR-17 cells and (B) TxR-miR-NC or TxR-miR-17 cells were treated either with 24 nM or 50 nM paclitaxel for another 24 h. Cells were then harvested for apoptosis analysis by annexin V- FITC/ PI staining and flowcytometry.

5.3.8. Overexpression of miR-17 induced disruption of mitochondrial membrane potential and release of cytochrome-c from mitochondria in A549-T24 cells

Mitochondrial pathway mediated apoptosis is often associated with the collapse of membrane potential $\Delta \Psi$ as a result of depolarization and leakiness of the inner mitochondrial membrane. To examine whether mitochondrial membrane integrity is damaged by miR-17 over- expression and subsequent paclitaxel treatment in A549-T24 cells, change in $\Delta \Psi$ was measured using JC-1 dye. As shown in Fig. 10A and 10B, inhibition of autophagy by miR-17 overexpression and subsequent paclitaxel treatment in A549-T24 cells induced collapse of $\Delta \Psi$ as observed by drop in red fluorescence intensity (JC-1). These data suggested that loss of mitochondrial membrane potential might be an early event of paclitaxel induced cell death.
Thereafter, we checked whether drop in mitochondrial membrane potential resulted in the release of cytochrome-c in the cytosol from mitochondria. **Fig. 10C** shows, compared to the respective negative controls, with miR-17 overexpression and subsequent paclitaxel treatment amount of cytochrome-c increased in the cytosol of T24-miR-NC cells.

**Figure 10.** (A-B) miR-17 overexpression and subsequent treatment with paclitaxel induced collapse of mitochondrial membrane potential in A549-T24. NC1, T1, NC2 and T2 represents T24-miR-NC cells treated with 24nM paclitaxel, T24-miR-17 cells treated with 24 nM paclitaxel, T24-miR-NC cells treated with 50 nM paclitaxel and T24-miR-17 cells treated with 50 nM paclitaxel respectively. (C) Western blot analysis to detect the release of the cytochrome-c in the cytosol from mitochondria in A549 T24 cells following miR-17 overexpression and subsequent paclitaxel treatment.

**Figure 11.** (A-B) miR-17 overexpression and subsequent treatment with paclitaxel induced collapse of mitochondrial membrane potential in H596-TxR. Western blot analysis to detect the release of the cytochrome-c in the cytosol from mitochondria in H596-TxR cells following miR-17 overexpression and subsequent paclitaxel treatment.
Moreover, analysis of mitochondrial membrane potential of TxR-miR-17 cells following paclitaxel treatment (50 nM) also revealed similar drop in the ΔΨ value compared to TxR-miR-NC cells (Fig. 11A and 11B) and that ultimately resulted in the release of cytochrome-c into the cytosol from mitochondria of the TxR-miR-17 cells (Fig. 11C).

5.3.9. Overexpression of miR-17 caused the change in expression of pro-apoptotic and anti-apoptotic proteins in paclitaxel resistant lung cancer cells

The effect of miR-17 overexpression and subsequent paclitaxel treatment on the mitochondrial membrane potential of A549-T24 cells intrigued us to study the expression status of the major protein component of the mitochondrial apoptotic pathway. Paclitaxel treatment following miR-17 transfection for 24 h resulted in the increase in Bax (pro-apoptotic)/Bcl-2 (anti-apoptotic) ratio in A549-T24 cells accompanied by an increase in p53 expression and increase in the amount of a 19 kDa caspase-3 cleavage intermediate as well as increase in cleaved poly (ADP-ribose) polymerase (PARP) (Fig. 12A-D).

![Figure 12](image_url)
Furthermore, to extend our finding we also measured mRNA expression levels of Bax, Bcl-2 and P53 proteins by qRT-PCR in H596-TxR cells following miR-17 overexpression. We found that overexpression of miR-17 and subsequent paclitaxel treatment resulted in significant upregulation of Bax and P53 mRNA expression (Fig. 13B and 13C) with a concomitant downregulation of Bcl-2 (Fig. 13A).

We also assessed the relative caspase3 mRNA level by qRT-PCR and found overexpression of miR-17 and subsequent paclitaxel treatment resulted in increased caspase3 mRNA level compared to the negative control (T24-miR-NC) (Fig. 14A).

Moreover, as shown in Fig. 14B, compared to TxR-miR-NC cells, overexpression of miR-17 and subsequent treatment with paclitaxel resulted in upregulation of caspase3 mRNA level in TxR-miR-17 cells indicating increase in cellular apoptosis via caspase3 mediated...
pathway. These results collectively suggested that autophagy inhibition by miR-17 overexpression and subsequent paclitaxel treatment resulted in induction of apoptotic cell death by caspase3 mediated pathway in paclitaxel resistant lung cancer cells.

5.3.10. Over-expression of miR-17 stimulates ROS generation, required for paclitaxel mediated apoptosis

Recent studies have shown that involvement of reactive oxygen species (ROS) following paclitaxel treatment in the induction of autophagy and apoptosis and also demonstrated the importance of ROS in cytochrome-c release from the mitochondria to cytosol [102,239-242]. So, we wanted to assess whether inhibition of autophagy by miR-17 overexpression and subsequent paclitaxel treatment could stimulate ROS generation in A549-T24 cells. T24-miR-NC and T24-miR-17 cells were treated with 24 nM and 50 nM paclitaxel for 24 h. The treated-cells were stained with cell-permeant fluorescent dye H2-DCFDA to detect the change in ROS generation.

Figure 15. (A-B) miR-17 overexpression and subsequent paclitaxel treatment stimulated ROS generation in A549-T24 cells. NC1, T1, NC2 and T2 represent the same as mentioned earlier. (C) Amelioration of paclitaxel induced cytotoxicity following miR-17 overexpression in A549-T24 cells by NAC. T24-miR-NC or T24-miR-17 cells were pre-incubated with 1 mM NAC for 4h and then treated with 24 nM or 50 nM paclitaxel for 24 h. Cell viability was measured by MTT assay. (D) miR-17 overexpression and subsequent paclitaxel treatment stimulated ROS generation in H596-TxR cells. (E) Amelioration of paclitaxel induced cytotoxicity following miR-17 overexpression in H596-TxR cells by NAC.
Fig. 15A and 15B shows that ROS levels were increased in T24-miR-17 cells compared to the respective negative controls (T24-miR-NC) following paclitaxel treatment. We also noticed increased accumulation of ROS following miR-17 overexpression and paclitaxel treatment in H596-TxR cells (Fig. 15D). Furthermore, we were interested to examine whether ROS inhibition could influence paclitaxel mediated cell death or not. Therefore, we pre-treated both T24-miR-NC and T24-miR-17 cells with 1mM N-acetyl-L-cysteine (NAC) for 4 h, followed by treatment with 24 nM or 50 nM paclitaxel for another 24h after changing the NAC containing media and cell viability was measured by MTT assay. It was observed that pre-treatment with NAC inhibited the cytotoxic effects of paclitaxel by scavenging ROS (Fig. 15C). Similar results were observed with TxR-miR-17 cells (Fig. 15E) where pre-treatment with 1 mM NAC and subsequent paclitaxel treatment resulted in increase in cell viability.
5.4. Discussion

Autophagy, an evolutionarily conserved intra-cellular self defence mechanism, which not only prevent toxic accumulation of damaged cellular counterparts but is responsible for the degradation and recycling of cytoplasmic constituents including organelles, long-lived misfolded proteins to sustain metabolic homeostasis [102,103,105,234-236,243]. Previously many studies have reported stimulation of autophagy in response to treatment with chemotherapeutic agents including paclitaxel and internal cellular needs to maintain metabolism, which in turn contributed to development of chemo-resistance in many cancer types including NSCLCs [102,105] and blocking cancer cell autophagy emerged as a novel approach to enhance the efficiency of chemotherapy in lung and other cancers [102,105,244,245]. Moreover, in recent years our most current knowledge of molecular mechanism of cancer drug resistance began to include not only dysregulation of expression of protein coding genes but also that of non- coding regulatory RNA, especially miRNA [51,156,162,216]. Previously, many groups have reported miRNA dysregulation following paclitaxel resistance in lung and other cancer types and tried to correlate particular miRNAs with the resistant phenotype. Here in this report, we were interested in investigating the role of miRNAs in regulating autophagy leading to paclitaxel resistance in lung non-small cancer cells. We performed miRNA arrays to compare the differential miRNA profiles of paclitaxel resistant and sensitive A549 cells (Fig. 1). We also observed that in vitro generated paclitaxel resistant lung cancer cells (A549-T24 and H596-TxR) exhibited heightened level of autophagy compared to their paclitaxel sensitive counterparts (A549 and NCI-H596 respectively) (Fig. 2 and Fig. 3). Our results clearly indicated that both beclin 1 and LC3-II which are considered as the most important autophagic markers were significantly upregulated in both A549-T24 and H596-TxR cells indicating increased autophagy in paclitaxel resistant lung cancer cells compared to sensitive cells (Fig. 2 and Fig. 3).

From the array results we found that miR-17 was significantly downregulated in A549-T24 cells (Fig. 1 and Fig. 4A). Moreover, analysis of of the miR-17 expression level in H596-TxR cells line also showed downregulation of miR-17 expression (Fig. 4B) indicating downregulation of miR-17 expression following paclitaxel resistance was not cell line specific. Furthermore, here we showed that miR-17 directly targeted beclin 1 in paclitaxel resistant lung cancer cells and its overexpression resulting in downregulation of beclin 1 expression (Fig. 6 and Fig. 7). Our results clearly indicated that inhibition of beclin-1 mediated autophagy by miR-17 overexpression significantly sensitized the paclitaxel resistant
lung cancer cells to paclitaxel (Fig. 5). We also observed that downregulation of beclin1 mediated cellular autophagy by miR-17 overexpression and subsequent paclitaxel treatment significantly increased the percentage of apoptotic cells in both A549-T24 and H596-TxR cells (Fig. 9) which was further confirmed by the change in expression of several pro-apoptotic and anti-apoptotic proteins (Fig. 12 and Fig. 13).

Paclitaxel was reported to affect mitochondrial apoptotic mechanism by altering mitochondrial permeability transition and changing the expression of several mitochondrial membrane proteins [102,105,210,246,247]. In this report, we also demonstrated that inhibition of cyto-protective autophagy by miR-17 overexpression and subsequent paclitaxel treatment caused change in mitochondrial membrane potential (Fig. 10 and Fig. 11) and expression of mitochondrial apoptotic marker proteins (Fig. 12 and Fig. 13). Furthermore, recently many reports have shown that suppression of autophagy by silencing ATG7 and beclin 1 results in increase in ROS generation leading to sensitization of cancer cells to drug induced apoptosis [102,103,243]. So, we checked the role of ROS in paclitaxel induced cell death in both A549-T24 and H596-TxR cells. We observed that inhibition of beclin 1 mediated autophagy by miR-17 overexpression and subsequent paclitaxel treatment resulted in the stimulated ROS formation and apoptotic cell death in paclitaxel resistant lung cancer cells (Fig. 15A-B and Fig. 15D). Moreover, inhibition of ROS accumulation by pre-treating cells with NAC following miR-17 overexpression inhibited paclitaxel induced cytotoxicity (Fig. 15C and Fig. 15E).