"Identifying novel miRNA targets of anticancer drugs in normoxic and hypoxic condition"

Research Project submitted to the Central University of Punjab

For the award of

Master of Science

In

Life Sciences with Specialization in Molecular medicine

By

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May, 2018

CERTIFICATE

I declare that the Research project entitled “Identifying novel miRNA targets of anticancer drugs in normoxic and hypoxic condition” has been prepared by me under the guidance of Dr. Sandeep Singh, Assistant Professor, Department of Human Genetics and Molecular Medicine, School of Health Sciences, Central University of Punjab. No part of this project work has formed the basis for the award of any degree or fellowship previously.

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Date:

CERTIFICATE

I certify that Sapna Goyal has prepared her Research project entitled “Identifying novel miRNA targets of anticancer drugs in normoxic and hypoxic condition”, for the award of M.Sc., degree of the Central University of Punjab, under my guidance. She has carried out this work at the Department of Human Genetics and Molecular Medicine, School of Health Sciences, Central University of Punjab.

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ABSTRACT

Identifying novel miRNA targets of anticancer drugs in normoxic and hypoxic condition

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Breast cancer has become the prevailing cause of death, so the research for their treatment has become advanced day by day. Breast cancer behaves differently in normoxic and hypoxic condition so does the anticancer drugs in both the condition in order to treat them. These drugs might have different effect on the expression of miRNAs in both normoxic and hypoxic conditions. In the present study, we evaluated the IC50 of three anticancer drugs-doxorubicin, α-Amanitin, DCA on MDA-MB-231 and after treatment with these drugs, have checked the expression of miRNAs- let-7b and miR-1275 in both hypoxic and normoxic condition by RT-PCR and results were analyzed by native PAGE.

(Sapna Goyal) (Dr. Sandeep Singh)

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(Sapna Goyal)

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

S.No.	Full Form	Abbreviation
1	Breast Cancer1,2	BRCA1,2
2	Human epidermal growth factor receptor 2	Her 2
3	Triple Negative Breast Cancer	TNBC
4	Micro RNA	miRNA
5	Untranslated Region	UTR
6	Doxorubicin	DOXO
7	Dichloro Acetic Acid	DCA
8	Reactive oxygen species	ROS
9	Pyruvate dehydrogenase kinase	PDK
10	Pyruvate dehydrogenase	PDH
11	DiGeorge syndrome critical region in gene 8	DGCR8
12	Exportin-5	Exp-5
13	RNA Induced Silencing Complex	RISC
14	Argonaute	Ago
15	Hypoxia Inducible Factor-1α	HIF-1α
16	Epithelial to Mesenchymal Transition	EMT
17	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide)	MTT
18	Triple negative breast cancer cell line	MDA-MB-231
19	Dulbecco's Modified Eagle's Medium	DMEM

20	Dimethyl Sulphoxide	DMSO
21	Ammonium per Sulphate	APS
22	Tetramethylethylenediamine	TEMED
23	Tris-Borate-EDTA	TBE
24	Deoxy Nucleotide Triphosphates	dNTPs
25	Reverse Transcriptase Polymerase Chain Reaction	RT-PCR
26	Inhibitory Concentration	IC50
27	Fetal Bovine Serum	FBS
28	Phosphate Buffer Saline	PBS

CHAPTER I

1. INTRODUCTION

Breast cancer is the most common type of cancer among women worldwide (Beiki *et al*., 2012). It is very heterogenous and complex when considering the aspect of histology, cellular origin, mutations, metastatic potential, disease progression, therapeutic response, and clinical outcome (Ossovskaya *et al*.,2011). Most important causes of breast cancer includes exposure to estrogen and progestrone hormones and inheritance of BRCA1 and BRCA2 genes (Martin and Weber, 2000; Colditz *et al*.,2004). Estrogen hormone exposure to breast tissue for long term leads to the tumor generation (Hebert, 2009). Based on the gene expression profiles of histological features, they are divided into five subtypes (Choi *et al*.,2012). These are luminal A, Luminal B, HER2 Overexpressing, TNBC, Normal breast like tumor (Boyle, 2012). TNBC patients can be benefitted by chemotherapy.

MiRNAs are small endogenous non-coding RNA molecules (18-22) nucleotide long (Bartel DP,2004). They regulate the gene expression by targeting different molecules. They regulate the gene expression at the post-transcriptional level by targeting 3'-UTR of target RNA sequence. One MiRNA can target 100 different molecules (Hummel *et al*., 2010). Important roles have been played by MiRNAs in different cellular processes such as survival, proliferation, differentiation, development and stress response. MiRNAs have shown to be aberrantly expressed in different types of cancer. Numerous MiRNAs are shown to be de-regulated in breast cancer, thus it is helpful in distinguishing normal and malignant breast tissues (Iorio *et al*., 2009; Leivonen *et al*., 2014). MiRNAs modulates the expression of known oncogenic and tumor suppressor genes, thus has a role in tumorigenesis (Garzon *et al*., 2006; Cowland *et al*., 2007; Cho 2007; Zhang *et al*., 2007; Hummel *et al*., 2010). MiRNAs are thought to be involved in differential treatment responses (Eroles *et al*., 2015; Tian *et al*., 2013). Different MiRNA expression profiles have been obtained by microarray analysis in drug resistant and drug-sensitive

cell lines (Chen *et al*., 2010; Pogribny *et al*., 2010; Zhou *et al*., 2010). MiRNAs can act as biomarkers and can be used as novel therapeutic anti-cancer targets.

Mechanism of action of anti-cancer drugs

Doxorubicin

The anthracycline antibiotics doxorubicin (DOXO) belongs to the most effective anti-cancer drugs. It has been widely used for the treatment of both solid tumors and hematological malignancies. It was first isolated from *Streptomyces peucetius* (Carvalho *et al*.,2009).

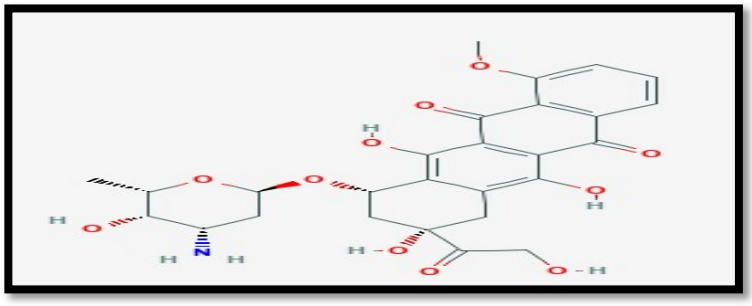

Figure1. Chemical Structure of doxorubicin

Mechanism: Inhibition of topoisomerase II activity (topoisomerase II binds to DNA and allows its cleavage but this covalent complex is trapped in the presence of doxorubicin and DNA cannot re-ligate thus subsequently blocking transcription and replication) (Nitiss, J.L.,2009; Pommier *et al*.,2010). Other mechanisms are DNA intercalation and production of ROS and appears to be responsible for serious side effects such as cardiotoxicity (Gewirtz, D.A.,1999).

α-Amanitin

α-Amanitin is a cyclic peptide isolated from Amanita mushrooms and responsible for extreme toxicity (Bushnell *et al*.,2002).

Mechanism: α-Amanitin binds with high specificity and affinity near the catalytic active site of RNA polymearse II (Bushnell *et al*.,2002). It traps a conformation of the enzyme

that prevents nucleotide incorporation and translocation of the transcript (Kaplan CD, Larsson KM, Kornberg RD.,2008 and Brueckner F, Cramer P.,2008). RNA polymerase II is highly sensitive to it (Kedinger C, Nuret P, Chambon P., 1971 and Weinmann R, Raskas HJ, Roeder RG.,1974), while RNA polymerase III is hunderd fold less sensitive than pol II (Listerman I *et al*., 2007 and Raha D *et al*., 2010). A-Amanitin is an irreversible inhibitor because it promotes polyubiquitination of Rpb1 and thus triggers degradation of Rpb1,the largest subunit of RNA polymerase II (Nguyen VT *et al*., 1996 and Lee KB, Sharp PA.,2004 and Jung Y, Lippard SJ.,2006).

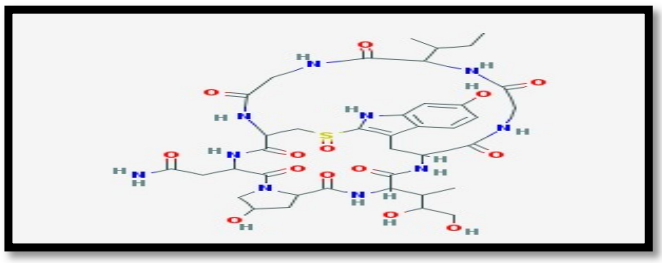

Figure2. Chemical Structure of α-Amanitin

DCA (Dichloroacetic Acid)

Cancer cells prefer to use glycolysis even in the presence of oxygen (*WARBURG* O, 1956). Warburg effect refers to the diversion of pyruvate metabolism from mitochondrial oxidation to lactate production (Bonnet S *et al*., 2006 and McFate T *et al*., 2008).

Mechanism: DCA is a pyruvate dehydrogenase kinase (PDK) inhibitor, thus reverses the Warburg effect by activating PDH and redirecting pyruvate metabolism back into the mitochondria (Bonnet S *et al*., 2006; McFate T *et al*., 2008).

DCA induces apoptosis in tumors via increased production of ROS due to increased mitochondrial activity, with decreased polarisation of the mitochondrial membrane restoring apoptotic processes (Bonnet S *et al*., 2006; Sutendra G *et al*., 2013).

DCA has been reported to be most effective when used in combination with agents that inhibit mitochondrial function (Sánchez-Aragó *et al*.,2010; Sun RC *et al*.,2011; Stockwin LH *et al*.,2010 and Sun W *et al*., 2009).

Michelakis and his colleagues reported that DCA had caused cell death in certain types of cancerous cells in vitro by inducing apoptosis. At lower concentrations, DCA brings about apoptotic changes but at higher concentrations, most cytotoxic effects of DCA are related to necrosis. Most prominent effects of DCA were seen on changing mitochondrial membrane potential (Δψm) and the production of reactive oxygen species (ROS). DCA is capable of interfering with the adaptation to tumor hypoxia by inhibiting function of the PDKs. Since oxygen deprivation also downregulates mitochondrial function,it is hypothesised that hypoxic cells would be more sensitive to DCA. DCA treatment led to inhibition of PI3K/Akt pathway and reduction of PDK1 protein level.

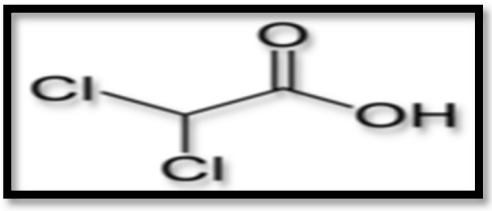

Figure3. Chemical Structure of DCA

CHAPTER II

2. Review of Literature

2.1 MiRNAs

MiRNAs are small endogenous non-coding RNA molecules (18-22) nucleotide long (Bartel DP,2004). MiRNAs are synthesized as hairpin-shaped precursors, which are then processed after synthesis (Ambros *et al*., 2003). In animals, primary MiRNA is formed by transcription of genes for MiRNA by RNA pol II. The pre- MiRNA formation is formed by (processing) cleavage of stem- looped structure of Pri-MiRNA in nucleus by Drosha (Class 2 RNase III enzyme). Pre-MiRNA is 60-70 nt long hairpin structure (Lee.Y *et al*., 2002; Zeng, Y., & Cullen, B. R. 2003). Drosha enzymes needs cofactor DiGeorge syndrome critical region in gene 8 (DGCR8) in humans and Pasha in *D. melanogaster* or *C. elegans* (Lee. Y *et al*., 2003; Han.J *et al*., 2004; Denli.A.M *et al*., 2004; Gregory, R. I. *et al*., 2004; Landthaler, M *et al*., 2004). Pre-MiRNA is transported to cytoplasm by nuclear transport receptor exportin-5 (EXP-5), which is dependent on Ran-GTP (Lund, E *et al*., 2004; Yi, R., Qin *et al*., 2003; Bohnsack, M. T., Czaplinski, K., & GÖRLICH, D. 2004) through large proteinaceous channels in the nuclear membrane (nuclear pore complexes) (Nakielny,S. & Dreyfuss G.1999). Pre-MiRNAs are then further processed in the cytoplasm by Dicer (RNase III type protein) to become mature MiRNA (Grishok, A *et al*., 2001; Hutvágner, G *et al*., 2001; Ketting, R. F *et al*., 2001; Knight, S. W., & Bass, B. L., 2001). The effector RNA-induced silencing complex (RISC) is formed by loading mature MiRNA on to the Argonaute (ago) protein.

MiR-1275 is a confirmed tumor-repressing miRNA, plays an important role in several diseases. Let-7b was found to be downregulated in invasive breast carcinomas of BRCA2 germ-line mutation carriers. It was showed by target gene analysis that let-7b is involved in many cellular pathways, such as p38 MAPK, p53, Wnt/β-catenin, apoptosis, tight junction, integrin and actin cytoskeleton. Low let-7b expression has been shown to be associated with poor prognosis in breast cancer (Quesne, J. L.*et al*., 2012; Ma, L., Li, G. Z. Wu, Z. S. & Meng, G. 2014).

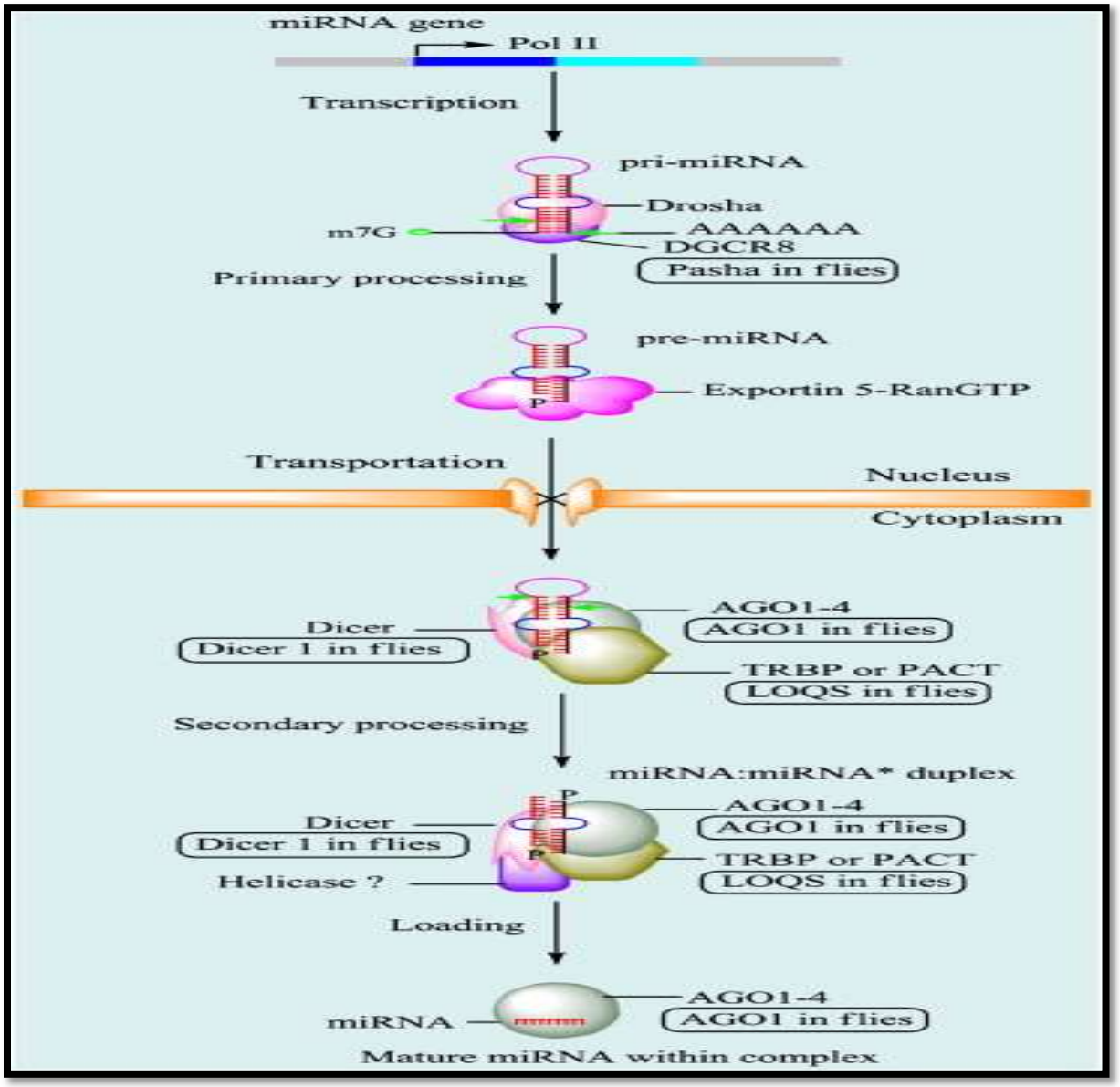

Figure 4. The figure shows the mammalian miRNA synthesis pathway and fly factors are in the squares.

2.2 HYPOXIA

Hypoxia has many known roles in various pathological and physiological conditions such as cancer, Parkinson's disease, myocardial ischemia/reperfusion and stroke. Mitochondrial dysfunction and activation of various signaling pathways through

hypoxia inducible factor 1α is caused by hypoxic microenvironment. Various biological events such as activation of the glycolytic pathway, increased blood vessel formation and downregulation of mitochondrial activity through pyruvate dehydrogenase kinase-1 are triggered by activation of HIF-1 α (Papandreou *et al*., 2006; Bristow, R. G., & Hill, R. P. 2008).

HIF-1α, which is the key factor in hypoxia regulates various miRNAs. MiR-210 is one of the most hypoxia-sensitive miRNA (Kulshreshtha *et al*., 2007). MiR-210 is upregulated by hypoxia.

2.3 Tumour Hypoxia: The Impact on Treatment

Various research have been done to study the effects of hypoxia to cancer treatments. Anti-cancer chemotherapeutics resistance has been reported in hypoxic cancer cells (Vaupel *et al*., 2001; Bertout *et al*., 2008). Proliferating cells are mainly targeted by majority of chemotherapy compounds but hypoxic cells have slower proliferation rates (Zolzer and Streffer 2002). Hypoxia resulted in accumulation of G1 cells by arrest in the G1 phase of cell cycle (Brown 2000; Amellem and Pettersen 1991). Cell cycle progression of hypoxia exposed S phase cells gets arrested after treatment for few hours (Amellem and Pettersen 1991), thus suggesting S phase cells are more-sensitive to hypoxia exposure than the other cell cycle phases. Additionally, it has been reported that HIF-1α inhibits Myc (an oncogene that drives proliferation in cancer cells) at the molecular level to prevent cell cycle progression (Koshiji *et al*., 2004).

2.4 CANCER CELLS BEHAVIOUR IN HYPOXIC CONDITION

It was known that, hypoxic cells have better ability to metastasize as they are more invasive and aggressive. As, multiple myeloma cancer cells cultured in hypoxic conditions in vitro and injected into mice were able to spread to the new bone marrow faster than the cells cultured in normoxic conditions (Azab *et al*.,2012; Muz *et al*.,2015).

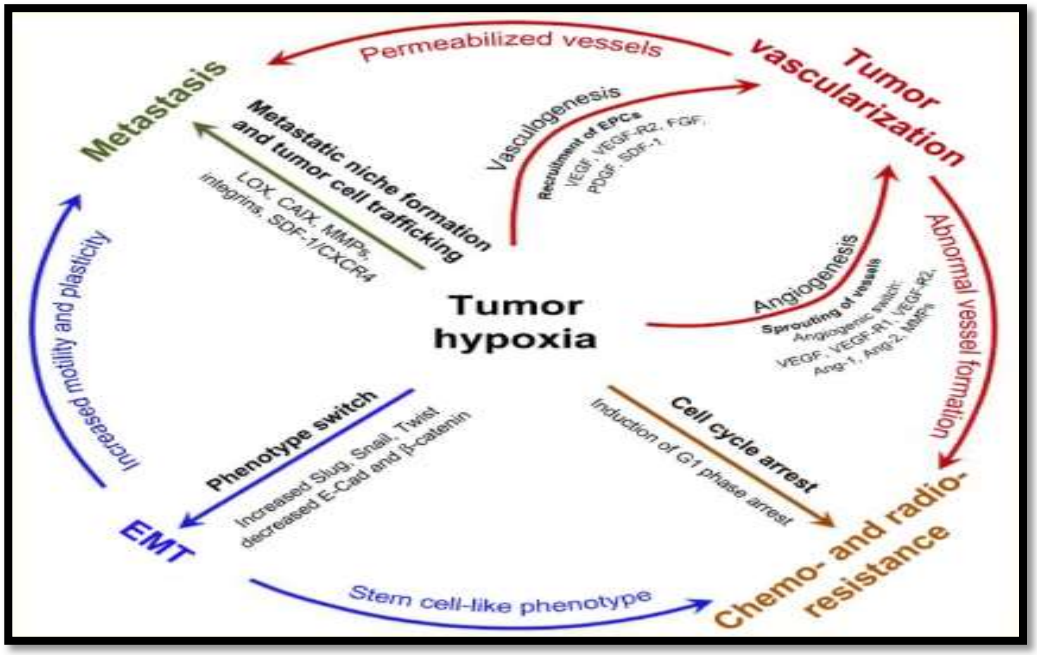

Figure5. Hypoxia as a driving force of tumor proression and metastasis (Azab *et al., 2009)*

2.5 Hypoxia regulated expression of miRNA

There was a significant reduction of proteins which are involved in miRNA biogenesis, such as Dicer, Drosha, TARBP2 and DCGR8 mRNA and protein levels in the cells exposed to hypoxia. This effect was dependent on HIF hydroxylase PHD2 but independent of HIF. But there were no modest changes in the production of mature miRNA (Bandara *et al*.,2014).

Some miRNAs were shown to be consistently induced in hypoxic condition in breast and colon cancer cells, such as miR-21, 23a, 23b, 24, 26a, 26b, 27a, 30b, 93, 103, 103, 106a, 107, 125b, 181a, 181b, 181c, 192, 195, 210 and 213 (Kulshreshtha *et al*., 2007) and some miRNAs were down-regulated in hypoxic condition, these are miR-122a, 565, 195, 30e-5p, 374, 19a, 101, 424, 29b, 186, 141, 320, 422b, and 197 in SCC cells, miR-15b, 16, 20a, 20b, 30b and 224 in CNE cells, and miR-424 in trophoblasts (Hua *et al*., 2006; Herbert *et al*., 2007; Donker *et al*., 2007).

Let-7 family have showed contrasting behavior in hypoxic condition upregulated in squamous cell carcinoma (SCC) and downregulated in CNE cells (from nasopharyngeal carcinoma) (Hua *et al*., 2006; Herbert *et al*., 2007). Cell cycle can be slow down or arrested by hypoxia therefore microRNAs that are selectively expressed in one phase of the cell cycle are expected to exhibit decreased levels (Donker *et al*., 2007).

2.6 Alternation of miRNA expression profile after treatment with drugs

5-FU is known to significantly alter the miRNA expression in MCF-7 cell line. Some miRNAs were expressed only in control cells while some expressed only in treated cells. Some miRNAs were expressed in both control and treated cells but their level of expression differs. Some of them get upregulated or downregulated after treatment as compared to control (Shah *et al*.,2011)

miR-1275 was known to be strongly affected in MCF-7 cells than in MDA-MB-231 by doxorubicin. Evaluation of miRNA expression in normal MCF-7 breast cell line, both with and without treatment with doxorubicin has showed contrasting results than that observed in breast cancer cell line. Thus the changes observed after treatment are specific to tumor cells and do not occur in normal breast cells (Tormo *et al*., 2015).

CHAPTER III

3. Objective of the Study

To check the effect of anticancer drugs on tbe expression of miRNA in normoxic and hypoxic condition.

3.1 Study design

The study was initiated by culturing breast cancer (MDA-MB-231) cells followed by determination of IC50 of doxorubicin, α-Amanitin, DCA. After that treatment with these drugs in normoxic and hypoxic conditions for 24 hours. The cells were harevsted and total RNA isolation was done followed by cDNA synthesis. The RT-PCR was performed with the primers of let-7b and miR-1275. The expression of let-7b and miR-1275 was analysed by running Native PAGE.

3.2 Materials and Methods

3.2.1 List of Equipments

The following equipments used for all the experimental purposes.

S. No.	Name of Instruments	Manufacturing company
1	Weighing balance	Mettler Tolendo
2	Autoclave	Narang Scientific Works
3	Hotplate	Tarsons
4	CO2 incubator	New Brunswick, UK
5	Inverted microscope	Olympus Magnus
6	Laminar air flow	NSW (Narang Scientific Works)
7	Microplate reader	Synergy H1
8	Oven	Samsung
9	ProFlex™ PCR System	Thermo Fisher Scientific
10	Refrigerated Centrifuge 5430R	Eppendorf, Germany
11	UV Transilluminator	Tarsons

12	Gel Doc™ XR+ Gel Documentation system	Bio-Rad
13	Vortex Shaker	Tarsons
14	Bench top Centrifuge	Tarsons
15	Deep Freezer (-20°C)	REM 600
16	Refrigerator	Sharp

3.2.2 List of Chemicals

The following chemicals used for conducting biological work.

S.No.	Materials	Manufacturing Company
1	DMEM	Gibco
2	Penicillin/ streptomycin antibiotic solution	Gibco
3	Fetal bovine serum	Gibco
4	DMSO	Gibco
5	Culture Plates and T25 Flask	Sigma falcon
6	MTT dye	Life Technology
7	PBS	Sigma
8	Trizol	ThermoFisher Scientific
9	Chloroform	Sisco Research Laboratories
10	Isopropanol	Sisco Research Laboratories
11	Drugs(Doxorubicin,α-Amanitin, Dichloroacetic Acid)	Sigma

3.3 Methodology

3.3.1 Cell culturing

The triple-negative breast cancer cell line MDA-MB-231 was cultured using complete media having DMEM, 10% FBS and antibiotics and incubated at 37^0C under 5% CO2 and humidified air. Cell sub-culturing was done when the cells attain 70-80% confluency, using trpsin-EDTA after washing the cells with fresh media, trypsinisation was stopped by adding fresh complete media and then cells were centrifuged at 1200rpm for 5 minutes, supernatant was discarded and cells were resuspended in fresh media.

3.3.2 MTT Assay for determination of IC50

The cytoxicity of anticancer drugs (doxorubicin, α-Amanitin and DCA) on MDA-MB-231 cells were determined by performing MTT Assay. In this assay viability of cells get reduced in a time and concentration dependent manner. 10^4 cells were seeded in three 96 well plates and incubated for 24 hours, then treated with doxorubicin with varying concentration (10nM to 50nM),treated with α-Amanitin (1nM to100nM) and treated with DCA (5mM to 50mM) and incubated for 48 hours, then media was discarded and MTT (5mg/ml) was added and incubated for 4 hours. After incubation DMSO was added and incubated for 4 hours for solubilising the formazon crystals. Then reading was taken at 570 nm. A graph of concentration vs percentage toxicity was plotted.

3.3.3 Cell treatment

The MDA-MB-231 cells were grown in 8 petri-plates. Out of them 2 were considered as controls and thus not treated with any drug. 2 were treated with 10nM doxorubicin, 2 with 25mM DCA, 2 with 30nM α-Amanitin. 4 of them were grown under normoxic condition (control, doxo treated, DCA treated , α-Amanitin treated) and 4 of them were grown under hypoxic condition (control, doxo treated, DCA treated , α-Amanitin treated) for 24 hours.

3.3.4 Cell harvesting

Treated cells were harvested after 24 hours ,using cell scraper cells were removed from the plates and transferred to eppendorffs and then centrifuged for 5 minutes. Then the media was discarded and cells were washed with 100µl PBS twice followed by centrifugation. Trizol (250µl) was then added and stored at -20^{0}C.

3.3.5 RNA isolation

Cells were suspended in 250µl Trizol, 40µl chloroform was then added and centrifuged at 12000rpm for 10 minutes. Then aqueous layer was collected carefully in new eppendorf and 100µl isopropanol was added to it and incubated at room temperature for 10 minutes followed by centrifugation for 10 minutes . Then pellet was washed twice with 400µl of ethanol. Then RNA was quantified by Nanodrop method.

3.3.6 CDNA Synthesis by kit method

4 microlitre of 5X Iscript reaction mix buffer was taken in effordorf, 12microlitre water was added, 3µl template RNA was added and 1µl RT enzyme added to make 20 µl reaction material. Eppendorfs were centrifuged at 1200rpm for 2 minutes at 4^{0}C and then kept in machine with reaction protocol set was priming at 25^{0}C for 5miutes followed by reverse transcription reaction at 46^{0}C for 20 minutes followed by inactivation of reverse transcriptase at 93^{0}C for 1 minute. After synthesis, CDNA was stored at -20^{0}C.

3.3.7 Reverse transcription (RT) PCR Reaction

14µl water was taken in PCR tube, 2µl of 10Xbuffer was added, 0.5µl forward and 0.5µl reverse primer was added, 0.25 µl MgCl2 was added, 0.25µl of 10 mM dNTPs were added, 0.5µl taq polymerase was added and 2 µl CDNA was added and then tubes were kept in PCR machine with reaction protocol set was denaturation at 94^{0}C for 1minute, annealing at 58^{0}C for 20 seconds and extension at 72^{0}C for 3 minutes.

3.3.8 NATIVE PAGE

7.5% gel was prepared using 3.5ml water, 2ml 50% glycerol, 2.5 ml 30% acrylamide, 2ml 5X TBE, 200 microlitre 10% APS, 15microlitre TEMED. Then there was pre-run for 15-20 minutes in 1XTBE buffer followed by loading of samples (2µl dye + 4µl PCR product) and run for 1.5 hours at 80 V, m A- 25. It was performed to visualise the microRNA expression.

CHAPTER IV

4. Results

4.1 The antiproliferative or cytotoxicity activity of doxorubicin on MDA-MB-231 cells

The anti-proliferative activity and toxicity of doxorubicin drug on MDA-MB-231 was determined by MTT assay. The IC50 of doxorubicin on MDA-MB-231 was determined by to be 10nM.

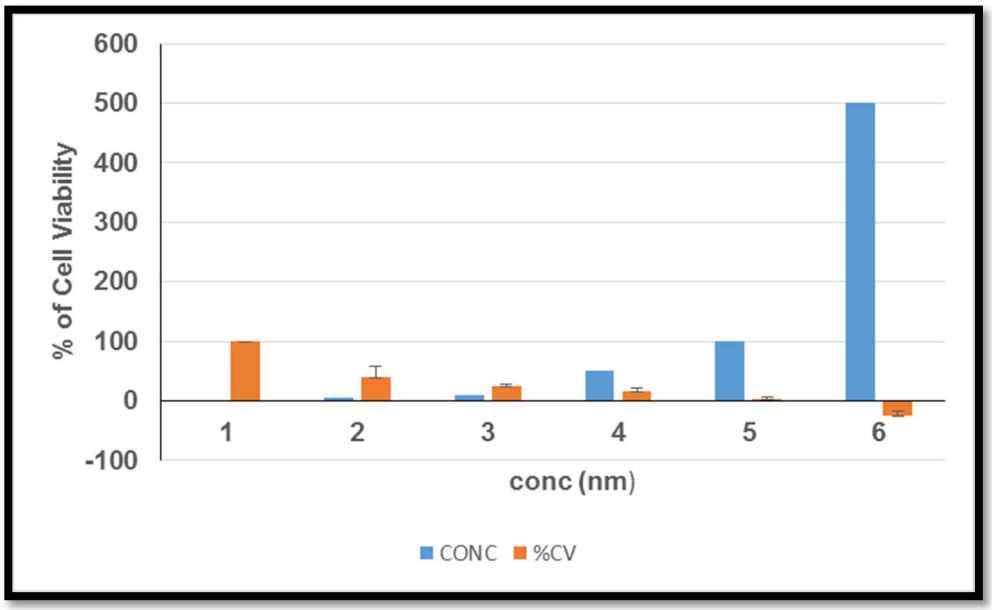

Figure 6. MDA-MB-231 (10^4) were seeded and incubated with varying concentration of Doxorubicin for 48 hrs. The IC50 value obtained by MTT assay is 10 nM. Here on x-axis (1=ctrl, 2=10nm, 3=20nm, 4=30nm, 5=40nm, 6=50nm)

4.2 The antiproliferative or cytotoxicity activity of α-Amanitin on MDA-MB-231 cells

The anti-proliferative activity and toxicity of drug α-Amanitin on MDA-MB-231 was determined by MTT assay. The IC50 of α-Amanitin on MDA-MB-231 was determined to be 30nM.

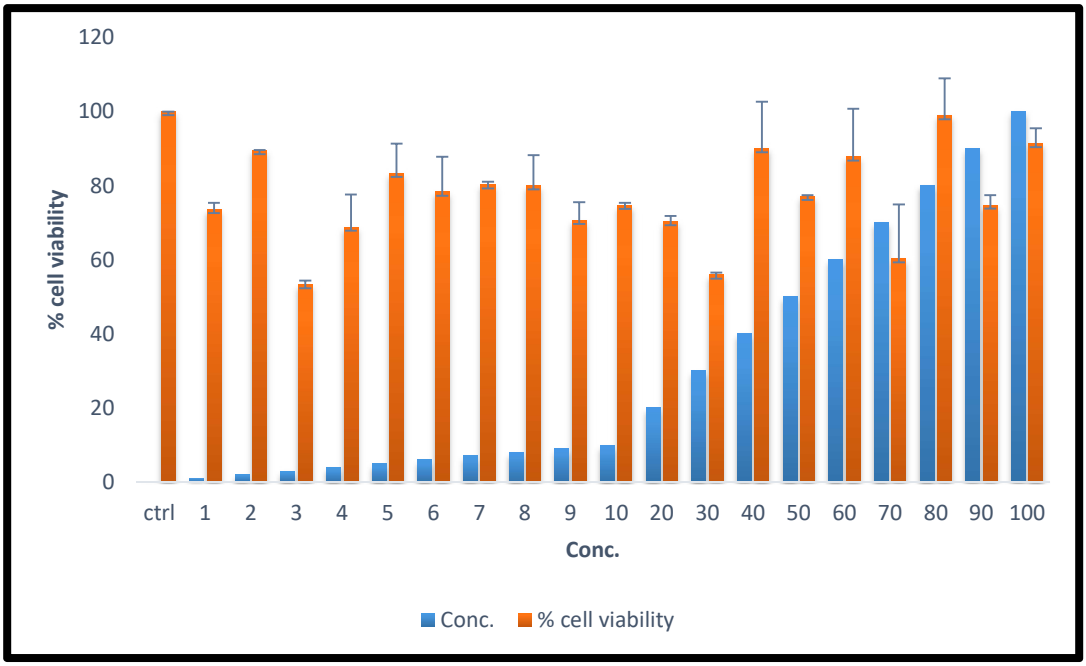

Figure 7. MDA-MB-231 (10^4) were seeded and incubated with varying concentration of α-Amanitin for 48 hrs. The IC50 value obtained by MTT assay is 30nM.

4.3 The antiproliferative or cytotoxicity activity of DCA on MDA-MB-231 cells

The anti-proliferative activity and toxicity of DCA drug on MDA-MB-231 was determined by MTT assay. The IC50 of DCA on MDA-MB-231 was determined to be 25mM.

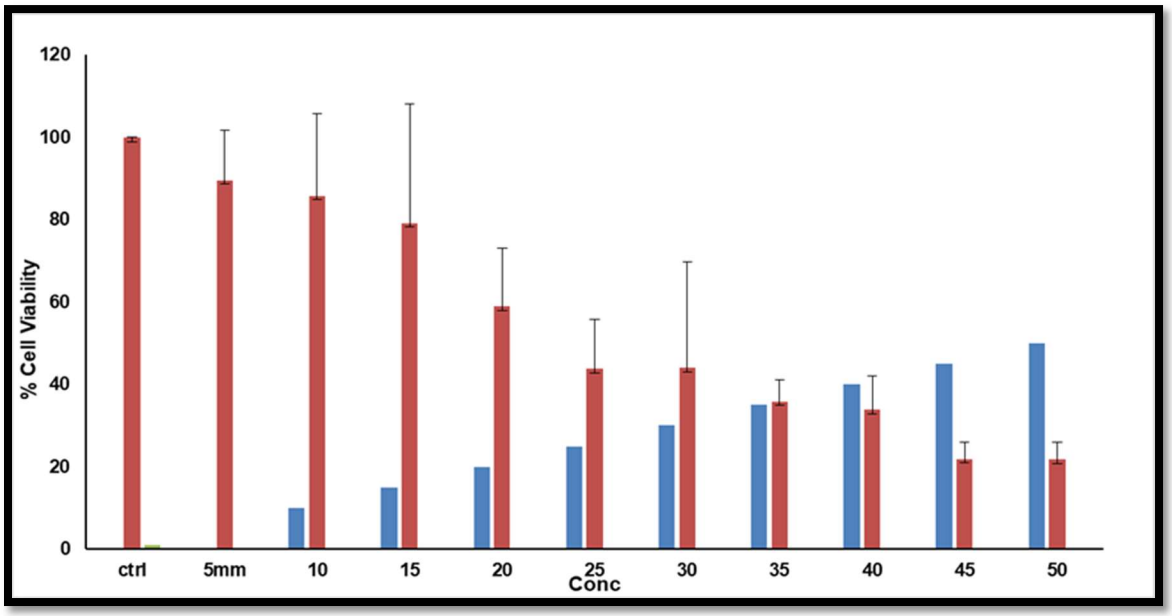

Figure8. MDA-MB-231 (10^4) were seeded and incubated with varying concentration of DCA for 48 hrs. The IC50 value obtained by MTT assay is 25 mM.

4.4 In normoxic condition, miRNA expression analysis by RT-PCR on Native PAGE

Upon determination of IC50, the cells were treated with these(doxorubicin, α-Amanitin and DCA) anticancer drugs and changes in expression of miRNA (let-7b and miR-1275) in MDA-MB-231 cells (untreated and treated) in normoxic condition were analysed by RT-PCR products on Native PAGE.

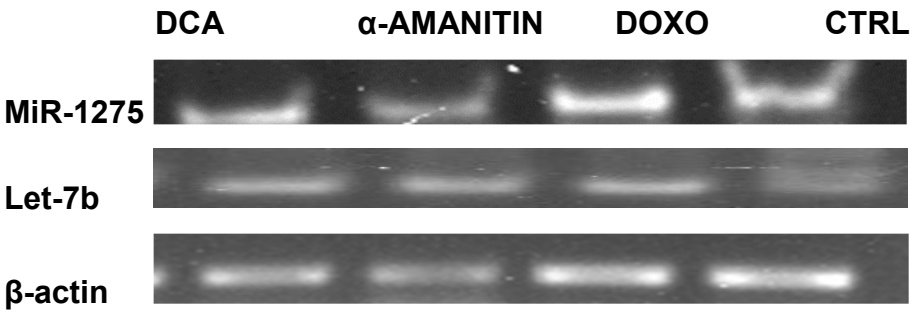

Figure 9(a). MiRNA expression was analysed by RT-PCR in MDA-MB-231 Cell line treated with Doxorubicin, DCA and α-Amanitin under normoxic condition.

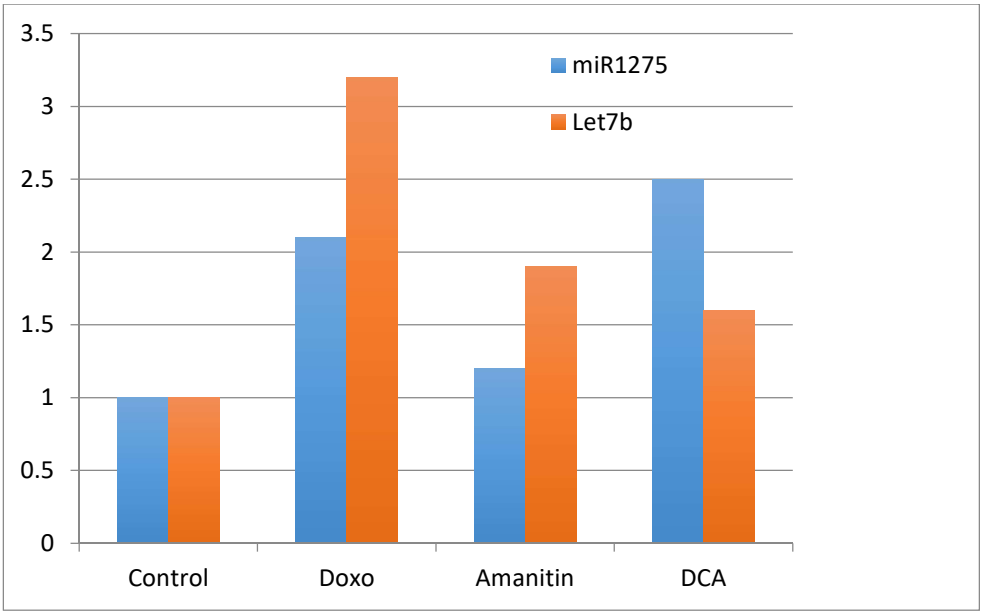

Figure 9(b). Densitometric analysis of MiRNA expression

4.5 In hypoxic condition, miRNA expression analysis by RT-PCR on Native PAGE

Upon determination of IC50, the cells were treated with these(doxorubicin, α-Amanitin and DCA) anticancer drugs and changes in expression of miRNA (let-7b and miR-1275) in MDA-MB-231 cells (untreated and treated) in hypoxic condition were analysed by RT-PCR products on Native PAGE.

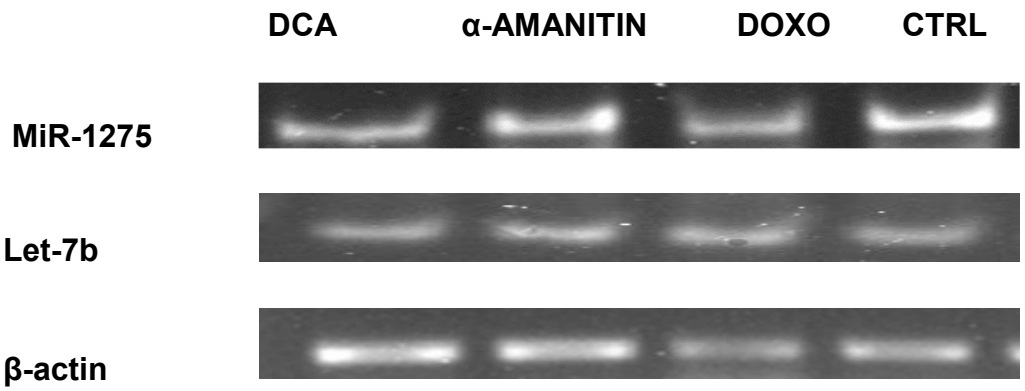

Figure 10(a). MiRNA expression was analyzed by RT-PCR in MDA-MB-231 Cell line treated with Doxorubicin, DCA and α-Amanitin under hypoxic condition.

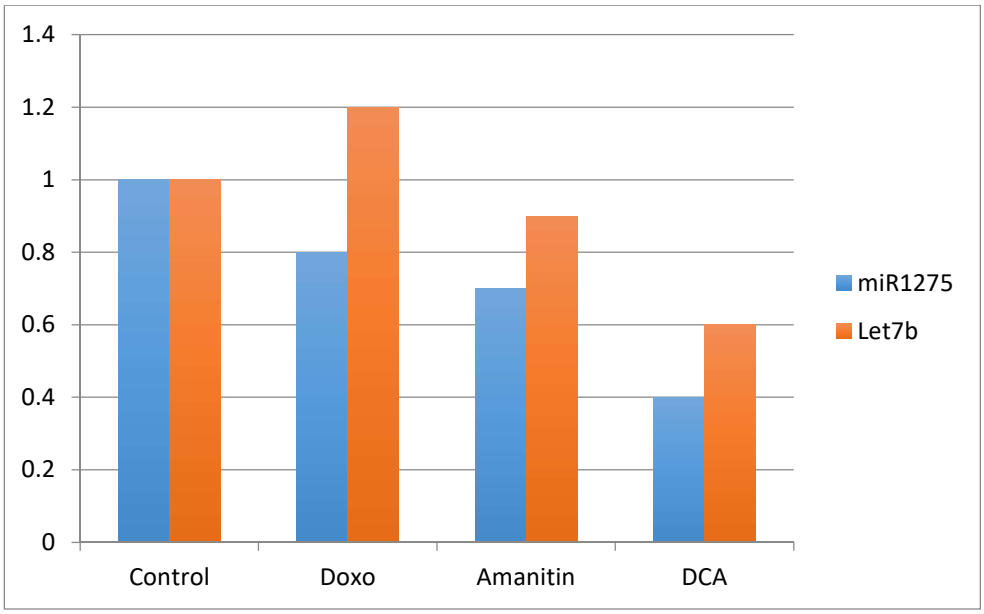

Figure 10(b). Densitometric analysis of MiRNA expression

CHAPTER V

5. DISCUSSION

Anticancer drugs are known to be used for long time for treating cancer, they can modulate the expression of oncogenic and tumor suppressor miRNAs and can provide a better option for treating cancer. miR-1275 was known to be affected by doxorubicin (Tormo *et al*., 2015). MiR-21 expression was significantly reduced in MCF-7 cells treated with doxorubicin(Tofigh *et al*., 2017). These anticancer drugs have different effect in normoxic and hypoxic condition, these drugs mainly stops proliferation of cells (Zolzer and Streffer 2002), thus can stop cancer progression in normoxic condition. While in hypoxic condition, cells get arrested in G1phase of cell cycle and become less sensitive to drugs(Brown 2000; Amellem and Pettersen 1991). MiRNA expression also gets affected in hypoxic condition , some miRNAs were shown to be consistently induced in hypoxic condition in breast and colon cancer cells, such as miR-21, 23a, 23b, 24 (Kulshreshtha *et al*., 2007) and some miRNAs were down-regulated in hypoxic condition, these are miR-122a, 565, 195, 30e-5p, 374 (Hua *et al*., 2006; Herbert *et al*., 2007; Donker *et al*., 2007). Let-7 family have showed contrasting behavior in hypoxic condition upregulated in squamous cell carcinoma (SCC) and downregulated in CNE cells (from nasopharyngeal carcinoma) (Hua *et al*., 2006; Herbert *et al*., 2007). Cell cycle can be slow down or arrested by hypoxia therefore microRNAs that are selectively expressed in one phase of the cell cycle are expected to exhibit decreased levels (Donker *et al*., 2007). Thus in the present study, we have investigated the effect of three anticancer drugs (Doxorubicin, α-Amanitin, DCA) on miRNA (let-7b and miR-1275) expression in normoxic and hypoxic condition. We found that in normoxic condition, there was an increased expression of both let-7b and miR-1275 in doxorubicin, α-Amanitin and DCA treated cells. Let-7b was most up-regulated in doxorubicin treated cells, while miR-1275 was most up-regulated in DCA treated cells. In hypoxic condition, except let-7b which has increased expression in doxorubicin treated cells, there was decreased expression of both miR-1275 and let-7b in doxorubicin, α-Amanitin and DCA treated cells. There was a slight decrease in miR-1275 expression in doxorubicin treated cells and major decrease in DCA treated cells. It could be inferred that doxorubicin was

responsible for increased expression of let-7b in both normoxic and hypoxic conditions. While DCA and α-Amanitin were responsible for increased expression of let-7b in normoxic condition and decreased expression in hypoxic condition. While for miR-1275, it has increased expression in normoxic condition in doxorubicin, α-Amanitin and DCA treated cells had highest expression in DCA treated cells but in hypoxic condition, there was decreased expression of miR-1275 in doxorubicin, α-Amanitin and DCA treated cells, had lowest expression in DCA treated cells. Thus the effect of anti-cancer drugs on miRNA expression was different in both normoxic and hypoxic condition.

Conclusion

This could be concluded as differential effect of these anti-cancer drugs in normoxic and hypoxic condition on the expression of miRNA. This study could be further expanded and the intermediate pathways to get these effects can be identified. This study can form the basis for further studies, to determine the targets of different anticancer drugs in different conditions.

CHAPTER VI

6. Summary

Background: MiRNAs have shown to be aberrantly expressed in different types of cancer. Numerous MiRNAs are shown to be de-regulated in breast cancer, thus it is helpful in distinguishing normal and malignant breast tissues (Iorio *et al*., 2009; Leivonen *et al*., 2014). MiRNAs modulates the expression of known oncogenic and tumor suppressor genes, thus has a role in tumorigenesis(Garzon *et al*., 2006; Cowland *et al*., 2007; Cho 2007; Zhang *et al*., 2007; Hummel *et al*., 2010). MiRNAs are thought to be involved in differential treatment responses(Eroles *et al*., 2015; Tian et al., 2013). Different MiRNA expression profiles have been obtained by microarray analysis in drug resistant and drug-sensitive cell lines(Chen *et al*., 2010; Pogribny *et al*., 2010; Zhou *et al*., 2010). MiRNAs can act as biomarkers and can be used as novel therapeutic anti-cancer targets. Some miRNAs were expressed in both control and treated cells but their level of expression differs. Some of them get upregulated or downregulated after treatment as compared to control (Shah *et al*.,2011). Some miRNAs get upregulated in hypoxic condition while some get downregulated in hypoxic condition. (Kulshreshtha *et al*., 2007)

Materials and methods: MDA-MB-231 cell line was cultured and then treated with doxorubicin, α-Amanitin, DCA and their IC50 value was determined by MTT assay. Then cells were treated with these anti-cancer drugs and incubated in normoxic and hypoxic conditions. Then RNA isolation, cDNA synthesis and RT-PCR was done followed by miRNA expression analysis by running Native PAGE.

Results and Discussion: In the present study, we have investigated the effect of three anticancer drugs (Doxorubicin, α-Amanitin, DCA) on miRNA (let-7b and miR-1275) expression in normoxic and hypoxic condition. We found that in normoxic condition, there was an increased expression of both let-7b and miR-1275 in doxorubicin, α-Amanitin and DCA treated cells. Let-7b was most up-regulated in doxorubicin treated cells, while miR-1275 was most up-regulated in DCA treated cells. In hypoxic condition, except let-7b which has increased expression in doxorubicin treated cells, there was

decreased expression of both miR-1275 and let-7b in doxorubicin, α-Amanitin and DCA treated cells. There was a slight decrease in miR-1275 expression in doxorubicin treated cells and major decrease in DCA treated cells. It could be inferred that doxorubicin was responsible for increased expression of let-7b in both normoxic and hypoxic conditions. While DCA and α-Amanitin were responsible for increased expression of let-7b in normoxic condition and decreased expression in hypoxic condition. While for miR-1275, it has increased expression in normoxic condition in doxorubicin, α-Amanitin and DCA treated cells, had highest expression in DCA treated cells but in hypoxic condition, there was decreased expression of miR-1275 in doxorubicin, α-Amanitin and DCA treated cells, had lowest expression in DCA treated cells. Thus the effect of anti-cancer drugs on miRNA expression was different in both normoxic and hypoxic condition. This can be concluded as differential effect of these drugs in normoxic and hypoxic condition with respect to the expression of miRNA. This study can be further expanded and the intermediate pathways to get these effects can be identified. This study can form the basis for further studies, to determine the targets of different anticancer drugs in different conditions.

REFERENCES

Ambros, V., Bartel, B., Bartel, D. P., Burge, C. B., Carrington, J. C., Chen, X., ... & Matzke, M. (2003). A uniform system for microRNA annotation. *Rna*, *9*(3), 277-279.

Azab, A. K., Hu, J., Quang, P., Azab, F., Pitsillides, C., Awwad, R., ... & Roccaro, A. M. (2012). Hypoxia promotes dissemination of multiple myeloma through acquisition of epithelial to mesenchymal transition-like features. *Blood*, *119*(24), 5782-5794.

Azab, A. K., Runnels, J. M., Pitsillides, C., Moreau, A. S., Azab, F., Leleu, X., ... & Alt, C. (2009). CXCR4 inhibitor AMD3100 disrupts the interaction of multiple myeloma cells with the bone marrow microenvironment and enhances their sensitivity to therapy. *Blood*, *113*(18), 4341-4351.

Bandara, V., Michael, M. Z., & Gleadle, J. M. (2014). Hypoxia represses microRNA biogenesis proteins in breast cancer cells. *BMC cancer*, *14*(1), 533.

Bohnsack, M. T., Czaplinski, K., & GÖRLICH, D. (2004). Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. *Rna*, *10*(2), 185-191.

Bristow, R. G., & Hill, R. P. (2008). Hypoxia and metabolism: hypoxia, DNA repair and genetic instability. *Nature Reviews Cancer*, *8*(3), 180.

Beiki, O., Hall, P., Ekbom, A., & Moradi, T. (2012). Breast cancer incidence and case fatality among 4.7 million women in relation to social and ethnic background: a population-based cohort study. *Breast Cancer Research*, *14*(1), R5.

Boyle, P. (2012). Triple-negative breast cancer: epidemiological considerations and recommendations. *Annals of oncology*, *23*(suppl_6), vi7-vi12.

Bushnell, D. A., Cramer, P., & Kornberg, R. D. (2002). Structural basis of transcription: α-amanitin–RNA polymerase II cocrystal at 2.8 Å resolution. *Proceedings of the National Academy of Sciences*, *99*(3), 1218-1222.

Brueckner, F., & Cramer, P. (2008). Structural basis of transcription inhibition by α-amanitin and implications for RNA polymerase II translocation. *Nature Structural and Molecular Biology*, *15*(8), 811.

Bonnet, S., Archer, S. L., Allalunis-Turner, J., Haromy, A., Beaulieu, C., Thompson, R., ... & Harry, G. (2007). A mitochondria-K+ channel axis is suppressed in cancer and its normalization promotes apoptosis and inhibits cancer growth. *Cancer cell*, *11*(1), 37-51.

Carvalho, C., Santos, R. X., Cardoso, S., Correia, S., Oliveira, P. J., Santos, M. S., & Moreira, P. I. (2009). Doxorubicin: the good, the bad and the ugly effect. *Current medicinal chemistry*, *16*(25), 3267-3285.

Cowland, J. B., Hother, C., & Grønbaek, K. (2007). MicroRNAs and cancer. *Apmis*, *115*(10), 1090-1106.

Cho, W. C. (2007). OncomiRs: the discovery and progress of microRNAs in cancers. *Molecular cancer*, *6*(1), 60.

Chen, G. Q., Zhao, Z. W., Zhou, H. Y., Liu, Y. J., & Yang, H. J. (2010). Systematic analysis of microRNA involved in resistance of the MCF-7 human breast cancer cell to doxorubicin. *Medical oncology*, *27*(2), 406-415.

Colditz, G. A., Rosner, B. A., Chen, W. Y., Holmes, M. D., & Hankinson, S. E. (2004). Risk factors for breast cancer according to estrogen and progesterone receptor status. *Journal of the National Cancer Institute*, *96*(3), 218-228.

Choi, J., Jung, W. H., & Koo, J. S. (2012). Clinicopathologic features of molecular subtypes of triple negative breast cancer based on immunohistochemical markers. *Histology and histopathology*, *27*(10), 1481.

Denli, A. M., Tops, B. B., Plasterk, R. H., Ketting, R. F., & Hannon, G. J. (2004). Processing of primary microRNAs by the Microprocessor complex. *Nature*, *432*(7014), 231.

Donker, R. B., Mouillet, J. F., Nelson, D. M., & Sadovsky, Y. (2007). The expression of Argonaute2 and related microRNA biogenesis proteins in normal and hypoxic trophoblasts. *MHR: Basic science of reproductive medicine*, *13*(4), 273-279.

E Asensio, P., T Martin, E., P Merlo, B., E Armas, E., & L Hernández, A. (2016). MicroRNAs in breast cancer: one More Turn in Regulation. *Current drug targets*, *17*(9), 1083-1100.

Gregory, R. I., Yan, K. P., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N., & Shiekhattar, R. (2004). The Microprocessor complex mediates the genesis of microRNAs. *Nature*, *432*(7014), 235.

Grishok, A., Pasquinelli, A. E., Conte, D., Li, N., Parrish, S., Ha, I., ... & Mello, C. C. (2001). Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. *Cell*, *106*(1), 23-34.

Garzon, R., Fabbri, M., Cimmino, A., Calin, G. A., & Croce, C. M. (2006). MicroRNA expression and function in cancer. *Trends in molecular medicine*, *12*(12), 580-587.

Gewirtz, D. (1999). A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. *Biochemical pharmacology*, *57*(7), 727-741.

Hua, Z., Lv, Q., Ye, W., Wong, C. K. A., Cai, G., Gu, D., ... & Zhang, Y. (2006). MiRNA-directed regulation of VEGF and other angiogenic factors under hypoxia. *PloS one*, *1*(1), e116.

Hebert, C., Norris, K., Scheper, M. A., Nikitakis, N., & Sauk, J. J. (2007). High mobility group A2 is a target for miRNA-98 in head and neck squamous cell carcinoma. *Molecular cancer*, *6*(1), 5.

Hutvágner, G., McLachlan, J., Pasquinelli, A. E., Bálint, É., Tuschl, T., & Zamore, P. D. (2001). A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science*, *293*(5531), 834-838.

Hummel, R., Hussey, D. J., & Haier, J. (2010). MicroRNAs: predictors and modifiers of chemo-and radiotherapy in different tumour types. *European journal of cancer*, *46*(2), 298-311.

Han, J., Lee, Y., Yeom, K. H., Kim, Y. K., Jin, H., & Kim, V. N. (2004). The Drosha-DGCR8 complex in primary microRNA processing. *Genes & development*, *18*(24), 3016-3027.

Hebert, J. R. (2009). Epidemiology: identifying cancer's causes. In 'The biology and treatment of cancer', Eds. Pardee AB, Stein GS.

Iorio, M. V., Casalini, P., Piovan, C., Di Leva, G., Merlo, A., Triulzi, T., ... & Tagliabue, E. (2009). microRNA-205 regulates HER3 in human breast cancer. *Cancer research*, *69*(6), 2195-2200.

Jung, Y., & Lippard, S. J. (2006). RNA polymerase II blockage by cisplatin-damaged DNA Stability and polyubiquitylation of stalled polymerase. *Journal of Biological Chemistry*, *281*(3), 1361-1370.

Ketting, R. F., Fischer, S. E., Bernstein, E., Sijen, T., Hannon, G. J., & Plasterk, R. H. (2001). Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. *Genes & development*, *15*(20), 2654-2659.

Knight, S. W., & Bass, B. L. (2001). A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in Caenorhabditis elegans. *Science*, *293*(5538), 2269-2271.

Kulshreshtha, R., Ferracin, M., Wojcik, S. E., Garzon, R., Alder, H., Agosto-Perez, F. J., ... & Calin, G. A. (2007). A microRNA signature of hypoxia. *Molecular and cellular biology*, *27*(5), 1859-1867.

Kaplan, C. D., Larsson, K. M., & Kornberg, R. D. (2008). The RNA polymerase II trigger loop functions in substrate selection and is directly targeted by α-amanitin. *Molecular cell*, *30*(5), 547-556.

Kedinger, C., Nuret, P., & Chambon, P. (1971). Structural evidence for two α-amanitin sensitive RNA polymerases in calf thymus. *FEBS letters*, *15*(3), 169-174.

Listerman, I., Bledau, A. S., Grishina, I., & Neugebauer, K. M. (2007). Extragenic accumulation of RNA polymerase II enhances transcription by RNA polymerase III. *PLoS genetics*, *3*(11), e212.

Leivonen, S. K., Sahlberg, K. K., Mäkelä, R., Due, E. U., Kallioniemi, O., Børresen-Dale, A. L., & Perälä, M. (2014). High-throughput screens identify microRNAs essential for HER2 positive breast cancer cell growth. *Molecular oncology*, *8*(1), 93-104.

Lee, Y., Jeon, K., Lee, J. T., Kim, S., & Kim, V. N. (2002). MicroRNA maturation: stepwise processing and subcellular localization. *The EMBO journal*, *21*(17), 4663-4670.

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., ... & Kim, V. N. (2003). The nuclear RNase III Drosha initiates microRNA processing. *Nature*, *425*(6956), 415.

Lee, K. B., & Sharp, P. A. (2004). Transcription-dependent polyubiquitination of RNA polymerase II requires lysine 63 of ubiquitin. *Biochemistry*, *43*(48), 15223-15229.

Landthaler, M., Yalcin, A., & Tuschl, T. (2004). The human DiGeorge syndrome critical region gene 8 and its D. melanogaster homolog are required for miRNA biogenesis. *Current biology*, *14*(23), 2162-2167.

Lund, E., Güttinger, S., Calado, A., Dahlberg, J. E., & Kutay, U. (2004). Nuclear export of microRNA precursors. *Science*, *303*(5654), 95-98.

McFate, T., Mohyeldin, A., Lu, H., Thakar, J., Henriques, J., Halim, N. D., ... & Zhou, S. (2008). Pyruvate dehydrogenase complex activity controls metabolic and malignant phenotype in cancer cells. *Journal of Biological Chemistry*, *283*(33), 22700-22708.

Ma, L., Li, G. Z., Wu, Z. S., & Meng, G. (2014). Prognostic significance of let-7b expression in breast cancer and correlation to its target gene of BSG expression. *Medical Oncology*, *31*(1), 773.

Martin, A. M., & Weber, B. L. (2000). Genetic and hormonal risk factors in breast cancer. *Journal of the National Cancer Institute*, *92*(14), 1126-1135.

Muz, B., de la Puente, P., Azab, F., Ghobrial, I. M., & Azab, A. K. (2015). Hypoxia promotes dissemination and colonization in new bone marrow niches in Waldenström macroglobulinemia. *Molecular Cancer Research*, *13*(2), 263-272.

Nitiss, J. L. (2009). Targeting DNA topoisomerase II in cancer chemotherapy. *Nature Reviews Cancer*, *9*(5), 338.

Nakielny, S., & Dreyfuss, G. (1999). Transport of proteins and RNAs in and out of the nucleus. *Cell*, *99*(7), 677-690.

Nguyen, V. T., Giannoni, F., Dubois, M. F., Seo, S. J., Vigneron, M., Kédinger, C., & Bensaude, O. (1996). In vivo degradation of RNA polymerase II largest subunit triggered by α-amanitin. *Nucleic acids research*, *24*(15), 2924-2929.

Ossovskaya, V., Wang, Y., Budoff, A., Xu, Q., Lituev, A., Potapova, O., ... & Daraselia, N. (2011). Exploring molecular pathways of triple-negative breast cancer. *Genes & cancer*, *2*(9), 870-879.

Pommier, Y., Leo, E., Zhang, H., & Marchand, C. (2010). DNA topoisomerases and their poisoning by anticancer and antibacterial drugs. *Chemistry & biology*, *17*(5), 421-433.

Pogribny, I. P., Filkowski, J. N., Tryndyak, V. P., Golubov, A., Shpyleva, S. I., & Kovalchuk, O. (2010). Alterations of microRNAs and their targets are associated with acquired resistance of MCF-7 breast cancer cells to cisplatin. *International journal of cancer*, *127*(8), 1785-1794.

Papandreou, I., Cairns, R. A., Fontana, L., Lim, A. L., & Denko, N. C. (2006). HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. *Cell metabolism*, *3*(3), 187-197.

Quesne, J. L., Jones, J., Warren, J., Dawson, S. J., Ali, H. R., Bardwell, H., ... & Caldas, C. (2012). Biological and prognostic associations of miR-205 and let-7b in breast cancer revealed by in situ hybridization analysis of micro-RNA expression in arrays of archival tumour tissue. *The Journal of pathology*, *227*(3), 306-314.

Raha, D., Wang, Z., Moqtaderi, Z., Wu, L., Zhong, G., Gerstein, M., ... & Snyder, M. (2010). Close association of RNA polymerase II and many transcription factors with Pol III genes. *Proceedings of the National Academy of Sciences*, *107*(8), 3639-3644.

Sánchez-Aragó, M., Chamorro, M., & Cuezva, J. M. (2010). Selection of cancer cells with repressed mitochondria triggers colon cancer progression. *Carcinogenesis*, *31*(4), 567-576.

Sutendra, G., Dromparis, P., Kinnaird, A., Stenson, T. H., Haromy, A., Parker, J. M. R., ... & Michelakis, E. D. (2013). Mitochondrial activation by inhibition of PDKII suppresses HIF1a signaling and angiogenesis in cancer. *Oncogene*, *32*(13), 1638.

Sun, R. C., Board, P. G., & Blackburn, A. C. (2011). Targeting metabolism with arsenic trioxide and dichloroacetate in breast cancer cells. *Molecular cancer*, *10*(1), 142.

Stockwin, L. H., Yu, S. X., Borgel, S., Hancock, C., Wolfe, T. L., Phillips, L. R., ... & Newton, D. L. (2010). Sodium dichloroacetate selectively targets cells with defects in the mitochondrial ETC. *International journal of cancer*, *127*(11), 2510-2519.

Sun, W., Zhou, S., Chang, S. S., McFate, T., Verma, A., & Califano, J. A. (2009). Mitochondrial mutations contribute to HIF1α accumulation via increased reactive oxygen species and up-regulated pyruvate dehydrogenease kinase 2 in head and neck squamous cell carcinoma. *Clinical Cancer Research*, *15*(2), 476-484.

Tian, W., Chen, J., He, H., & Deng, Y. (2013). MicroRNAs and drug resistance of breast cancer: basic evidence and clinical applications. *Clinical and Translational Oncology*, *15*(5), 335-342.

Shah, M. Y., Pan, X., Fix, L. N., Farwell, M. A., & Zhang, B. (2011). 5-fluorouracil drug alters the microrna expression profiles in MCF-7 breast cancer cells. *Journal of cellular physiology*, *226*(7), 1868-1878.

Tofigh, R., Akhavan, S., Tarban, N., Sadrabadi, A. E., Jalili, A., Moridi, K., & Tutunchi, S. (2017). Doxorubicin Induces Apoptosis through down Regulation of miR-21 Expression and Increases miR-21 Target Gene Expression in MCF-7 Breast Cancer Cells. *Journal of Clinical Medicine*, *8*, 386-394.

Tormo, E., Pineda, B., Serna, E., Guijarro, A., Ribas, G., Fores, J., ... & Eroles, P. (2015). MicroRNA profile in response to doxorubicin treatment in breast cancer. *Journal of cellular biochemistry*, *116*(9), 2061-2073.

Weinmann, R., Raskas, H. J., & Roeder, R. G. (1974). Role of DNA-dependent RNA polymerases II and III in transcription of the adenovirus genome late in productive infection. *Proceedings of the National Academy of Sciences*, *71*(9), 3426-3430.

Yi, R., Qin, Y., Macara, I. G., & Cullen, B. R. (2003). Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes & development*, *17*(24), 3011-3016.

Zhou, M., Liu, Z., Zhao, Y., Ding, Y., Liu, H., Xi, Y., ... & Riker, A. I. (2010). MicroRNA-125b confers the resistance of breast cancer cells to paclitaxel through suppression of pro-apoptotic Bcl-2 antagonist killer 1 (Bak1) expression. *Journal of Biological Chemistry*, *285*(28), 21496-21507.

Zeng, Y., & Cullen, B. R. (2003). Sequence requirements for micro RNA processing and function in human cells. *Rna*, *9*(1), 112-123.

Central University of Punjab

Declaration

I declare that all the changes suggested by the VC nominee examiner in the Research Project entitles “Identification of novel miRNA targets of anticancer drugs in normoxic and hypoxic conditions” submitted by me for the award of degree of Master’s in Science in Life Sciences with specialization in Molecular Medicine in the Department of Human Genetics and Molecular Medicine has been incorporated in the Research Project.

(Sapna Goyal)

Department of Human Genetics and Molecular Medicine

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Student Approval Form

Name of the Author	Sapna Goyal
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Degree	M.Sc. Life Sciences with specialization in Molecular Medicine
University	Central University of Punjab
Guide	Dr. Sandeep Singh
Project Title	Identification of novel miRNA targets of anticancer drugs in normoxic and hypoxic conditions.
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