Preparation, characterization and comparative miRNA microarray expression analysis of paclitaxel resistant lung cancer cells.
Cover Image:
Graphical abstract showing paclitaxel resistant cells (A549-T24 and H596-TxR) exhibited increased cell viability compared to their respective paclitaxel sensitive counterparts (A549 and NCI-H596) when exposed to increasing concentration of paclitaxel. Also shown is the heat Map and supervised hierarchical clustering of differentially expressed miRNAs in A549-T24 cells compared to A549 cells.
4.1. Introduction:

Lung cancer is one of the most common malignancies in this world. In 2012 lung cancer was the most commonly diagnosed cancer worldwide making up 13.0% of the total incidence of cancer. It was also reported to be the most common cause of cancer related deaths, accounting for nearly one in five cancer deaths (19.4% of the total) [217]. Almost 85% of lung cancer cases belong to non-small cell lung cancer (NSCLC) [218]. Tubulin-binding agents (TBA) such as taxens, vinblastin and vinorelbine are widely used in the treatment of aggressive NSCLCs, either as single agent or in combination with other platinum compounds. Taxens, especially paclitaxel is commonly used in combination chemotherapy for solid tumours. Paclitaxel exerts its cytotoxic effect through binding with the β- tubulin subunits of αβ- tubulin heterodimer, stabilizes microtubule and thereby interfere with the proper formation of mitotic spindle causing G2/M phase cell cycle arrest and ultimately drives the cancer cells to apoptotic death, activating spindle- mitotic check point [50,51].

Since its approval by the Food and Drug Administration (FDA) in 1992 for the treatment of ovarian cancer, paclitaxel is now being routinely used in the adjuvant, neoadjuvant and metastatic setting for a wide range of solid malignancies, including those of the breast, prostate, ovary, lung, head and neck [25,51,156,210,219]. However, the prognosis for the patients remains dismal since 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. High abundance of P-glycoproteins (P-gp) efflux transporters [207,211] and β- tubulin isotypes [206,213] are the two predominant mechanisms which are mostly reported for clinical resistance to paclitaxel. However recent studies indicate that miRNAs also play a critical role in the acquisition of paclitaxel resistance in cancer cells.

MicroRNAs are a group of endogenous small, non-coding RNAs which regulate gene expression at the posttranscriptional level [127,220,221]. Dysregulation of miRNA expression has been frequently reported in various human cancers including NSCLC [222,223]. So, we wanted to understand how differential miRNA expression can be correlated with the development of paclitaxel resistance. Furthermore we also wanted to correlate dysregulation of particular miRNA expression with tumor responsiveness to paclitaxel. For this purpose, we prepared and characterized two paclitaxel resistant lung cancer cell lines (A549-T24 and H596-TxR) from two paclitaxel sensitive lung cancer cell lines (A549 and NCI-H596) by continuous exposure of the paclitaxel sensitive cells to paclitaxel. We performed differential miRNA analysis between paclitaxel sensitive and resistant A549 cells to identify miRNAs which are significantly deregulated in paclitaxel resistant cells.
4.2. Methods

4.2.1. Cell lines and cell culture

Human non-small lung epithelial adenocarcinoma cell line Type II, A549 and human lung adenosquamous carcinoma cell line NCI-H596 were maintained in DMEM media at 37°C in a humidified atmosphere containing 5% CO₂ as mentioned earlier. A549 and H596 cells were selected for resistance to paclitaxel in a stepwise manner essentially as described [205,209,224]. Briefly, A549 cells were initially exposed to 2 nM of paclitaxel and once normal growth was achieved the drug dose was increased in the multiples of two until a final concentration of 24 nM paclitaxel (A549-T24) was reached. NCI-H596 cells which were a little tolerant to paclitaxel, were first exposed 5 nM of paclitaxel and maintained at this concentration until normal growth was reached. Thereafter the drug dose was enhanced in the multiples of three until a final dose of 20 nM of paclitaxel (H596-TxR) was achieved.

4.2.2. Trypan blue exclusion assay of cell viability

A549 and H596 cells along with their paclitaxel resistant counterparts were treated with 24 nM paclitaxel for 24 h. Trypan blue exclusion assay [225] was used to determine the number of viable and dead cells. After 24 h cells were harvested through trypsinization, washed twice with 1X PBS and then stained with 0.4% trypan blue in PBS. Cells were then prepared for analysis by flow cytometry.

4.2.3. Cell cycle analysis

Cultured A549 and H596 cells along with their paclitaxel resistant derivatives, A549-T24 and H596-TxR cells were grown at a density of 10⁶ cells /mL and treated with 24 nM paclitaxel for 24 h. There after cells were harvested, fixed in ice cold methanol for at least 30 min in 4°C and incubated for 4 h at 37 °C in a PBS solution containing 1 mg/mL RNase A. Then nuclear DNA was labeled with propidium iodide (PI). Cell cycle analysis was performed using a Becton Dickinson FACS Caliber flow cytometer, and the data were analyzed using the Cell Quest program from Becton Dickinson.

4.2.4. Flow cytometric analysis for apoptotic cells:

A549 and H596 cells along with their paclitaxel resistant counterparts were treated with 24 nM paclitaxel for 24 h. Approximately 1x10⁵ cells were then stained for 15 min at room temperature in the dark with FITC-conjugated annexinV (1 µg/ml) and propidium iodide (PI) (0.5 µg/ml) in a Ca²⁺-enriched binding buffer and analysed by a two color flow cytometric assay. AnnexinV and PI emissions were detected in the FL1 and FL2 channels of a
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4.2.5. miRNA micro array expression analysis:

Differential miRNA profiling of A549 and A549-T24 cells were done from Exiqon (Vedbaek, Denmark). Total RNA including miRNA was extracted from A549 and A549-T24 cells using Qiagen miRNeasy mini kit following the manufacturer protocol. RNA integrity was assessed with an Agilent Bioanalyser 2100 (Agilent, Palo Alto, CA, USA). The total RNA samples having adequate quality for analysis by miRCURY LNA miRNA microarray platform were labeled using the miRCURY LNA microRNA Hi-Power Labelling Kit, Hy3/Hy5 and pairs of sample were hybridized on the miRCURY LNA microRNA Array (6th gen - hsa, mmu & rno) and the array was read in Agilent G2505B Micro array scanner system in an ozone free environment following manufacturers protocol [226]. In successful arrays, signal intensities were background corrected using the normexp method [227] with offset value k= 10 and normalized using the global Lowess (locally weighted scatterplot smoothing) regression algorithm (Cleveland, 1979). The log2 median ratio (LMR) value was calculated per miRNA probe set by log2-transformation of the mean Hy3/Hy5 ratio. The ΔLMR values were subsequently used to calculate the fold change per miRNA, using the following formula: $2^{\Delta LMR}$. For subsequent analysis, we used miRNAs, which passed the filtering criteria on variation across samples, that is, LMR $\geq$ 2.0. Only those miRNAs which were dysregulated by at least two fold ($\Delta LMR \geq 2$) were taken into account for further studies [205].

4.2.6. 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.

4.2.7. Cell Proliferation inhibition assay (MTT assay):

A549-T24 and H596-TxR cells were transfected with either 100 nM pre-miR-17 or 100 nM pre-miR-16 or 100 nM pre-miR-101 or 100 nM pre-miR-106a and were plated in 96-well culture plates ($1 \times 10^4$ cells per well). 24 h following transfection, cells were treated with different concentrations (0- 100 nM) of paclitaxel for another 24 h. MTT (5 mg/mL) was dissolved in PBS and filter sterilized. Then 20 μL of the prepared solution was added to each well. This was incubated until purple precipitate was visible. Subsequently 100 μL of Triton-
X100 was added to each well and incubated in darkness for 2 h at room temperature. The absorbance was measured on an microplate reader (VersaMax Absorbance Microplate Reader, Molecular Devices, California, USA) at a test wavelength of 570 nm and a reference wavelength of 650 nm. Data were calculated as the percentage of inhibition by the following formula:

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\text{% inhibition} = \left[100 - \left( \frac{A_t}{A_s} \right) \times 100 \right] \% \quad \text{------------- (1)}
\]

\(A_t\) and \(A_s\) indicated the absorbance of the test substances and solvent control, respectively [205].

**4.2.8. Western blotting**

Cells were lysed in lysis buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X100, 0.1% (w/v) sodium dodecyl sulphate, 200 mM DTT and a cocktail of protease inhibitor (complete Mini; Roche Applied Science) for 30 min at 4°C and the protein concentration was measured by Bradfords method. Cell lysates containing ~50 µg of total protein were separated by 12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA) and probed with anti-βIII-tubulin monoclonal and GAPDH antibodies. Bands on the Western blots were visualized by adding SuperSignal West Pico Chemiluminescent Substrate (Pierce, Thermo Scientific, USA) and imaged by Bio-rad ChemiDoc XRS+ molecular imager.
4.3. Results:

4.3.1. Chemical Structure and Absorption spectrum of paclitaxel

The chemical structure of paclitaxel was given in Fig. 1 and the absorption spectrum of 20 µM ligand in methanol was given where it gave an absorption maximum at 230 nm. The molar excitation coefficient method was used for the determination of concentration of paclitaxel. The molar extinction coefficient of paclitaxel in methanol was reported to be 3400 M- cm⁻¹ at 230 nm [228]. The paclitaxel concentration was accurately determined using this molar extinction coefficient.

![Chemical structure of paclitaxel]

Figure 1: The chemical structure and the absorption spectrum of paclitaxel in methanol.

4.3.2. Paclitaxel resistant lung cancer cells exhibited increased cell survival when exposed to paclitaxel

![Viability assay results]

Figure 2: (A) A549 and A549-T24 cells were seeded into 96 well plates at a density of 1x 10⁴ cells per well. Cells were treated with 0-250 nM paclitaxel for another 24 h. The cell viability was assessed by MTT assay. (B) Cells (A549 and A549-T24) were subsequently treated with 24 nM paclitaxel for 24h and then subjected to FACS analysis after being stained by Trypan blue.
To address our objectives we first prepared paclitaxel resistant lung non-small cancer cell line A549-T24 from paclitaxel sensitive lung cancer cells A549 by continuous exposure of paclitaxel sensitive cells to paclitaxel. Then to investigate the change in cell viability, we performed both MTT assay and trypan blue exclusion assay with A549 and A549-T24 cells (Fig. 2). We found compared to the paclitaxel sensitive A549 cells, paclitaxel resistant A549-T24 cells exhibited much tolerance to paclitaxel (Fig. 2).

Figure 3: (A) H596 and H596-TxR cells were seeded into 96 well plates and were treated with 0-200 nM paclitaxel for another 24 h. The cell viability was assessed by MTT assay. (B) Cells (H596 and H596-TxR) were subsequently treated with 20 nM paclitaxel for 24h and then subjected to FACS analysis after being stained by Trypan blue.

Moreover to extend our finding we also prepared another paclitaxel resistant cell line H596-TxR from paclitaxel sensitive lung adenosquamous carcinoma cell line NCI-H596 cells. *in vitro* and compared the cell viability of H549-TxR cells with that of parental H596 cells by both MTT assay (Fig. 3A) and trypan blue exclusion assay (Fig. 3B). We found like A549-T24 cells, H596-TxR cells exhibited higher cell survival than paclitaxel sensitive H596 cells when exposed to paclitaxel.
4.3.3. Paclitaxel resistance was associated with increase in expression of βIII-tubulin isotype

Since paclitaxel resistance is reported to be associated with the increased expression of multi drug resistant protein -1 (MDR-1) and related drug efflux pumps [207,211] and βIII-tubulin isotype [212-214], we compared the status of both MDR-1 and βIII-tubulin expression in paclitaxel sensitive and resistant A549 cells by Western blotting. We found that compared to the control A549 cells, paclitaxel resistant A549-T24 cells exhibited much higher levels of both MDR-1 and βIII-tubulin (Fig. 4A). Moreover, to extend our finding, we also checked the status of both MDR-1 and βIII-tubulin isotype in H596 and H596-TxR cells by Western blotting and observed that there is considerable increase in the expression of these marker proteins in H596-TxR (Fig. 4B) cells compared to paclitaxel sensitive H596 cells confirming development of paclitaxel resistance.

4.3.4. Paclitaxel resistant lung cancer cells exhibited no G₂/M cell cycle arrest when treated with paclitaxel

Effects of paclitaxel (24 nM) on cell cycle progression of paclitaxel sensitive and resistant lung cancer cells were analyzed by flow cytometer and the data were presented in the Fig. 5. We found when A549 cells were treated with 24 nM paclitaxel for 24 h they exhibited significant G₂/M cell cycle arrest, characteristics of paclitaxel treatment. Whereas under similar condition no such G₂/M arrest was observed in case of A549-T24 cells confirming normal cell cycle progression (Fig. 5).
Figure 5: Flow cytometric analysis of cell cycle distribution of A549 and A549-T24 cells treated with 24 nM paclitaxel for 24 h. M1, M2, M3 and M4 are indicating sub-G1, G1, S and G2/M phase respectively.

Similar results were obtained when H596 and H596-TxR cells were treated with 24 nM paclitaxel for 24 h and the cell cycle progression was investigated (Fig. 6).

Figure 6: Flow cytometric analysis of cell cycle distribution of H596 and H596-TxR cells treated with 24 nM paclitaxel for 24 h. M1, M2, M3 and M4 are indicating sub-G1, G1, S and G2/M phase respectively.
We observed that like A549-T24, H596-TxR cells exhibited no G2/M cell cycle arrest when treated with paclitaxel for 24 h (Fig. 6). On the contrary, under similar condition paclitaxel sensitive H596 cells exhibited significant G2/M arrest when treated with 24 nM paclitaxel offr 24 h (Fig. 6).

4.3.5. Paclitaxel resistant lung cancer cells exhibited reduced apoptosis when treated with paclitaxel for 24 h

Paclitaxel exerts its cytotoxic effect by inducing apoptosis [104]. During paclitaxel resistance, cells become resistant to paclitaxel induced apoptosis. So to check whether in vitro generated paclitaxel resistant lung cancer cells exhibited reduced apoptosis or not, both A549 and H596 cells and along with their respective paclitaxel resistant counterparts (A549-T24 and H596-TxR) were treated with 24 nM paclitaxel for 24 h and % of early apoptotic and late apoptotic cells were determined by annexin V and PI staining and flow cytometry.

Figure 7: A549-T24 and H596-TxR cells exhibited reduced apoptosis compared to parental A549 and H596 cells when treated with 24 nM paclitaxel, respectively.
Fig. 7 demonstrated that when A549 and H596 cells were treated with 24 nM paclitaxel for 24 h they showed almost 33.5% and 18% apoptosis, respectively. However under similar conditions A549-T24 and H596-TxR cells showed negligible apoptosis, confirming development of paclitaxel resistance.

4.3.6. miRNA profiling by miRCURY LNA™ microRNA microarray platform

Since our main objective was to identify miRNAs responsible for the development of paclitaxel resistance, after preparing and characterizing paclitaxel resistant lung cancer cell lines we wanted to compare the miRNA expression patterns between paclitaxel sensitive and resistant cell A549 cells. So, search for the critical miRNAs involved in the development of paclitaxel resistance, we screened miRNA profiles of A549-T24 cells and compared it with that of A549 cells using miRCURY LNA™ microRNA Array (Exiqon, Denmark) (Fig. 8).

We found 23 miRNAs to be dysregulated by at least two fold (ΔLMR ≥ 2) in paclitaxel resistant A549-T24 cells compared to the parental A549 cells (Fig. 8). 14 miRNAs were found to be downregulated and 9 miRNAs were upregulated in A549- T24 cells compared to A549 cells.
4.3.7. Overexpression of miR17 and miR16 decreases the viability of paclitaxel resistant lung cancer cells

From the expression matrix we have initially selected 4 candidate miRNAs, miR-17, miR-16, miR-101 and miR 106a which showed greater abundance (higher copy number) and higher correlation with the paclitaxel resistance phenotype as predicted from different miRNA target prediction databases such as miRBase (www.mirbase.org), TargetScanHuman 6.2 (www.targetscan.org), PicTar (http://pictar.mdc-berlin.de/), microRNA.org (www.microrna.org) etc. Since these miRNAs were downregulated in A549-T24 cells compared to the parental A549 cells, we overexpressed these miRNAs individually into A549-T24 cells and measured cell viability following treatment with different doses of paclitaxel for 24 h (Fig. 9A). We observed that among the four miRNAs over expression of miR-17 and miR-16 into A549-T24 cells reduces cell viability following paclitaxel treatment in a dose dependent manner.

Moreover, to show that this effect of miR-17 and miR-16 overexpression and subsequent paclitaxel treatment on the viability of A549-T24 cells was not limited to a particular cell line we overexpressed these four miRNAs into H596-TxR cells and measured cell viability by MTT assay following paclitaxel treatment for 24 h. We observed that when H596-TxR cells were overexpressed with miR17 or miR16 and subsequently treated with paclitaxel for 24 h, there was dose dependent loss of cell viability which was absent in case of miR-101 or miR-106a overexpression (Fig. 9B).
Figure 10. A549-T24 and H596-TxR cells were either transfected with pre-miR-negative control RNA (T24-miR-NC and TxR-miR-NC) or with pre-miR-17 (T24-miR-17 and TxR-miR-17) or with pre-miR-16 (T24-miR-16 and TxR-miR-16) RNA and 24 h following transfection the status of βIII–tubulin was evaluated by Western blotting.

Since overexpression miR-17 and miR-16 into paclitaxel resistant lung cancer cells sensitized the cells to paclitaxel we wanted to see whether this overexpression of miRNAs could alter the expression status of βIII–tubulin. So, we checked the cellular levels of βIII -tubulin isotype following miR-17 and miR-16 (100 nM each) overexpression into A549-T24 (Fig 10A) and H596-TxR (Fig. 10B) cells. It was observed that there was no significant change in βIII -tubulin isotypic expression level following miR-17 or miR-16 overexpression.
4.4. Discussion

In lung cancer, paclitaxel based combination chemotherapy is widely used to cure and extend prospective survival in patients diagnosed with lung cancer. However, the clinical effectiveness of paclitaxel based chemotherapeutic agents in the treatment of aggressive NSCLC is often negated by the emergence of paclitaxel resistance. In the previous decade considerable amount of research was done to elucidate the mechanisms underlying paclitaxel resistance. However, despite all these efforts paclitaxel resistance still continues to be a global problem for a wide range of cancer histopathologies [51]. Therefore, identification of the molecular pathways involved in primary and acquired drug resistance plays an important role to establish rational therapeutic approaches aimed to circumvent or retard the acquisition of the paclitaxel- resistant phenotype. In this context the recent identification of the regulatory roles of miRNAs extend the spectrum of possible key factors involved in such biological processes. In fact, miRNAs are involved in various biological processes, including cell proliferation, cell death, stress resistance and fat metabolism though fine tuning of gene expression. Moreover, altered miRNA expression has already been shown to be associated with paclitaxel resistance in various human malignancies including non-small cell lung cancer (NSCLC) [51,205,223].

In this study we wanted to investigate the correlation between differential expressions of miRNAs with paclitaxel resistant phenotype using non-small cell lung cancer as model. We prepared two paclitaxel resistant lung cancer cell lines (A549-T24 and H596-TxR) in vitro by continuous exposure of paclitaxel sensitive lung cancer cells (A549 and NCI-H596) to an increasing dose of paclitaxel. We observed that paclitaxel resistant lung cancer cells (A549-T24 and H596-TxR) exhibited higher degree of tolerance compared to their respective paclitaxel sensitive counterparts (A549 and NCI-H96) when exposed to varying dose of paclitaxel (Fig. 2 and Fig. 3). Since paclitaxel resistance was reported to be associated with the upregulation of MDR-1 and βIII-tubulin expression [209], we analysed cellular status of both MDR-1 and βIII-tubulin isotype and observed that both MDR-1 and βIII-tubulin expressions were significantly increased in paclitaxel resistant lung cancer cells thereby confirming development of paclitaxel resistance (Fig. 4). Paclitaxel is known to induce G2/M cell cycle arrest by stabilizing microtubule, reducing its dynamicity [210,219]. During paclitaxel resistance, cells escape from this mitotic arrest and assume normal cell cycle progression. Likewise, comparative cell cycle analysis of these paclitaxel resistant cells revealed no characteristic G2/M cell cycle arrest when exposed to paclitaxel while under similar condition both A549 and H596 cells exhibited significant cell cycle arrest suggesting development of paclitaxel resistance (Fig. 5 and Fig. 6). During paclitaxel resistance, cells
become resistant to paclitaxel induced apoptosis. Since, paclitaxel resistant lung cancer cells exhibited no significant $G_2/M$ cell cycle arrest upon paclitaxel exposure, we further checked whether these cells were resistant to apoptosis or not. We observed that, compared to the respective control cells, both A549-T24 and H596-TxR cells exhibited inhibition of apoptosis when exposed to paclitaxel for 24 h (Fig. 7). These results clearly suggested that we successfully prepared and functionally characterized two paclitaxel resistant cell lines A549-T24 and H596-TxR from paclitaxel sensitive A549 and NCI-H596 cells respectively.

However, our major objective was to screen differential miRNA profiles of paclitaxel resistant and sensitive cells. So, we performed miRNA micro-array analysis with total RNA isolated from A549-T24 and A549 cells and found 23 miRNAs were significantly dysregulated, in paclitaxel resistant A549-T24 cells compared to parental A549 cells (Fig. 8). Among the downregulated miRNAs we selected 4 candidate miRNAs, miR-17, miR-16, miR-101 and miR 106a which were more abundant and exhibited higher correlation with the paclitaxel resistance phenotype as predicted from different miRNA target prediction databases. To check whether downregulation of these miRNAs were responsible for the development of paclitaxel resistant phenotype, we individually overexpressed these miRNAs into both A549-T24 and H596-TxR cells and subsequently treated the cells with paclitaxel. We observed that for among these miRNAs only miR-16 and miR-17 was capable of sensitizing paclitaxel resistant lung cancer cells to paclitaxel in a dose dependent manner (Fig. 9). However, analysis of cellular βIII- tubulin expression level, following miR-17 or miR-16 overexpression resulted in no change in the cellular βIII- tubulin level indicating that overexpression of miR-17 or miR-16 sensitized the paclitaxel resistant cells by βIII- tubulin independent pathway (Fig. 10).