GENOMIC AND PROTEOMIC ANALYSIS OF CHEMORESISTANCE IN BREAST CANCER CELL LINES: MCF-7 AND MDA-MB-231

by

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Abstract

In this study, cisplatin-resistant adenocarcinoma breast cancer cell lines, labeled MCF-7/R6 and MDA-MB-231/R2 were established by 6 replicative sequential treatment of low doses (1 micromolar) over high doses (30 micromolar) of cisplatin and chemoresistance was evaluated as apoptotic response to 30 micromolar cisplatin for 48 hours. Throughout the study parental cells were used as control.

RNA was isolated to conduct microarray study to analyze 48000 gene probes (Illumina Human WG-6 BeadChip). Gene expression data was analyzed by MATLAB 2009 and identification of differentially expressed genes were further studied by proteomic analysis to understand the drug resistance mechanisms.

Our data indicated that transcription of drug resistant marker genes, such as GSTP1 and ABCB6 were upregulated in MCF-7/R6 cell line. Both intrinsically resistant cell line: MDA-MB-231 and acquired resistant cell line: MCF-7/R6 were similar in activating NF- kappa-B pathway but not MAP kinase pathway. In MCF-7/R6 cell line NF-kappa-B pathway was transactivated through p50 subunit, as well as translocation of Foxo3a transcription factor into the nucleus.

Genes responsible for cell death, Foxo family and MAPK levels were found to change significantly at protein and genetic level in MCF-7/R6 and MDA-MB-231/R2 cells. Phosphorylation levels in transcription factor Foxo1 and Foxo3a were found to change MCF-7/R6 cells, indicating resistance to apoptosis. Moreover, cell cycle, tumor suppressor and estrogen receptor levels were found to be altered in MCF-7/R6 cell line.

Our data clearly identify several proteins and pathways which have distinct roles in chemoresistance mechanism. This knowledge can be further validated in a clinical setting.

KEMORESİSTANT MCF-7 VE MDA-MB-231 HÜCRE HATLARINDA PROTEOMİK VE GENOMİK ANALİZLERİN YAPILMASI

TUĞBA MEHMETOĞLU

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Anahtar kelimeler: Sisplatin, Dirençli, MCF-7, MDA-MB-231, MAPK, NF-κB, FOXO, Mikroarray

Özet

Bu çalışmada, 6 tekrarlı ardışık düşük doz (1 mikromolar) ilaç muamelesini takiben yüksek doz (30 mikromolar) ilaç verilerek, MCF-7/R6 ve MDA-MB-231/R2 olarak adlandırılan sisplatine dirençli adenokarsinom hücre hattı oluşturulmuş ve 48 saat sonunda 30 mikromolar ilaç verilerek apoptotik cevaba bakılmış ve kemoresistans durumu değerlendirilmiştir.

Microarray çalışması için RNA izole edilmiş, analiz için 48000 gen probu (Illumina Human WG-6 BeadChip) kullanılmıştır. Gen ekspresyon verileri MATLAB 2009 kullanılarak analiz edilmiş ve fark gösteren genler belirlenmiş ve ilaç resistant mekanizmalarını anlamak için proteomik analiz yapılmıştır.

Verilerimiz ışığında ilaca dirençliliğin oluşmasını sağlayan GSTP1 ve ABCB6 gibi markör genlerin transkripsiyonlarının MCF-7/R6 hücrelerinde arttığı görülmüştür. Kendiliğinden dirençli olan: MDA-MB-231 ve sonradan dirençli olan MCF-7/R6 hücreleri NF-kappa-B yolağını aktive etmede benzer fakat MAPK yolağında ise farklı davranmışlardır. MCF-7/R6 hücreleri NF-kappa-B yolağını p50 alt birimi ile aktive etmekle birlikte Foxo3a transkripsiyon faktörünü çekirdeğe geçişini sağlamıştır.

Hücre ölümü, Foxo ailesi ve MAPK seviyelerinden sorumlu olan genler, MCF-7/R6 ve MDA-MB-231/R2 hücrelerinde protein seviyesinde de anlamlı bir şekilde değişmiştir. Transkripsiyon faktörü olan Foxo1 ve Foxo3a fosforilasyon seviyelerindeki değişim MCF-7/R6 hücrelerinin apoptosise olan rezistantını göstermektedir. Hücre döngüsü, tumor baskılayıcı, ve östrojen reseptör seviyelerinin MCF-7/R6 hücrelerinde değiştiği görülmüştür.

Datalarımız açık olarak farklı rolleri olan kemoresistant mekanizmasına müteakip birçok protein ve yolak teşhis etmiştir. Bu bilgiler klinikte kullanılmak üzere doğrulanabilir.

"To My Family"

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Tuğba Mehmetoğlu

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ABBREVIATIONS

Annexin-V: Annexin A5

AP-1: Activator protein-1

Apaf-1: Apoptosis protease activating factor-1

ASK: Apoptosis stimulating kinase

BER: Base excision repair

C-terminus: Carboxyl terminus

cDDP: Cisplatin

CDK: Cyclin dependent kinase

CDKI: Cyclin dependent kinase inhibitor

DD: Death domain

DMSO: Dimethylsulfoxide

DR: Death receptor

EGFR: Endothelial growth factor receptor

ER: Endoplasmic reticulum

ERK: extracellular signal-regulated kinase

FADD: Fas-associated death domain

FBS: Fetal bovine serum

FOXO: forkhead box, sub-group O

GSH: Glutathione

GST: Glutathione S-transferase

HR: Homologous recombination

IKK: inhibitor kappa kinase

IκB: I kappa B

JNK: c-Jun N-terminal kinase

MAPK: Mitogen-activated protein kinase

MCP-1: Monocyte chemoattractant protein-1

MMR: Missmatch repair

MW: Molecular weight

NER: Nucleotide excision repair

NF-κB: Nuclear factor-kappa B

NHEJ: Non-homologous end joining

N-terminus: Amino terminus

PAK2: p21(CDKN1A)-activated kinase

PARP: Poly-(ADP-ribose) polymerase

PI3K: Phosphatidylinositol-3 kinase

PKC: Protein kinase C

RIP: Receptor interacting protein

ROS: Reactive oxygen species

RTK: Receptor tyrosine kinase

SAPK: Stress-activated protein kinase

TNF: Tumor necrosis factor

TRADD: TNF-R-associated death domain

TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand

UV: Ultraviolet

XIAP: X-linked Inhibitor of Apoptosis Protein

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1. INTRODUCTION

Cancer arises from sequential changes that accumulate in the cell over time. One of the most implications is having combination of genetic, epigenetic and environmental changes which lead to transcriptional deregulation of genes, activation of different signal transduction pathways and altered gene expression. Cancer cells have some points in common; at the genetic level both loss and gain of function of genes, at the chromosomal level, abnormal chromosome number (aneuploidy, is universal in solid tumors), centrosome amplification, chromosome imbalance and loss of heterozygosity [26-28].

1.1 Breast Cancer

Breast cancer is the most frequently seen cancer type among women and mortality rate is considerably high compared to other cancer types in both developing and developed countries. According to the World Health Organization statistics, nearly 1.2 million people worldwide were diagnosed with breast cancer in 2007. The American Cancer Society estimates for women that 27% of new cancer cases resulted to death after attributing to invasive breast cancer in the United States in 2009. The probability of developing invasive breast cancer during a woman's lifetime is approximately 1 in 8. Although there is a slight chance of common, approximately 1,700 cases of male breast cancer occur each year [1].

Breast cancer arises from the mutation of genes controlling cell growth, suppressing tumor development and repairing DNA. One of the genes that are mutated in breast cancer is BRCA1 and BRCA2. In addition to that, breast cancer developed by hormone deregulation such as progesterone and estrogen, both promotes breast tumorigenesis. In

breast cell cultures studies; strong correlation between progesterone receptors and cell growth is observed [35, 36].

In the long run, patients who are diagnosed with breast cancer begin to have distant metastasis. The prognosis still stays unclear. Breast cancer diagnosis level is crucial for patient. Stage IV patients have poor survival rate, whereas those with Stage I, usually have a normal life span. The involvement of axillary lymph nodes is an important indicator for overall survival in breast cancer. Nearly, 20 to 30% of node-negative patients relapse within 10 years, compared with about 70% of patients with axillary nodal involvement [64-68].

Adjuvant chemotherapy is highly recommended to people who have breast cancer with node positive case, but for those who are node negative, adjuvant therapy create a risk of disease recurrence and death.

1.1.1 Role of BRCA genes in breast cancer

BRCA is a tumor suppressor gene that plays major role in breast cancer development. This gene somehow becomes disabled in progressing phase of the cancer. Two variants of BRCA do exist; BRCA1 and BRCA2. They have a wide range of function that is sensing DNA damage, repairing DNA, stabilizing chromosome. Once BRCA1 gene is mutated in breast cancer cells, double strand breaks that happen at basal state in G1 phase of the cell cycle cannot be removed and some part of the DNA sequences are bypassed during replication and lost or moved to another place in the chromosome where similar sequence is found. Moreover, when BRCA gene mutation happened, instead of “error free” DNA repairing mechanism that is homologues recombination (HR), “error prone” DNA repair mechanism that are non-homologous end joining (NHEJ), and single-strand annealing (SSA) become to be preferred as default [11].

Poly (ADP-ribose) polymerase-1 (PARP-1) is an enzyme that is involved in base excision repair which is an essential mechanism for single strand break (SSBs). Inhibition of PARP-1 leads to accumulation of SSBs and leading to Double strand

breaks (DSBs) and subsequently contributing BRCA gene mutation in breast cancer. This involvement inhibits HR (homologous recombination) type repair and NHJE (non-homologous end joining) on the other hand, promotes SSA (Single-Strand Annealing) type repair mechanisms [15].

1.2 Chemotherapeutic resistance

1.2.1 Anti cancer agent: Cisplatin

Cisplatin is a chemotherapeutic drug used in the treatment of a variety of neoplasms and various cancers. Cisplatin induces DNA damage, inhibits DNA synthesis, suppresses RNA transcription, affects the cell cycle, and induces apoptosis in cancer cells by interfering with the resistance mechanism [155]. One proposed mechanism for major cisplatin activation is that, it becomes activated inside the cell by replacing two *cis*-chloro "leaving" groups with water and subsequently attaches to DNA with a covalent bond, results DNA adducts. Once it is activated, it becomes positively charged. The cytotoxicity of cisplatin is primarily comes from its interaction with nucleophilic N7-sites of purine bases which are common in all cells' DNA and results DNA–protein and DNA–DNA interstrand and intrastrand crosslinks [156].

Cisplatin exerts its cytotoxic action through binding to DNA; mostly to guanine (G) and lesser extent to adenine (A) base [157]. Binding mostly occur on the same strand of DNA, even in between adjacent bases known as intrastrand adducts, interstrand adducts or crosslinks namely GpG 1,2 intrastrand (60-65%) and ApG 1,2 intrastrand (20-25%). In rare cases, same strand adducts can occur, namely GpXpG intrastrand crosslink where X represents any type of nucleotides (2%). Eventually 2% of adducts are G-G interstrand crosslinks that involves G on opposite strands [158]. In all cases two amine groups in cisplatin stay together with platinum.

Generation of DNA adducts activates various signaling pathways in the cell, such as cell cycle arrest, programmed cell death /apoptosis if cell inadequately repaired [155]. By existence of this adducts; bending, winding, unwinding and transcription of DNA is

inhibited so; this is recognized by many cellular proteins that can also be involved in DNA repair mechanism [159].

1.2.2 Cisplatin Resistance

Apoptosis effect which is resulting from cisplatin induced DNA damage can be attenuated by de novo established drug resistance mechanism. That mechanism creates limitations for cisplatin-based chemotherapy [160].

Reasons for gaining resistance are; first, insufficient amount of drug is delivered to target cells; decreased accumulation or increased efflux of the drug may happen and second, Pt-DNA adducts may be repaired fast enough to impede apoptosis pathway [161, 162].

Cisplatin resistance exists as a natural mechanism against drug called intrinsic resistance or can be gained during cycles of application along therapy by some effected organs with cancer such as ovarian, lung or colorectal. In other way, some cells always show hypersensitivity to cisplatin such as seen in testicular cancer cells. All in all, cisplatin resistance is cell type and tissue dependent. An excellent example to acquired resistance case occurs in ovarian cancer, which generally responds well to cisplatin-based therapy at the beginning. Unfortunately, the initial response rate of cancer cells are up to 70% but not durable, and to the end of the therapy, decrease of survival rate down to 15–20% is seen because tumors become resistant to therapy [163].

1.2.2.1 Resistance through insufficient DNA binding

Resistant tumor cell lines have less amount of Platinum accumulation than parental tumor cells [164]. Also upregulation or downregulation of certain proteins influence uptake or efflux of drug.

In multidrug resistance mechanism, ATP dependent efflux pumps such as P-glycoprotein’s are overexpressed; in cisplatin resistance, this pumps works for both decreasing uptake of drug and increasing efflux of drug [165]. Example to this efflux proteins are ATP-binding cassette protein subfamily and copper transporters; MDR1,

MRP2, MRP3 and ATP7A, ATP7B subsequently. ATPase genes are overexpressed in cisplatin resistant tumor cells [166]. ATP7B gene is used as a clinical marker of chemoresistance to cisplatin in ovarian cancer [167]. Copper transporter-1 (CTR1) role in cisplatin efflux has been shown clearly [168, 169].

Cisplatin or carboplatin treatment causes cell to express more thiol-containing species such as glutathionine or metallothionein [Figure 1.1]. These two compounds are rich in methionine and cysteine and that leads to detoxification of drug because platinum can bind to sulphur strongly when compared to DNA. Cisplatin conjugated with glutathionine is catalyzed by glutathione S-transferases (GSTs) that makes compound more anionic so it can be pumped out to the cell more easily by ATP dependent glutathionine S-conjugate export (GS-X) pump namely, MRP1 and MRP2 [126]. In translational studies, cisplatin or other DNA alkylating agent application to tumor biopsies resulted, glutathionine metabolic pathway modulation, *de novo* formation and inheritance [170]. Overexpression of low-molecular-weight thiol containing proteins or metallothioneins results heavy metal binding and detoxification so this leads to cisplatin resistance which is specific to cell types at many folds [171, 172]. Resistance due to elevated GSH is acquired by overexpression of c-Jun gene and it is a reversible step [8].

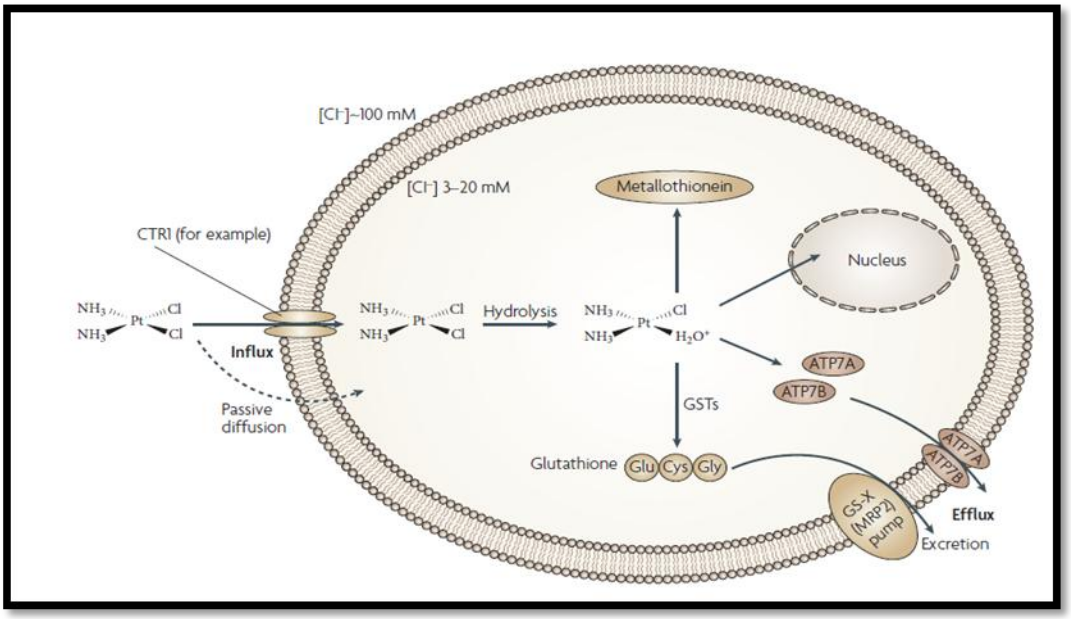


Figure 1. 1: Cisplatin resistance during delivery of drug to the cell-prior to entering to nucleus (adapted from Kelland et al, 2007 ref 173).

1.2.2.2 Resistance mediated after DNA binding

Cisplatin binding to DNA forms adducts that inhibit normal cellular functions, but cells can compensate this phenotype by activating DNA repair enzymes or removing these adducts. Testicular cancer cell has DNA repair deficiency by contrast in many resistant tumor cell lines have increased DNA repair capacity [174]. Once DNA is damaged, nearly 20 individual candidate proteins can recognize this adduct and bind physical distortions [175].

There are 4 major ways for DNA repairing; Nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR) and double strand break repair. NER is the major pathway to remove cisplatin lesions from DNA, for the last years many groups have particular attention on NER endonuclease protein called ERCC1 (Excision repair cross complementing 1). ERCC1 forms heterodimer with Xeroderma Pigmentosum complementation group F (XPF), and make 5' incision near to platinated DNA to repair. Resistant tumor cells have increased level of ERCC1 and XPF protein expression [176]. Downregulation of ERCC1 via siRNA showed enhanced cellular sensitivity against cisplatin and decreased level of action of NER for removing DNA lesions [177]. In patient who suffers from resistant ovarian cancer has increased ERCC1 mRNA expression, nearly 2.6 folds [178]. MMR and HMG1, demonstrate greater preference for cisplatin adducts than for adducts induced by distinct platinum analogs [Figure 2]. Actual role of the DNA damage recognition proteins are; transducing DNA damage signal to downstream effectors, in this way appropriate action takes place but by means of HMG1 protein, case is different. It binds to the damaged DNA part but then shields this area from repair enzyme so it promotes cytotoxicity [179].

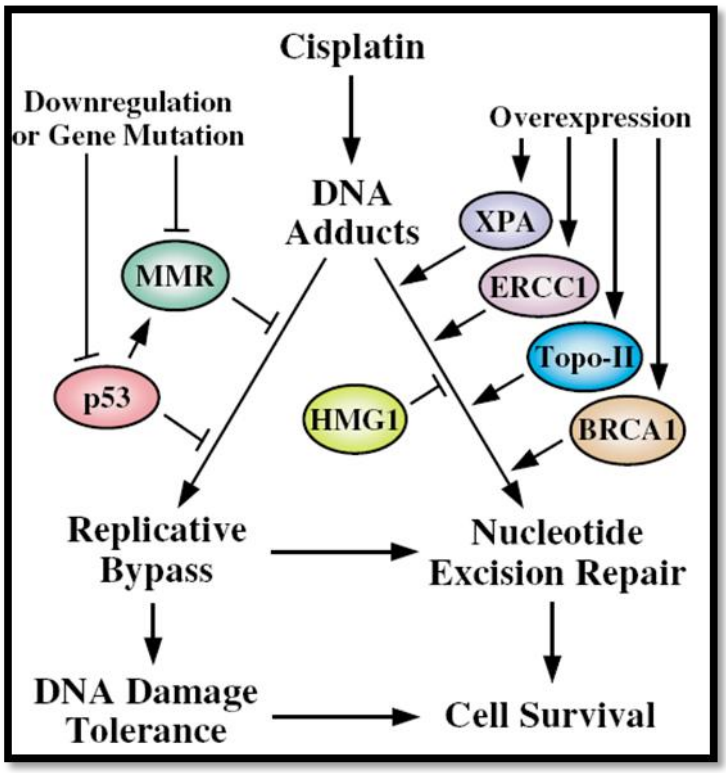


Figure 1. 2: Regulated mechanisms for DNA adducts during cisplatin resistance-mostly takes place inside the nucleus [adapted from Stordal et al 2007, ref 180].

Once the cells are treated with cisplatin, RAD51 foci formation, that show DNA damage is revived by homologues recombination, nearly diminished in BRCA1 mutant cells. Because of cells that cannot restore DNA damages, they are led to apoptosis [3]. BRCA1 gene transfection assays showed that; reconstitution of the gene to normal level or to wild type level, make cells resistant to cisplatin [4].

The most realistic breast cancer mouse model was achieved with deletion of both $p53^{k14cre}$ gene and $BRCA1^{k14cre}$ gene together only in epithelial cells; it is called as Jonkers mouse [5]. When Jonkers mouse is treated with anti-cancer agent; cisplatin, docetaxel and doxorubicin, tumors never developed drug resistance but except doxorubicin. In doxorubicin resistant tumors, gene expression study showed that; Mdr1a and Mdr1b genes upregulated. That result was documented by increased transportation of doxorubicin out of doxorubicin resistant tumors [6]. Cells were also initially sensitive to cisplatin but after treatment of tumor with cisplatin, cancer recurred. Hypothesis for cisplatin resistance cells to survive: cisplatin damages DNA, surviving cells against cisplatin treatment has to develop ability to produce repair

proteins/ or survival proteins to prepare itself for the next insult. Continuous exposure of tumor to cisplatin selected only cisplatin resistant stem cell and these cells become aggressive after some time of treatment and began to invade [7].

Patel et al. established breast cancer cell line that is over-expressing IκB, and found that these mutant cells, when compared to parental cells, express fewer anti-apoptotic proteins and are more sensitive to taxol induced apoptosis [181]. Thus, NF- κB - regulated genes seem to have effect on resistance against chemotherapy via the expression of anti-apoptotic proteins.

Drug resistant and metastatic cancer cells behave in similar way. By means of resistance and enhanced invasiveness, both type of cells show great analogy [153].

1.3 Nuclear Factor kappa (NF-κB)

NF-κB was discovered in the nuclei of mature B lymphocytes as a transcription factor that binds to 10 base pairs long sequence on DNA in the kappa immunoglobulin light-chain enhancer by a group of scientist Sen *et al* in 1986 [69]. Inappropriate NF κB expression is known to be the reason of coupling one transcription factor to aberrant, upstream signaling pathways affecting specific DNA promoter(s) involved in inflammation, cell survival, oncologic transformation or apoptosis because NF κB is a pleiotropic transcription factor. [144].

1.3.1 Canonical and non-canonical NF-κB pathway

The NF-κB/Rel family comprised of 5 subunits; p65 (RelA), p50/p105 (NFκB1), p52/p100 (NFκB2), c-rel and RelB. NF-κB1 is derived from proteolytic processing of its precursor p105 and liberates p50. NF-κB2 is derived from proteolytic processing of its precursor p100 and liberates p52. RelA, RelB, c-rel proto-oncogene products contain transcriptional activation domains on the other hand p50 and p52 do not [49, 145]. These 3 members, v-rel oncogene and the drosophila morphogen dorsal-encoded protein also belong to Rel family of protein [49, 145]. These subunits are identified and cloned

in mammalian cells [70, 71]. All NF-κB proteins involve 300-amino acid N-terminal Rel Homology Domain (RHD) that enables them to bind DNA and IκB also make dimmers with Rel family proteins and target to nucleus [72, 144].

NF-κB can be activated by either canonical or non-canonical pathway [reviewed in reference (144,146)].

In canonical pathway; there is activation and degradation of IKK and IκB subsequently. IκB Kinase (IKK) initiates degradation of IκB by phosphorylating it from 2 conserved serines in the N-terminal. Phosphorylated IκB becomes dissociated from NF-κB, unmasking the NLS. Phosphorylation also results in IκB ubiquitination and targeting to the proteasome to degrade rapidly by the 26S proteasome [79, 80]. This lets NF-κB to translocate to the nucleus and activate the transcription process [Figure 1.3A].

In non-canonical pathway; B cell-activating factor receptor (BAFF), CD40, and the lymphotoxin-βκ-receptor (LTβR) stimulated NF-κB-inducing kinase (NIK) and IKKα. This induction results with processing of p100 to p52 and facilitating the nuclear translocation of RelB/p52 dimmers into the nucleus as showed in Figure 1.3B [146]. Compared to p52 whose proteolytic processing is tightly regulated, the proteolysis of the precursor protein p105 to p50 is constitutive in unstimulated cells [144]. Increasing evidence of dysregulated NF-κB-associated pathways in various human breast cancer cell lines and primary tumors shows that NF-κB has an important role in neoplastic transformation [147]. Abnormal proliferation and branching of the mammary epithelium is observed in IκBα gene knock out mouse [148]. Inhibition of NF-κB restores the sensitivity of endocrine resistant breast cancer to tamoxifen [149]. P50/p65 is the major increased NF-κB dimmer in breast cancer cell lines [150]. Zhou et al. claimed that the activation of the p50 homodimer might be used as a prognostic marker in a subset of ER positive breast cancer patients [149, 151].

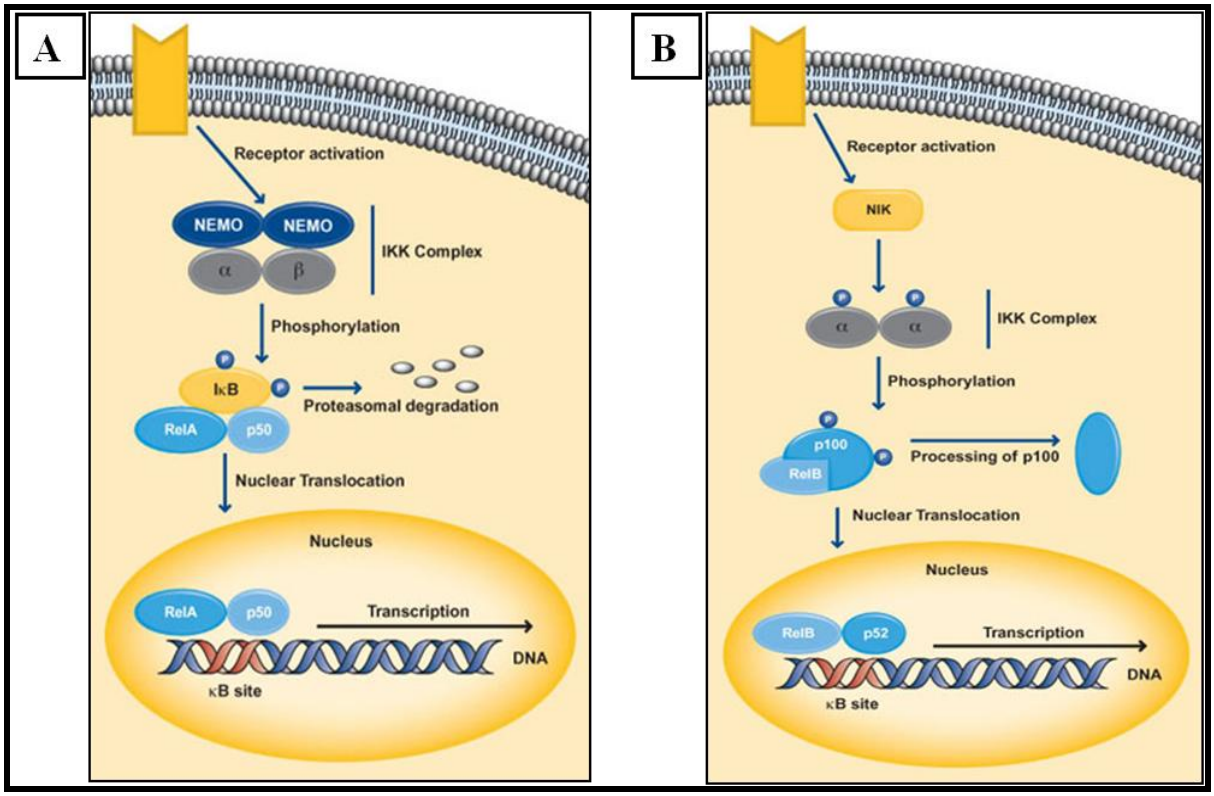


Figure 1. 3: Pathway of NF-κB activation. A: Canonical pathway B: Non-canonical pathway. [Retrieved from: www.abcam.com].

Before activation of NF-κB subunits, they can undergo either homo- or heterodimerization in the cytoplasm in this way that binds to promoter regions which is decameric sequences of both cellular and viral genes [45-48, 72].

1.3.2 IκB family of proteins

In resting cells, NF-κB dimmers are sequestered in the cytoplasm in a passive form via association with IκB (inhibitor of κB) family. The IκB family involves structurally related proteins defined as IκB-α, IκB-β, IκB-γ, IκB-ϵ, IκB-ζ, p105, p100, bcl3, among which IκB-α is the most abundant inhibitor protein for NF-κB [72–75]. These proteins characteristically include a stretch of five to seven ankyrin repeat domains concealing the nuclear localization sequences within the RHD of dimeric NF-κB. Activation of the NF-κB signaling cascade results in complete degradation of IκB by the 26S proteasome, or partial degradation of the carboxyl termini of p105 and p100 precursors, via cleavage

of ankyrin domains revealing the nuclear localization sequences of NF-κB proteins [72]. These modification processes of IκB and p105/p100 enable NF-κB to translocate into nucleus in a dimeric form, thereby affecting target gene expression [76].

1.3.3 IKK family of proteins

When cells are stimulated with proper ligand, signaling pathways of NF-κB converge on a family of proteins termed IκB kinase (IKK) kinases. IKK contains members of the mitogen-activated protein kinase kinase (MAPKK) family, such as NF-κB-inducing kinase (NIK) and mitogen-activated protein kinase/ERK kinase kinase 1 (MEKK1), 2, and 3 [77]. These proteins activate IκB kinase (IKK) complex, a heterotrimeric cytoplasmic protein including two catalytic subunits, IKK-α (IKK1) and IKK-β (IKK2), and a structural component termed IKK–γ (NEMO) [78]. IKK–α and IKK–β subunits are catalytically active and the IKK–γ subunit serves a regulatory function.

1.3.4 Mechanism of NF-κB Specificity

NF-κB has been connected to various metabolism processes and diseases, including developmental processes, inflammation and immune responses, cell growth, cancer, atherosclerosis, AIDS, diabetes, and the expression of certain viral genes [81]. Interestingly, NF-κB is induced by more than 150 different extracellular stimuli, enclosure but not limited to bacteria, virus, interleukins, growth factors, chemotherapeutic agents, and various stress stimuli (physiological, physical, and oxidative) [82]. Similarly, the activation of NF-κB promotes the expression of more than 150 target genes; Figure 1.4 illustrates particular genes [82].

Immune response	Immunoglobulin ε & κ Chains
	Interleukin 2 Receptor
	Major Histocompatibility Antigen Class I & II
	β2 Microglobulin
	T Cell Receptor
Regulators of apoptosis	Pro-Survival Bcl-2 Homologue
	cCD95 (Fas)
	Inducers of Apoptosis (IAPs)
Stress response genes	Angiotensin II
	Cyclooxygenase-2
	Lipoxygenase
	Inducible Nitric Oxide Synthase
	Phospholipase A2
Transcription factors	c-myc
	c-rel
	Inhibitor of Rel/NF-κB (IκB)
	Interferon Regulatory Factor 1 & 2
	junB
	NF-κB Precursors (p100 & p105)
	p53
Miscellaneous	Apolipoprotein C III
	Cyclin D
	Factor VIII
	Vimentin
	α1-Antitrypsin

Class	
Acute phase proteins	Angiotensinogen
	Complement factor B & C4
	C-Reactive protein
	Urokinase-type plasminogen activator
Cell adhesion molecules	Endothelial cell leukocyte adhesion molecule
	Intracellular Adhesion Molecule
	Platelet Adhesion Receptor
	Vascular Cell Adhesion Molecule
Cytokines	Interleukin 1, 2, 6, 8, 9, 11, 12 & 15
	Interferon γ
	Lymphotoxin α & β
	Tumor Necrosis Factor α & β
Enzymes	Collagenase I
	Glucose-6-Phosphate Dehydrogenase
	Lysozyme
	Transglutaminase
	Xanthine Oxidase
Growth factors	Colony Stimulating Factors
	Insulin-Like Growth Factor Binding Protein 1 & 2
	Platelet-Derived Growth Factors
	Thrombospondin
	Vascular Endothelial Growth Factor

Figure 1. 4: Brief list of NF-κB target genes

The aggregation of NF-κB inducers and its target genes suggests that NF-κB may function as a "central mediator of the human stress response" [82]. For NF-κB, the specificity may depend on the cell type targeted; not all cell types respond equally to a given stimulus, either because they devoid of the cognate receptor or because they lack the required signal transduction molecules [77]. NF-κB has a combinatorial regulation of target genes. Sometimes increment of NF-κB only is not enough for target gene expression it also requires other transcription factors. In this way NF-κB becomes very specific to the events and can have opportunity to merge with other signaling pathways [82].

1.3.5 Effect of NF-κB on breast cancer

Several studies were conducted on effect of NF-κB on breast cancer development and progression. ER-negative cell lines, MDA-MB-231, in general, contained elevated levels of AP-1 and NF-κB compared to MCF-7 cells at constitutional level [55]. NF-κB physically associates with transcription factor AP-1 and can indirectly increase expression of AP-1 regulated genes [44]. The NF-κB –DNA complex in ER-negative cell lines is comprised of the RelA subunit of NF-κB, confirmed by super-shift assay. Nakshatri et al. reported that; RelA band but not c-Rel antibody band super-shifted [55]. Estrogen receptor (ER) interferes with; constitutional transcriptional activation function of NF- κB and binding of it to decameric promoters in MDA-MB-231cells [55]. A posttranslational modification of NF- κB is crucial for optimal transcriptional activation

through NF- κB, and such modification takes place merely in ER-negative cell lines [55]. There is a parallel correlation between constitutional activation of NF- κB and DNA binding activity; on the other hand there is an inverse relationship between NF-κB activation and estrogen receptor (ER) presence status in breast cancer cells [55].

As the cancer cell become more vigorous and cell growth become deregulated, in parallel NF- κB is hyper-activated. Upon switching from inducible to constitutive NF-κB activation, cell become resistant to apoptosis and become to secrete chemokines, cytokine and growth factors and also have tendency to metastasize [39, 40]. Aberrant regulation of NF- κB modulates the response against to the cytotoxic effects of TNF-induced or radiation-induced apoptosis [61]. The proliferation of both normal and transformed rat Mammary Epithelial Cells (MEC) was accompanied by increased DNA-binding activity of the NF-κB p50 homodimer [143]. NF-κB down regulation is probably required for TP53-dependent apoptosis [62]. Because p53 is known to trigger apoptosis in cells with damaged DNA and conversely NF- κB promotes the survival of these mutated cells and leads to neoplastic transformation [152].

Target gene activation of NF-κB is not only dependent upon nuclear translocation but also dependent on tyrosine kinase transactivation process. In addition to that, increased level of expression of NF- κB does not have to be finalized with increased DNA binding [56]. NF-κB regulates the expression of many genes including; integrin-α_v which is required for metastatic protein activation MMP2 [37, 38] and many cell surface receptors, among them the Mdr-1 gene, which encodes the multiple drug resistance mediator [154].

Treatment of cancer cells with Cisplatin result in degradation of IκB proteins, thus releasing the bound NF-κB, which translocates to the nucleus and upregulates target gene expression [50]. Taken together, these results suggest that the NF- κB system plays a critical role in balancing survival and death of mammary epithelia, and that aberrant NF- κB signaling is responsible, relatively, for breast cancer progression.

1.4 MAP kinases

Mitogen-activated protein kinases ubiquitous and highly complex intracellular signaling network that is ultimately responsible for regulating gene expression in response to a variety of extracellular stimuli. MAPK pathways activate diverse fundamental processes: metabolism, survival, mitosis and apoptosis [197, 198]. In mammalians, MAPKs are categorized to 3 groups according to function: stress-activated protein kinase/c-Jun N-terminal kinases (SAPK/JNKs 1, 2 and 3), p38 (isoforms a, b, c and d) and extracellular signal-regulated kinases (ERKs 1, 2, 3, 4 and 5).

Each family of MAPKs is consisting of a set of three evolutionary conserved, consecutively acting kinases: MAPKK kinases (MAPKKKs or MAP3K), MAPK kinases (MAPKKs or MAP2K) and MAPKs. The MAP3K is a serin–threonine kinase. It is a contact molecule that senses active signals from the membrane-spanning receptors, then phosphorylates thus activates its downstream substrate which is MAP2K. MAP2K is an intermediate kinase with dual-specificity. It phosphorylates threonine and tyrosine residues on MAPKs. MAPKs are also serine-threonine kinase which phosphorylates and enables cytoplasmic proteins to transmit the signal from cytoplasm to the nucleus [199].

SAPK/JNK and p38 are activated throughout stress and inflammation evoked by a variety of physical, chemical and biological stress stimuli whereas ERK1/2 cascade is mostly activated by being exposed to growth factors. Despite differences of stimuli that activates them, these pathways merge and crosstalk [198]. MAPKs are induced by consequential stimulation of uppermost kinase cascade [197, 198].

Wide range of substrates are phosphorylated by MAPK such as; phospholipases, cytoskeletal proteins, several protein kinases (MAPK-activated protein kinases; MKs) and transcription factors in this way controls expression of different genes [Figure 1.5].

SAPK/JNK (stress activated protein kinase/Jun N-terminal kinase) as the name implies, is activated through cellular stress. It is a serin–threonine kinase which phosphorylates the amino-terminal domain of the Jun transcription factor. It has a resulting function as; cell survival, oncogenic transformation, growth, differentiation and cell death. SAPK/JNK is encoded by three separate genes; jnk1, jnk2 and jnk3. Alternative splice version of them creates at least ten isoforms with alternating substrate preferences. The substrates of SAPK/JNK are c-Jun, JunB, JunD and the related ATF2 transcription factor, as well as p53, NFAT4, NF-ATc1, HSF-1, STAT3 and Elk-1 [197, 200]. PI3-K/Akt signaling survival pathway inhibits cDDP-induced SAPK/JNK and p38 activation by phosphorylating ASK1 [122].

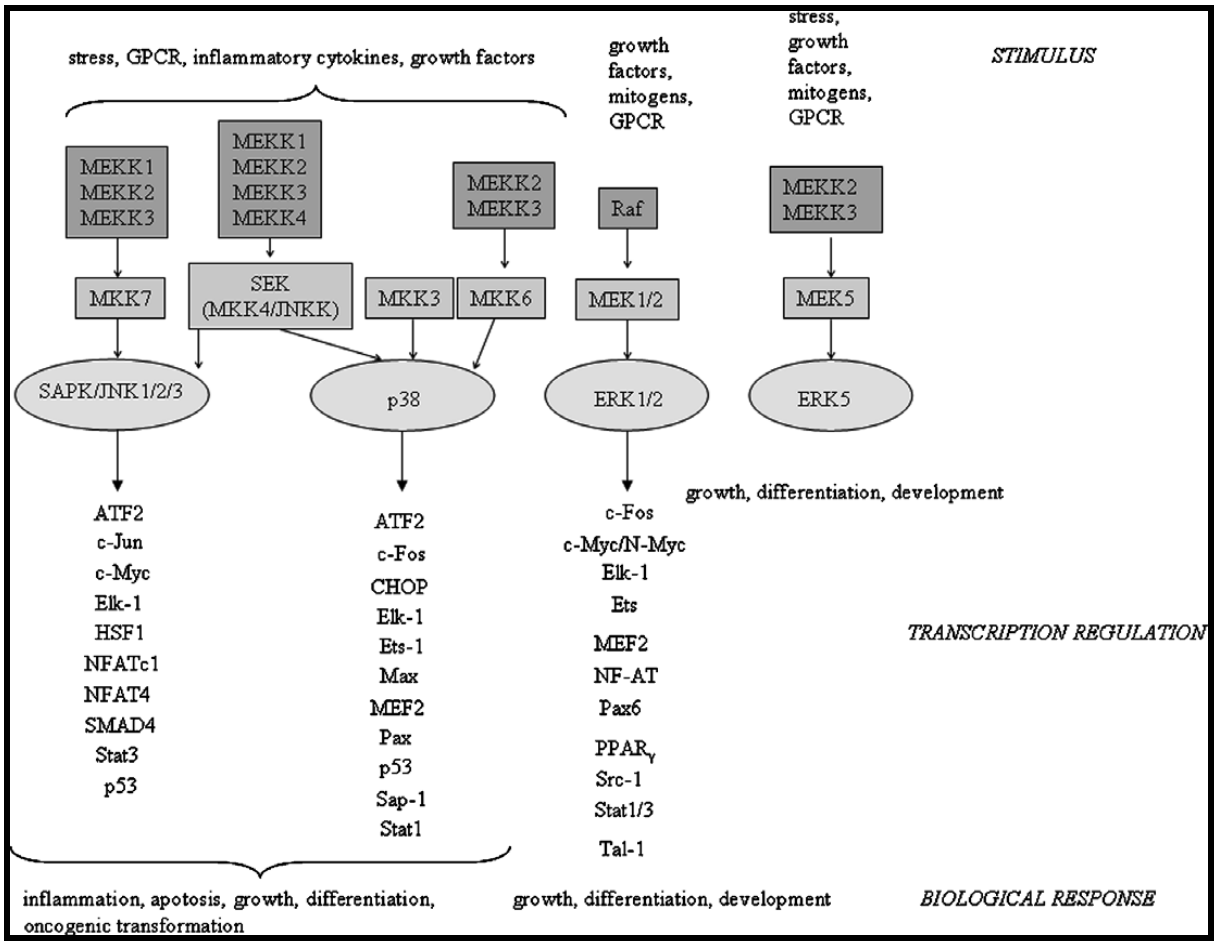


Figure 1. 5: MAPK signaling pathway

In mammalian cells, p38 is stimulated throughout environmental stress and inflammatory cytokines, less by mitogens. Most activators which induce p38 also stimulates SAPK/JNK cascade simultaneously. The p38 substrates are: cytosolic

phospholipase A_2, the microtubule-associated protein Tau, transcription factors ATF1-2, MEF2A, Sap-1, Elk-1, NF-κB, Ets-1 and p53, and several MAPK-activated protein kinases (MKs) [198].

ERK1/2, are involved in cell proliferation and activated through growth factors, serum, phorbol esters, ligand of heterotrimeric G protein-coupled receptors, cytokines, osmotic stress, and by microtubule disorganization [198,199]. ERK1/2 substrates are; CD120a, Syk and calnexin, SRC-1, Pax6, NF-AT, Elk-1, MEF2, c-Fos, c-Myc, STAT3, some cytoskeletal proteins and several MKs [198,199].

Cisplatin given to the cell activates ras mediated transduction pathway. Ras activates a complex signaling network of MAP3K and ERK1/2 leading to cell death in breast carcinoma cells. Members of ras also involved in regulation of p38 [201, 202]. Inhibition of ERK and SAPK/JNK sensitizes human ovarian papillary adenocarcinoma cells (Caov-3) and ovarian cancer cells (A2780) to cisplatin induced cell death [123].

One of the common crucial downstream substrate of SAPK/JNK and ERK activation cascade is activator protein-1 (AP-1), AP-1 components (including ATF2 and c-Jun), and transactivation sites of AP-1 that might be required for DNA repair mechanism. AP-1 provides molecular basis for enhanced DNA repair [121].

1.5 Foxo family

FOXO family of proteins function as transcriptional factors that interact with the core consensus DNA sequence GTAAA(C/T)A to modulate target gene expression [183]. The mammalian FOXO family of transcription factors are; FOXO1, FOXO3a, FOXO4 and FOXO6. These are major substrates of the protein kinases PKB (protein kinase B) and SGK (serum and glucocorticoid-induced protein kinase), which are part of PI3K signaling pathway [183–185]. The newly identified FOXO member, FOXO6, always stays in the nucleus due to lack of C-terminal PKB consensus phosphorylation motif [186].

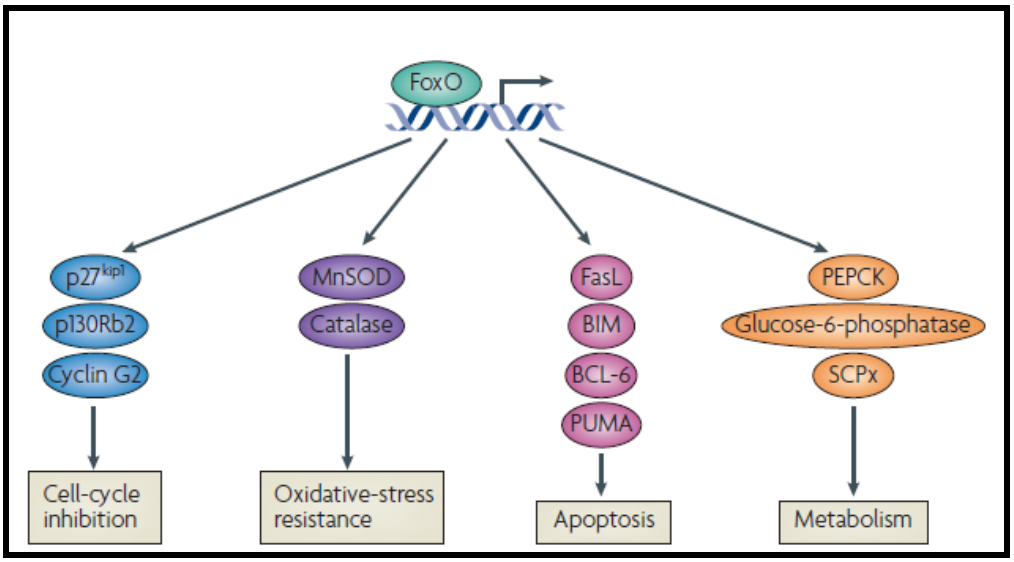


Figure 1. 6: Target genes of FOXO [Adapted from Host et al., ref 9]

FOXO transcription factors are thought to be as tumor suppressor genes because they mediate cell cycle arrest, DNA repair and apoptosis [183]. Low level of FOXO in the cell may result tumor development and expansion. The target genes of FOXO are involved in; cell cycle progression ($p27^{Kip1}$, p130 (RB2), cyclin D1/2 and Bcl-6 (B-cell lymphocytic leukemia proto-oncogene 6)) and apoptosis (Bim, Fas ligand, TRAIL (tumor-necrosis-factor-related apoptosis inducing ligand) and Bcl-XL) as showed in Figure-6 [183,187-189]. PKB phosphorylates FOXOs and this results sequestration of FOXO in the cytoplasm and inability to activate cell death inducing genes due to

degradation of FOXO therefore as a result cell survival is seen [187-189]. Phosphorylated FOXOs are retained in the cytoplasm by 14-3-3 chaperone. JNK can phosphorylate 14-3-3 in this way FOXO relocates into nucleus [192].

In neurons, FOXO3a triggers cell death circuitously by activating the expression of Fas Ligand, which stimulates programmed cell death through the death receptor pathway [183].

FOXO3a increase the expression of the antioxidant enzymes, mitochondrial MnSOD (manganese superoxide dismutase) and catalase, which are scavengers of oxygen-free radicals) and results oxidative resistance, another target is multidrug resistance transporter genes (MDR1, ABCB1) [190].

FOXO modifications is not limited only to phosphorylation, it can also be acetylated by SIRTs (Sirtuins) in this way FOXO is activated and resulting effect of activation is being away from cell death and cell becomes stress resistance in humans (Figure-7) [193].

FOXO1 gene expression level is enhanced in paclitaxel-resistant and cisplatin-resistant ovarian cancer cells and cells are sensitized to drugs when FOXO1 is silenced by siRNA technology [195]. Knocking out studies on FOXO1 shows embryonic lethality whereas FOXO3a and FOXO4 not [196].

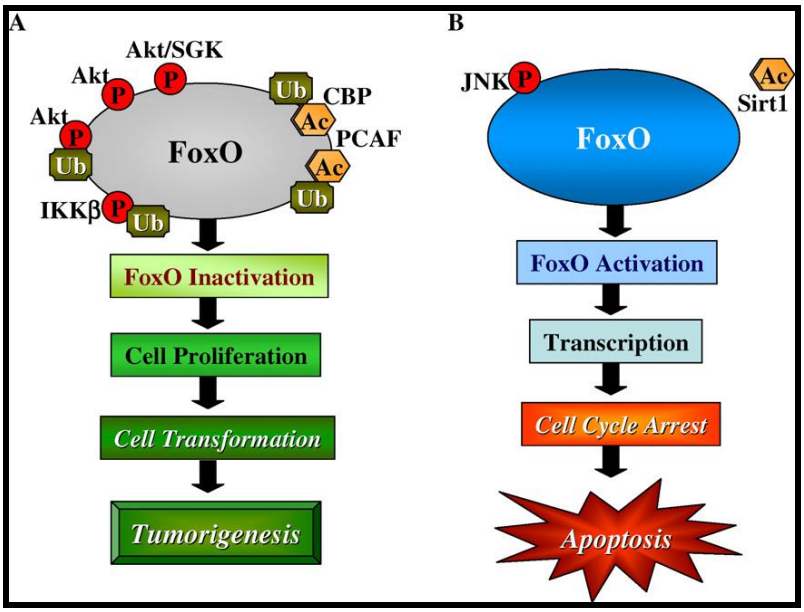


Figure 1. 7: FOXO modifications and different consequences

β-catenin, a transcription factor that plays a major role in development and tissue self-renewal, physically interact with FOXO proteins and enhances their transcriptional activity [194].

PTEN (phosphatase and tensin homologue deleted on chromosome 10) is an antagonist of PI3K pathway and somatic deletion or mutation elucidates human tumours at a large ratio 12-60% [182]. PTEN mutation leads to constitutive activation of PI3K and a resultant loss of FOXO1 and FOXO3a [191].

1.6 p53 gene

P53 is transcription factor which have a central role in tumor suppression by inducing apoptosis [16, 17] or promoting normal cell growth and regulate cell cycle [24, 25]. TP53 gene is mutant or inactivated in 50% case of human tumors [58]. Most of the breast cancer cells have high grade TP53 gene mutation or modulation and this enables gene to inactivate [12, 18]. MCF-7 human breast cancer cell line harbors wild-type p53 gene in the genome and functionalized p53 protein is located at the nucleus [19]. In breast cancer cells, high degree of mutation rate is seen in p53 gene and this reasons to high proliferation rate which is expected in view of the role of p53 as a negative regulator of cell proliferation [20-22].

The level of p53 protein is highly sensitive to hormones such as progesterone because progesterone induces cells to differentiate rather than growth and p53 protein level decreases as the level of exposed progesterone increases in MCF-7 cells [13].

In mammary epithelial cell model that lacks p53 gene, tumorigenesis, chromosome instability (aneuploidy), and centrosome amplification is seen [23, 29-31]. In developmental phase, p53 null mammary epithelial cells show normal development but high rate of tumorigenesis incidence. The mammary tumors that lacks p53 gene increased their DNA content by the hormone stimulation; especially progesterone rather than estrogen, it only expanded tumor rate [32-34]. DNA repair ability is not directly related to TP53 activity at G1/S-phase [59, 60].

ATM and ATR are two kinases that regulates and stabilize the p53. Cisplatin preferentially activates ATR kinase then it phosphorylates p53 from at serine-15 and activate [119, 120]. MAPK is also involved in p53 phosphorylation and inhibition of the MEK–ERK pathway leads to cisplatin resistance [117]. Akt also phosphorylates Mdm2 onco-protein that is inhibitor of p53 in this way drug resistance phenotype is achieved [118].

1.7 p21 gene

P21 is a cell cycle progression inhibitor whose expression is controlled by the p53 tumor suppressor gene [97]. P21 have a role in cellular differentiation, senescence and inhibition of apoptosis [98, 99]. Cyclins and CDKs bind to p21 through the consensus site CRRL (p21 amino acids [aa] 18 to 21) at the N-terminal end, and this motif is required for inhibition of cell cycle progression [96].

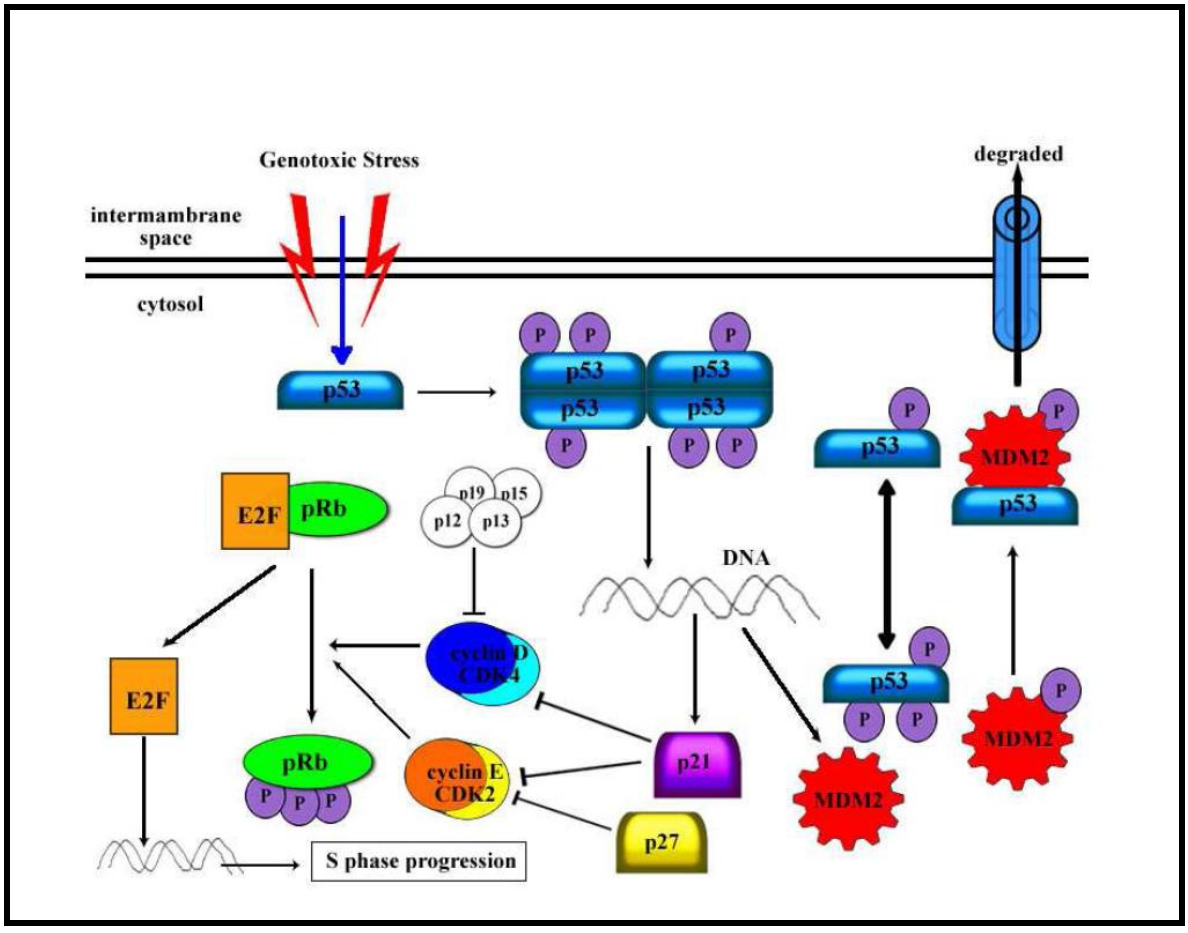


Figure 1. 8: p21 and p53 pathway

Interaction of Cyclin-CDK with p21 induces association of this complex with PCNA which stimulates DNA polymerase δ and ε. These polymerases enable DNA to replicate at high fidelity and conduct excision repair [94, 95].

Function of p21 is dependent on its subcellular localization. Akt has been reported to phosphorylate p21 and p21 retains in the cytoplasm whereas cytoplasmic retention of p21 in HER-2/neu-overexpressing cells results loss of cell cycle inhibition and gain of apoptosis inhibitory activity [90, 91]. It has also been reported that upon differentiation status of U937 cells into monocytes, p21 translocates from the nucleus to the cytoplasm and rescues cell death [83]. Furthermore, p21 mutant that lacks nuclear localization signal sequence did not induce cell cycle arrest or monocytic differentiation [90]. Subsequent exposure to alkylating agents, p21 localizes into nucleus and in addition to that promotes cell cycle arrest and prevent S-phase progression, p21 also colocalizes with PCNA to instigate nucleotide excision repair [85, 86].

1.8 Wnt Signaling Pathway

The Wnt signaling pathway is substantial for cell as having a role in cell differentiation and proliferation, cell movement and polarity, and for maintenance of self-renewal in hematopoietic stem cells [reviewed in ref. 84], and defects in this pathway are inclusive of the pathogenesis of several tumor types, including breast cancer [reviewed in refs. 92 and 93].

Wnt signals are transduced through two distinct pathways, the canonical and the non-canonical [84] substantially due to the fact that Wnt is a family of closely related, secreted glycoproteins with at least 19 members that can bind to different cell surface receptors with different combinations that eventually determine which specific pathway is activated [57, 63].

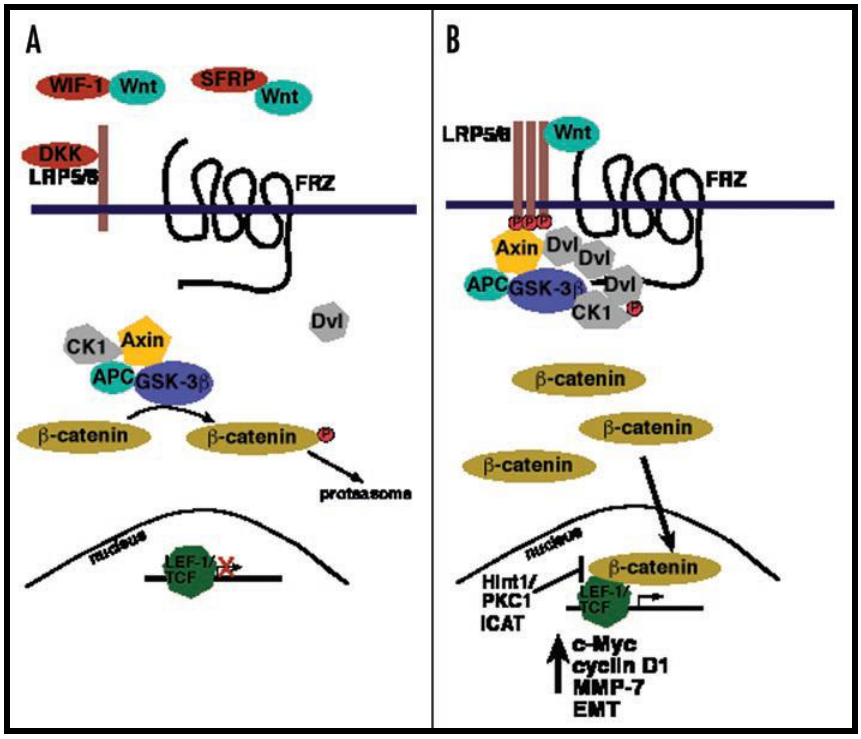


Figure 1. 9: Wnt pathway

On the canonical pathway, secreted Wnt ligands are transduced through the Frizzled (FRZ) family of transmembrane receptors and the low-density lipoprotein receptor-related protein (LRP5/6) co-receptor to eventually stimulate β-catenin, the important transcription regulator of growth-promoting genes. There are numerous positive and negative regulators of this pathway, and loss of negative regulation, which activates constitutive signaling and growth, is an important contributor to tumor development [87-89]. Binding of Wnt to Frizzled is followed by Dishevelled (Dvl) aggregates assembling a scaffold-like structure at the plasma membrane [54]. This triggers formation of clusters of LRP5/6 that are then phosphorylated by casein kinase I (CKIγ). On the following, Axin, in complex with glycogen synthase kinase 3 β (GSK-3β) and the associated APC proteins, binds to complete the LRP signalosome [54]. Thus the activity of GSK-3β is inhibited and β -catenin aggregated in the cytoplasm [53]. β -catenin is then able to translocate into the nucleus where it activates T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors that subsequently activate expression of a number of genes (in part by displacing Groucho and HDAC) including those involved in epithelial-to-mesenchymal transition and cell proliferation such as c-myc, c-jun, cyclin D1 and CD44 [Figure 1.9B]. In the absence of Wnt

binding, complex of GSK-3β along with CKIα, APC and Axin, phosphorylates β -catenin, targeting it for proteolytic degradation [Figure 1.9A]. However, Lee et al. recently demonstrated that Dvl overexpression compensate absence of Wnt ligand and promote transcriptional activation of TCF/LEF-responsive genes, reporting that if the concentration of Dvl is high enough to support self-association near the plasma membrane, LRP5/6 can assemble and form the LRP signalosome even in the absence of a Wnt ligand [52]. Wnt has an important contribution to tumorigenesis in breast cancer [87].

1.9 NFAT pathway

The nuclear factor of activated T cells (NFAT) signaling axis is a vertebrate-specific pathway important for a variety of cellular functions such as; development and activation of lymphocytes and differentiation of cardiac muscle cells [51, 44]. In the canonical pathway which was firstly elucidated in immune cells, NFAT is induced as a result of calcium efflux from endoplasmic reticulum stores and from the extracellular environment through the activation of store-operated channels in the plasma membrane. In the steady-state, NFAT is hyper-phosphorylated and remains in the cytoplasm. Subsequent to cell stimulation and calcium release, NFAT is dephosphorylated by the phosphatase calcineurin and translocates to the nucleus where it work together with other factors and co-activators to provoke de novo gene transcription.

NFAT1 (also known as NFATc2 and NFATp) null mice show hyperproliferation of splenic B and T cells due to absence of FasL expression and therefore do not undergo cell death [43].

NFAT2 (also known as NFATc1 and NFATc) null mice show defects in development of heart valve morphogenesis related to an abnormal cardiac septum, providing evidence for an irrevocable role for NFAT2 in cardiac development [42].

NFAT4 (also known as NFATc3 and NFATx) null mice confer abnormal development of myofibers as well as having reduced thymocyte numbers due to suppression of Bcl-2 expression [41].

Constitutively active NFAT1 stimulated cell to halt cell cycle and undergo apoptosis and inhibited RasV12 induced transformation, whereas constitutively active NFAT2 increased cell proliferation and transformation. NFAT1 null mice are more sensitive to chemically-induced carcinogenesis [14]. In human breast epithelial cells, the non-canonical Wnt ligand, Wnt5a which is known to inhibit metastatic progression blocks NFAT activation coincident with attenuated migration by a mechanism that depends in part on the binding of CK1 [2].

2. PURPOSE

Cells confer resistant to drug either intrinsically or extrinsically. Extrinsic resistance also called acquired resistance. Continuous and periodic treatment of cells with the same drug either with increasing amount or with the same dose of drug began to be ineffective against cancer cells. Cells adapt to this drug and start to provide a new mechanism to escape apoptosis such as enhanced DNA repair mechanisms, survival pathway activation and for breast cancer cells; ER positive (+) cells began to grow without needing estrogen. Resistant cells outline an obstacle for treatment of cancer.

This is the first study reporting, *de novo* established cisplatin resistant cell line of MCF-7 and MDA-MB-231. Research reports on drug resistant mechanism is always emanated from other cell lines especially from ovarian cancer cell. Cisplatin resistance mechanism in MCF-7 is unexplored on the other hand, ER resistance mechanism that means cell become to grow without needing estrogen receptor and estrogen, paclitaxel and tamoxifen resistance are well-defined.

For this purpose, after establishment of *de novo* cisplatin resistant cell line, we wanted to investigate differentially expressed genes between control MCF-7 and MCF-7/R6 cell line by using microarray. And for further investigation of which signaling pathways become activated in cisplatin resistant breast cancer cell line; total protein levels of MCF-7/R6, MDA-MB-231/R2 and parental cells of both are checked with specific antibodies. For further study of proteins, activation of some transcription factors is demanded to check through nuclear protein isolation.

3. MATERIALS

3.1 Chemicals

All chemicals that are used are listed in Appendix A.

3.2 Antibodies

All Antibodies that are used are listed in Appendix B.

3.3 Molecular biology and cell culture

Molecular biology kits used for immunoblotting experiments and cell culture materials are listed in Appendix C.

3.4 Equipments

Equipment that is used for general laboratory procedures are listed in Appendix D.

3.5 Buffers and Solutions

3.5.1 Buffers and Solutions for Cell death assays

Annexin V-FITC incubation buffer: 10 mM HEPES, 140 mM NaCl and 2.5 mM $CaCl_2$ were dissolved in 500 ml of ddH_2O. The buffer is kept at 4^oC.

3.5.2 Buffers and Solutions for protein isolation

3.5.2.1 Total protein isolation

Cell Lysis Buffer: 150 mM NaCl, 1% NP-40 and 50 mM Tris dissolved in ddH_2O; afterwards pH is adjusted to 8.0 by using 5M HCl solution. The buffer was stored at 4^0C.

Complete cell lysis buffer: was prepared by adding 1X protease inhibitors, 1X phosphatase inhibitors and 0.5 M PMSF freshly to cell lysis buffer.

10X PBS (Phosphate Buffered Saline): 80 g NaCl, 2.0 g KCl, 14.4 g Na_2HPO_4 and 2.4 g KH_2PO_4 were dissolved in 1L of ddH_2O and pH is adjusted to 7.4.

3.5.2.2. Cytoplasmic and Nuclear protein fractionation

T1 buffer: 10mM HEPES-KOH (pH:7.9), 2mM MgCl2.6H2O, 0.1mM EDTA, 10mM KCl, 1% NP-40 and freshly added DTT, 0.5mM PMSF, complete protease and phosphatase inhibitor (Roche).

T2 buffer: 20 mM HEPES-KOH (pH:7.9), 1.5 mM MgCl2, 0,2 mM EDTA, 650 mM NaCl, glycerol (25%,v/v) with freshly added 1 mM of DTT, 0,5 mM of PMSF, 1X protease and phosphatase inhibitors.

3.5.2.3 Buffers and Solutions for SDS polyacrylamide gel electrophoresis

1.5 M Tris-HCl pH 8.8: 1.5 M Tris was dissolved in ddH_2O and pH was adjusted at 8.8 with HCl.

0.5M Tris-HCl pH 6.8: 0.5M Tris was dissolved in ddH_2O and pH was adjusted at 6.8 with HCl.

3.5.2.4 Buffers and Solutions of Immunoblotting

1X PBS-Tween20 (PBS-T): 10X PBS was diluted to 1X, 0.2% Tween20 was added to 1X PBS.

10X Running Buffer: 30.3 g Tris, 144.1 g Glycine, 10 g SDS were dissolved in 1L of ddH_2O and pH was adjusted at 8.3.

10X Transfer Buffer (TB): 30.3 g Tris and 144 g Glycine were dissolved in 1L of ddH_2O.

1X Transfer Buffer: Before conducting transfer step, 1X TB is freshly prepared. 20% (v/v) methanol was added into 1X TB and the remaining volume was completed with ddH_2O.

Blocking Solution: 0.05% (w/v) dried milk powder was dissolved in 1X PBS-T.

Stripping Buffer: 2.5 mM Tris-HCl and 2% SDS (w/v) were dissolved in 500 ml ddH_2O and pH was adjusted to 6.7. 352.1 µl of β-mercaptoethanol was added for 50 ml of solution prior to use.

3.5.3 Buffers for RNA integration test

10X MOPS buffer: 0.4 M MOPS, pH 7.0, 0.1 M sodium acetate, 0.01 M EDTA was dissolved in ddH_2O.

3.5.4 EMSA buffers

T4 polynucleotide kinase (Promega): T4 Polynucleotide Kinase catalyzes the transfer of the γ-phosphate from ATP to the 5′-terminus of polynucleotides or to mononucleotides bearing a 5′-hydroxyl group. The enzyme, purified from recombinant E. coli, may be used to phosphorylate DNA and synthetic oligonucleotides prior to subsequent manipulations such as EMSA.

T4 Storage buffer: 20mM Tris-HCl (pH 7.5), 25mM KCl, 2mM DTT, 0.1mM EDTA, 0.1µM ATP and 50% (v/v) glycerol.

T4 10X Reaction Buffer: 700mM Tris-HCl (pH 7.6 at 25°C), 100mM $MgCl_2$, and 50mM DTT.

Gel shift binding buffer: 20 mM HEPES/KOH, [pH 7.9], 5 mM EDTA, 25% glycerol (v/v), 2.5 mM $MgCl_2.6H_2O$, 0.5 M KCl and freshly added, 10 mM DTT, final volume 10 µl.

Binding Reaction mix: ddH_2O, 4 µl binding buffer, 3.45 glycerol, 1 µl of BSA, 1.5 µl of poly [dI-dC], 5-8 µl nuclear protein extract and 1 µl γ-^{32}P-labeled NF-κB and Foxo3a probe .

10X TBE: 108 g Tris base, 55g Boric acid, and 40 ml of 0.5M EDTA (pH 8.0) in 1L dH_2O.

EMSA loading dye: 30% (v/v) glycerol, 0.25% (w/v) bromophenol blue

4. METHODS

4.1 Cell Culture

The MCF-7 human adenocarcinoma breast cancer cell lines were obtained from the American Type Culture Collection (HTB-22, ATCC, USA) and MDA-MB-231 cells (HTB-

26, ATCC) and were maintained in Dulbecco's Modified Eagle Medium (Pan Biotech, Germany) supplemented with 10% Fetal Bovine Serum (Pan Biotech, Germany), 2 mM glutamine (Pan Biotech, Germany), 50 U/ml penicillin, 50µg streptomycin (Pan Biotech, Germany). All cells were incubated in a humidified atmosphere of 37^{o}C and 5% CO_2 in T75 cm^2 flasks as an attached layer. MCF-7 drug-resistant (MCF-7/ Cisplatin) cell line was established by 2 step selection procedure after prolonged (> 6 months) treatment of cisplatin. These cyclic treatments were repeated for 6 months. MCF-7 cells were pretreated with 1µM Cisplatin (Sigma) for 4 days. Afterward, cells were treated with 30µM (Sigma) Cisplatin for 4 hours. For recovering phase (approximately 2 weeks), medium was replaced with fresh drug-free complete medium. Surviving populations of parent MCF-7 cells were used for cell death analysis by performing Flow Cytometry (BD Biosciences).

When cells are grown to confluency, subculturing to new passage numbers is done with 0.05 % Trypsin/EDTA solution. As soon as cells are detached, in 37^{o}C incubator, medium containing 10% serum is added to inhibit further activity of trypsin that might damage cells. Cells are counted with hemacytometer by using the following formula;

$$Cell\frac{number}{ml} = Average\ count\ per\ quare \times Dilution\ factor \times 10^4$$

Cell suspensions are passaged to new flask and wells in appropriate seeding densities as follows;

Table 3. 1: Seeding density. The number* of cells on a confluent plate, dish or flask will vary with cell type

Dimension	Seeding Density	Cells at Confluency*	Trypsin	Growth Medium
6-well	0.3 x 10^6	1.1 x 10^6	2	3-5 ml
100mm	1.7 x 10^6	6.8 x 10^6	8	10 ml
25cm^2	0.7 x 10^6	2.8 x 10^6	3	3-5 ml
75cm^2	2.1 x 10^6	8.5 x 10^6	5	8-15 ml

4.2 Cryopreservation

For long-term storage, cells are cryopreserved as follows; cells are trypsinized, spinned down at 13.400 rpm for 30 seconds and washed with 1X sterile PBS. Cells are resuspended in freezing medium which contains 10% tissue culture grade sterile DMSO as a cryoprotective agent in FBS alone. Cells in freezing medium are transferred into cryovials with each vial containing 10^6 cells/ml, equivalent to the seeding density of 25cm^2 flasks. Cryovials are frozen initially at -80^{o}C for 24 hours afterwards, in liquid nitrogen tank for months and years.

When cryopreserved cells are needed, they are thawed rapidly and residual DMSO is washed off with complete growth medium prior to seeding.

4.3 Cell death analysis

Cells were seeded in 6-well culture plates at determined number for flow cytometry analysis. After cells are attached to surface; they were treated with 30µM cisplatin, 50nm docetaxel* and 50nm paclitaxel* for 48 hours. At 48 hours, cells were harvested through trypsinization and washed twice with cold 1X PBS. The cells were centrifuged at 300g for 5 min, then the supernatant was discarded and the pellet was resuspended in 100µl 1X Annexin-V binding buffer then cell suspension was incubated

with 2µl of FITC-conjugated Annexin V (Pharmingen) for 15 min at room temperature in the dark. Five hundred µl of 1X Annexin-V binding buffer was added to each sample tube, and the samples were analyzed by FACS (Becton Dickinson) using FACS BD software.

* This final concentration of cisplatin is based upon reported clinically achievable plasma concentrations as well as our own best dose response used in experiments and *in vitro* study.

4.4 Total protein isolation

Treated and control MCF-7, MCF-7/R6 and MDA-MB-231 cells were harvested, washed in ice cold PBS and lysed on ice in 200 µl complete lysis buffer containing freshly added 1 mM PMSF, protease inhibitor cocktail (Roche, Mannheim, Germany) and phosphatase inhibitors (Roche, Mannheim, Germany). After lysis of the cells for 30 min on ice, cell debris was removed by cold centrifugation (4^{o}C) for 10 min at 13200 rpm. Supernatant contain total protein extracts which are immediately frozen and stored at -80 ^{o}C. Protein concentrations were determined with Bradford Protein assay.

4.5 Cytoplasmic and nuclear protein isolation

After treatment of MCF-7 and MCF-7 R6 cells with cisplatin for defined time points, nuclear proteins isolated from 60mm dish as follows; medium above the cells is sucked out, then cells are washed with ice-cold 1X PBS, after that scrapped within 1ml 1X PBS and harvested by cold (4^{o}C) centrifugation at 13200 rpm for 30 sec. Pellets are resuspended in 120µl of T1 buffer [10 mM HEPES/KOH, (pH 7.9), 10 mM KCl, 2mM $MgCl_2$, 0.1 mM EDTA, 1% NP-40 with freshly added of 1 mM dithiothreitol (DTT) 0,5 mM of PMSF, 1X proteases and phosphatase inhibitors]. Resuspended cells are chilled on ice for at least 20 min, then briefly vortexed and followed by cold centrifugation at 13200 rpm for 1 min. supernatants contain cytoplasmic protein fraction. After supernatants are transferred to a new appendorf tube and stored in -80 ^{o}C, protocol is carried on with the remaining pellets for nuclear lysis process. Pellets are resuspended

in 20 all of T2 buffer [20 mM HEPES/KOH (pH 7.9), 1.5 mM MgCl2, 0,2 mM EDTA, 650 mM Nail, glycerol (25%,v/v) with freshly added 1 mM of DTT, 0,5 mM of PMSF, 1X protease and phosphatase inhibitors] and chilled on ice for at least 20 min. Subsequently, cold centrifugation at 13200 rpm for 20 min is done to resuspends and resulting supernatant contain nuclear protein extract and immediately stored in -80 ^{o}C.

4.6 Protein Content Assay

Concentration of cytoplasmic and nuclear protein fraction is determined by Bradford assay which relies on the binding of the dye Coomassie Brilliant Blue G-250 to protein, especially alkalic and aromatic amino acids found in protein.

For measurement we diluted 1µl of protein sample with 4 µl distilled water in 95 µl of 1X Bradford reagent. In order to obtain a standard curve*, serial dilutions in 96-well plate are done. BSA with standard concentration; 5, 2.5, 1.25, 0.625, 0.313, 0.1570.078 µg of is used. By dividing the obtained optical density at 595 nm (OD595) to the slope of the calibration curve, the protein concentration is calculated in µg/µl. Calibration curve is used as long as R^2 is smaller than 1.0 and larger than 0.9.

*Standard curve is calculated for each experiment separately.

4.7 SDS- polyacrylamide gel electrophoresis of proteins (SDS-PAGE)

SDS-PAGE is the most widely used technique for analyzing mixtures of proteins. By this method proteins are separated on the basis of their molecular weight. They react with the anionic detergent sodium dodecylsulfate (SDS), present in the polyacrylamide gel and the loading buffer which gives negative charges to the protein complexes. SDS binds to the hydrophobic side chains of proteins and breaks non-covalent interactions. Before loading the sample onto the gel, it is mixed with loading-dye (2X), which contains β-mercapto-ethanol. The gel consists of polyacrylamide chains crosslinked with bisacrylamide. The polymerization of acrylamide and bisacrylamide is initiated by ammoniumpersulfate (APS) and catalyzed by N,N,N,N'-tetramethylenediamine (TEMED).

The gel consists of two parts, a stacking gel on top of a separation gel, which has a different pH and percentage of acrylamide. At the transition of the stacking to the separation gel, proteins are stacked to a narrow band caused by the difference in pH of the gels. Depending on the molecular weights of the proteins to be separated, different percentages of separating gels are used. Bio-Rad mini protean gel systems were used as protein gel electrophoresis.

9, 12, 15% SDS-PAGE was used throughout the experiments; variation emanating from resolution of the vicinity of protein bands. 12% Separating gel is prepared by mixing; 3.4ml distilled water, 2.5ml pH 8.8 1.5M Tris-HCl, 50µl 20% (w/v) SDS, 4ml 30% Acrylamide/ 0.8 bis-Acrylamide solution, 50 µl 10% APS and 5 µl TEMED in the given order (for 2 SDS-PAGE gels in 1mm glasses). Then gels were overlaid with distilled water to initiate air blockage and polymerization. Once the gel was polymerized, distilled water was removed and stacking gel was poured by mixing 3.075ml distilled water, 1.25ml 0.5M Tris-HCl, 25µl 20% (w/v) SDS, 670 µl 30% Acrylamide/ 0.8 bis-Acrylamide solution, 25 µl 10% APS and 5µl TEMED in the given order (for 2 SDS-PAGE gels in 1mm glasses). Finally well combs were placed and waited for the formation of wells. Fully polymerized gels were put into gel tanks containing 1X running buffer. On the other hand, protein samples as being at equal concentration (20-30µg) was prepared with 2X loading dye. To break the disulfide bridges present in the protein, the mix is boiled at 95˚C for 3 min and then samples are shortly centrifuged. Together with the protein samples, a protein marker are loaded (Fermentas, Germany). This is a mixture of standard proteins with known molecular weights, which are coupled to a dye. In this way the standard proteins are visible on the gel and molecular weights of proteins in the loaded samples can be derived. SDS-PAGE was run for about 2 hours at room temperature with a constant voltage of 100V.

4.8 Immunoblotting analysis

Total protein lysates were separated on a 12% SDS–PAGE typically was removed from the tank for being transferred onto PVDF membranes.

4.8.1 Blotting

PVDF transfer membrane (Roche) is firstly soaked in Methanol for 30 sec to be activated. Whatman papers, sponge which are soaked into freshly prepared 1X Transfer buffer are placed above and below of SDS gel and nitrocellulose membrane within cassettes. The cassettes were placed into the transfer tank which is filled to the top with 1X TB. Transfer tank was also containing an ice-block to prevent heating and to ensure a constant voltage of 100V during 1.5 hours at 4 ^{0}C.

4.8.2 Blocking

To remove residual methanol, membrane is washed with distilled water or 1X PBS-T briefly. Blocking solution (5% (v/v) dried milk powder in PBS-T) is used to block membrane; 1 hour incubation at room temperature was done.

4.8.3 Antibody

After a quick washing of the blocked membrane with PBS-T, membrane is incubated with primary antibodies in 1:2000 (v/v) dilution for 2 hour at room temperature or overnight at cold room (4^{o}C). Primary antibodies are listed in Appendix B.

Then membranes are washed with 1X PBS-T for 3 times for 15min. Afterwards, Membranes incubated with secondary antibody, HRP coupled anti-rabbit or anti-mouse IgG in 1:5000 (v/v) dilution for 2 hour at room temperature. Then second washings are done; membranes are washed with 1X PBS-T for 3 times for 15 min. Then substrate for HRP (ECL Advance Chemiluminescence) incubation is added at 1:1 and placed in cassette. In the dark room, membranes are exposed to Hyperfilm-ECL and developed manually to visualize proteins band clearly.

ECL Advance Chemiluminescence Substrate is a highly sensitive nonradioactive, enhanced luminol-based chemiluminescent substrate for the detection of horseradish peroxidase (HRP) on immunoblots. ECL Advance Chemiluminescence Substrate enables the detection of picogram amounts of antigen and allows for easy detection of

HRP using photographic or other imaging methods. Blots can be repeatedly exposed to X-ray film to obtain optimal results or stripped of the immunodetection reagents and re-probed.

4.9 Electrophoretic mobility Shift Assay (EMSA)

Electrophoretic mobility Shift Assay is based on reduced electrophoretic mobility of protein interacted with DNA (radioactively labeled probe) through a-non denaturing poly acrylamide gel compared to unbound labeled DNA probes.

4.9.1 Labeling of Probes

The oligonucleotides with 5'-OH (*) blunt ends (NF-κB and Foxo3a) were labeled with γ-^{32}P-dATP (3000 Ci/mmol) using T4 polynucleotide kinase (Promega). The reaction mix is assembled in 50 µl in total which contains 10.25 µl ddH_2O, 15 µl NF-κB oligonucleotide (1.75 pmol/µl, final: 26.25 pmol), 5µl T4 kinase buffer (10X, final: 1X), 15.75 µl γ-^{32}P-dATP (3000 Ci/mmol, final: 52.5 pmol, 170 µCi) and T4 kinase (5U/µl final: 20 Units). The mix is incubated in a 37^{o}C waterbath for 10 min.

Labeled oligonucleotide was purified on a Sephadex G-25 column (Roche). Column was washed completely off its buffer first by gravitation, then by centrifugation twice at 3500 rpm for 2 min. then 50 µl of reaction mix is applied to the center of the column in an upright position. After the column is placed in a new collection tube, the collection is centrifuged at 2300 rpm for 4 min. Eluate in the collection tube contains γ-^{32}P-labeled NF-κB and Foxo3a consensus oligonucleotides.

4.9.2 Binding Reaction

The binding reaction mix is assembled 20 µl in total and probe is added last. After addition of probe, binding reaction is allowed to proceed for 30 min at room temperature. Total volume is completed to 20 µl with distilled water. For supershift

experiment; 1.5 µl anti-p50 and anti-p65 are added to the binding reaction mix after 10 min before addition of ^{32}P-labelled oligonucleotide probe.

4.9.3 Vertical gel electrophoresis

6% non-denaturing polyacrylamide gel containing; 24 ml dH2O, 10ml of 1X TBE, 6 ml 30% Acrylamide/ 0.8 bis-Acrylamide solution, 280 µl 10% APS, and 36 µl TEMED. The comb inserted carefully and gel is left to polymerize for 1 h. then gel is pre-run in cold 1X TBE at 180 V at least for 30 min. In the meantime; 3 µl EMSA loading dye is added to the binding reaction mixes which has just completed 30 min period of incubations at room temperature at dark. Then mixtures of protein and DNA are loaded into gel and run at 300 V for 3hrs until bromophenol blue migrates ¾ distance of the gel.

Next, the gel was dried at 80^{o}C for 45 min - 1 h. Dried gel is put into a film cassette and kept at -80^{o}C and then autoradiographed on Kodak X-ray film.

The consensus sequences of the oligonucleotides used in this work were as following; For NF-κB;

5’- AGT TGA GGG GAC TTT CCC AGG C-3’

For Foxo3a;

5’- ATTGCTAGCAAGCAAAACAAACCGCTAGCTTA-3’

4.10 RNA isolation

MCF-7 and MCF-7/R6 cells lysed in TRIzol (Invitrogen, Germany) and total RNA was isolated according to the manufacturer's protocol.

4.11 RNA integration test

10X MOPS (final: 1X) buffer and 37% formaldehyde (12.3M, final: 18% of total volume) was used for preparation of 1% agarose gels. 1X MOPS running buffer to cover the gel gels was used. In the meantime, 2 μg of RNA in 2X RNA loading dye containing intercalating 0.025% ethidium bromide (Fermentas) was prepared. 2X RNA loading dye contains the tracking dyes bromophenol blue and xylene cyanol FF. After removing the combs, and loading RNAs to the gel, gel was run at 100 V for 45 minutes. RNA was visualized under UV rays by using UVITEC (UVIdoc Gel Documentation System, UK) machine.

Quantity assessment of RNA was done via spectrophotometer at an optical density at 260 nm (OD_{260}). Just prior to shipping to Cleveland Clinic (Ohio, USA), concentration was calculated using the $OD_{260/280}$ ratio.

4.12 Statistical analysis of cell death graphics

All samples were evaluated statistically using an excel function toolbar (TTEST). Relative cell death of treated cells compared to control was shown as mean ± standard deviation, and the student's t-test was applied to understand if values are significant or not. $P \leq 0.05$ (*) and $P \leq 0.01$ (**) were considered as significant.

4.13 Microarray

cDNA and biotin-labeled cRNA synthesis was generated from 250 ng total RNA using the Illumina® TotalPrep™ RNA Amplification Kit (Applied Biosystems, Darmstadt, Germany). cRNA was quantified using a nanodrop spectrophotometer and the cRNA quality (size distribution) was further analyzed on a 1% agarose gel. cRNA (1.5 μg) was hybridized to Human WG-6 Expression BeadChips V1 and V2 Illumina, (San Diego, CA) and scanned on Illumina BeadArray reader. The data was subsequently imported into Illumina's BeadStudio software and probe and gene level output files were generated as an excel format.

4.13.1 Data and statistical Analysis of microarray

Microarray data were analyzed by using MATLAB 2009b. The method used is explained in detail in the result section.

5. RESULTS

5.1 Establishment of cisplatin resistant MCF-7 cell line

5.1.1 The unique method for generation of cisplatin resistant breast cancer cell line

In order to obtain cisplatin resistant cell line, both MCF-7 and MDA-MB-231 cells are first treated with a low dosage of the drug medium which corresponds to 1μM cisplatin for 4 days, and then after sucking the low dosage drug medium from the cells, cells are treated with a high dosage of the drug medium which corresponds to 30 μM cisplatin for 4 hours [represented in Figure 5.1]. Cells are washed with a drug free medium in between the low to high dosage treatment transition. This treatment is repeated periodically until cell death declines to low rate. Using this pre-treatment method we aimed by that: only drug resistance genes will be stimulated rather than anti-apoptotic or survival genes. In this way we could see which genes were responsible for resistance status. Also by applying this treatment, we aimed for less cell death because resistant genes were already targeted to express at high rate by applying a low dosage of the drug during the 4 days period. Therefore, cisplatin treatment which is 30 times more compared to pre-treatment (1μM-30μM) for short period of time will not affect cells and they will survive at a high rate.

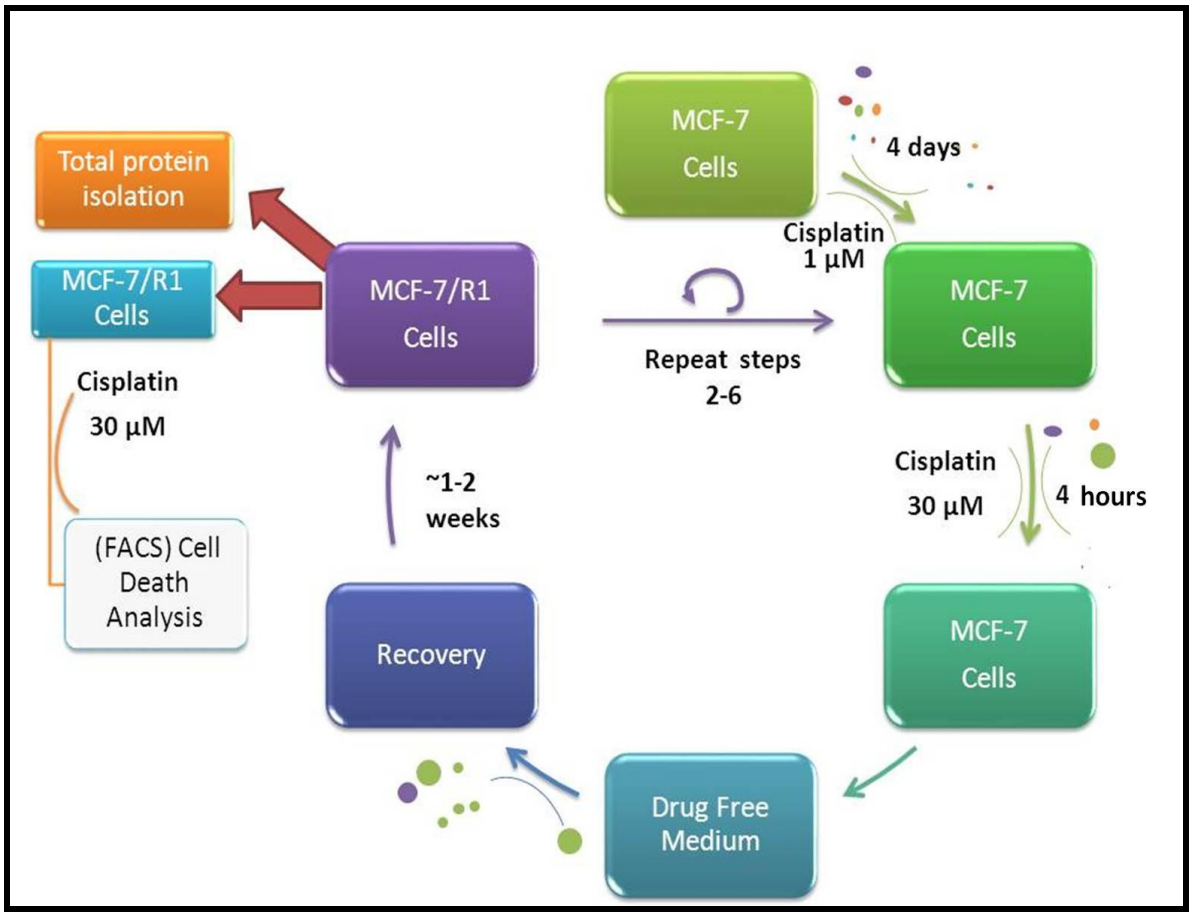


Figure 5. 1: Diagram representing our unique method for generating resistant cells.

5.1.2 Testing cells for cell death with flow cytometry

After each period of treatment, cells were seeded to 6 well plates for performing cell death analysis. In order to make cells adhere, one day of waiting time was given to the cells. After cells were treated with 30µM cisplatin for 48 hours, a flow cytometry experiment was conducted. A graph of Flow cytometry data after applying the t-test is shown in Figure 5.2 and 5.3.

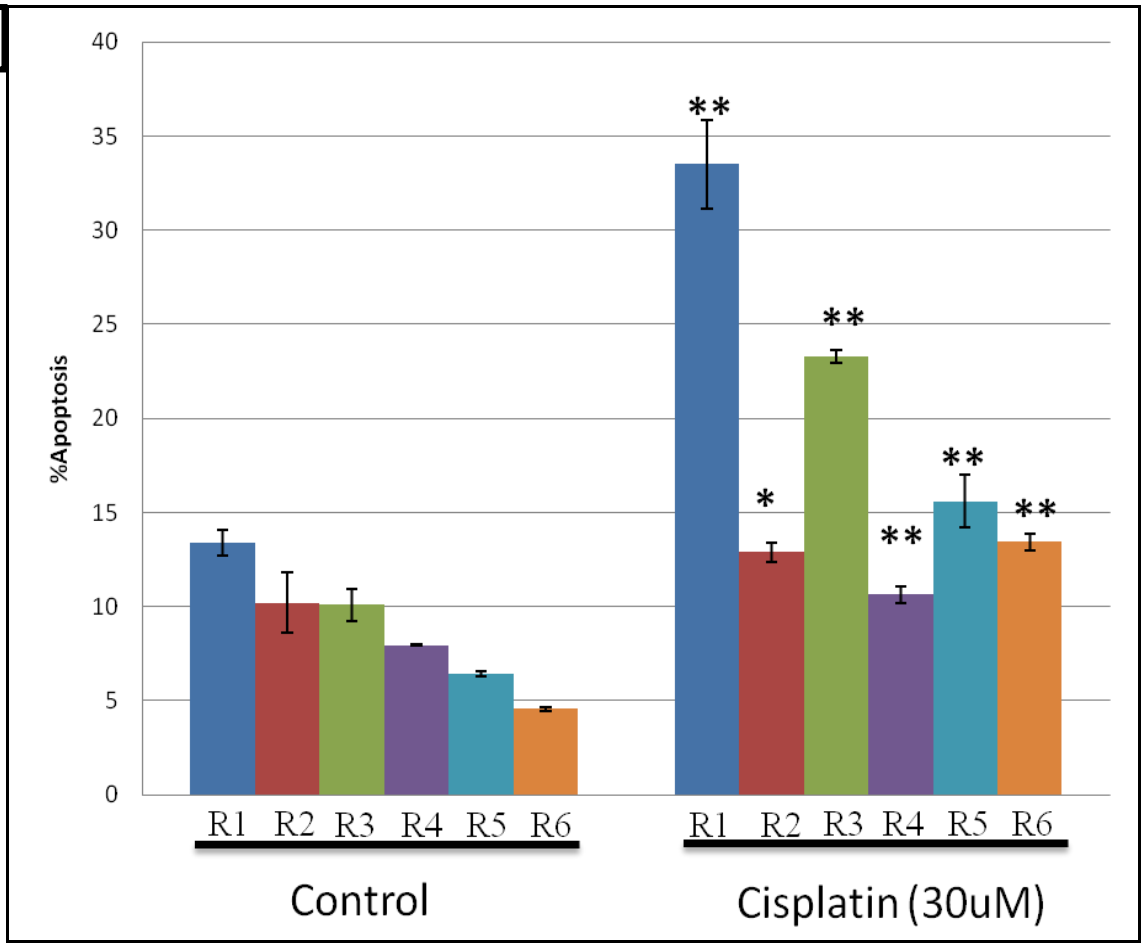
In addition, the total proteins of the newly established cell lines (R1, R2…) were isolated and for back up purpose, each cycle of treated cell was frozen and stored at -80^{o}C. At the end of each treatment cycle, we expected to see some cell clones survive. After 6 months of continuous treatment for MCF-7 cells and after one and half month for MDA-MB-231, cells became relatively more resistant to the cisplatin treatment. In

the flow cytometry experiment, Annexin V positivity level, which represents the apoptosis rate in cell clones, was checked and this rate declined after periodic drug treatment [Figure 2, 3]. Cells that retained drug and showed low rate of death were called “MCF-7 R6” for MCF-7 cells and “MDA-MB-231 R2” for MDA-MB-231 cells.

5.1.3 Examination of MCF-7 cell line derivatives

In Figure 5.2B, the level of apoptosis rate change by the time is depicted. Typically, control MCF-7 cells undergo apoptosis between 10-15% without drug treatment for 48 hours. On the other hand, MCF-7 cells treated with 30μM cisplatin for 48 hours undergo apoptosis between 30-35 %. The average apoptosis percent values at the end of each cycle of cisplatin treatment are shown in Table 5.1. As the cell line progresses, the apoptosis rate of control cells become less. For the cisplatin treated cells, there is no such direct relationship. They behave individually. The first line of the clone cell, called “R1”, act like a pair of non-resistant MCF-7 cells. But this graph only shows numerical data, in fact R1 cells experienced crucial changes biologically that affect the metabolism and also attachment of the cell. After the cycle of drug treatment, we give cells time to recover. Recover, in this case means that cells that have initiated death signaling and start to secrete death molecules should be washed away from the flask. Cells that adhere to the bottom of the flask should cover approximately up to 80% of total area. In the recovery phase, we changed the medium regularly with 10% FBS DMEM until only adherent cells were left. This recovery phase is different for each cycle of cell. For “R1” clone cells this recovery time period was approximately 1 month. In normal MCF-7 cells, passaging of cell should be done twice a week so this significant difference shows that substantial changes start to occur in parental MCF-7 cell lines. Table 5.1 shows the recovery time for each cycle of treatment.

A



B

	MCF-7/R (% Apoptosis)	
	Control	Cisplatin
R1	13,4	33,5
R2	10,2	12,9
R3	10,1	23,3
R4	7,97	10,63
R5	6,43	15,6
R6	4,57	13,43

Figure 5. 2: A: Graph of all cell line is combined. Diminishing apoptosis rate of the control cell (R1-R6) is shown at the right and fluctuating values of treated cells shown at the left of the graph. B: Mean values of triplicate data showing apoptosis rate in percent.

In the “R2” and “R4” cell lines, there is very small variance between the control and drug treatment. This 2-3% difference is not normal for a resistance experiment, according to drug resistance cell line experiments reported previously. The apoptosis rate should be grater after drug treatment otherwise data is considered as not significant. As a result we did not trust these 2 cell lines (R2 and R4) as being resistance and we maintained the treatments. In “R5”, the expected value was achieved but our control cell, non resistant MCF-7 cells, did not work well due to over-confluency and we did not consider “R5” cell line was reliable either. In “R1” and “R3”, there is significant difference between the two cases and clearly they are not resistant. Last, “R6” provides a match so we used this cell line as a keystone for our further studies. Its control, MCF-7 also worked pretty well in the same set-up of experiment.

Table 5. 1: Recovery time required for each cycle of treatment. The right column shows the days required for MDA-MB-231 cell line derivatives to recover. The left column shows days required for MCF-7 cell line derivatives to recover.

	MCF-7		MDA-MB-231
R1	27days	**R1**	43 days
R2	11 days	**R2**	17 days
R3	10days	**R3**	14 days
R4	21 days	**R4**	6 days
R5	6 days		
R6	10 days		

The fluctuation of the apoptosis rate shows that the resistance status is not stable for each cell line. This was confirmed by prolonging treatments to “R11” and by conducting flow cytometry. Once the cells became cisplatin resistant, protein isolation for EMSA and western blotting and also cryopreservation were done. Freezing and thawing sometimes kills resistant cells, as stated in the literature, however resistant cell death did not occur in this study. After thawing the cells for different drug trials, the cells showed nearly the same reaction to the cisplatin treatment. Thawing of the cells was done after 6 months; they were not affected by the temperature.

5.1.4 The MCF-7 "R6" cell line:

The apoptosis rate caused by cisplatin decreased from 32.7 % to 13.4 % [Figure 5.3A]. In the histogram graphs, shift of the peak entering to the gate (P2) is seen obviously in Figure 5.3B.

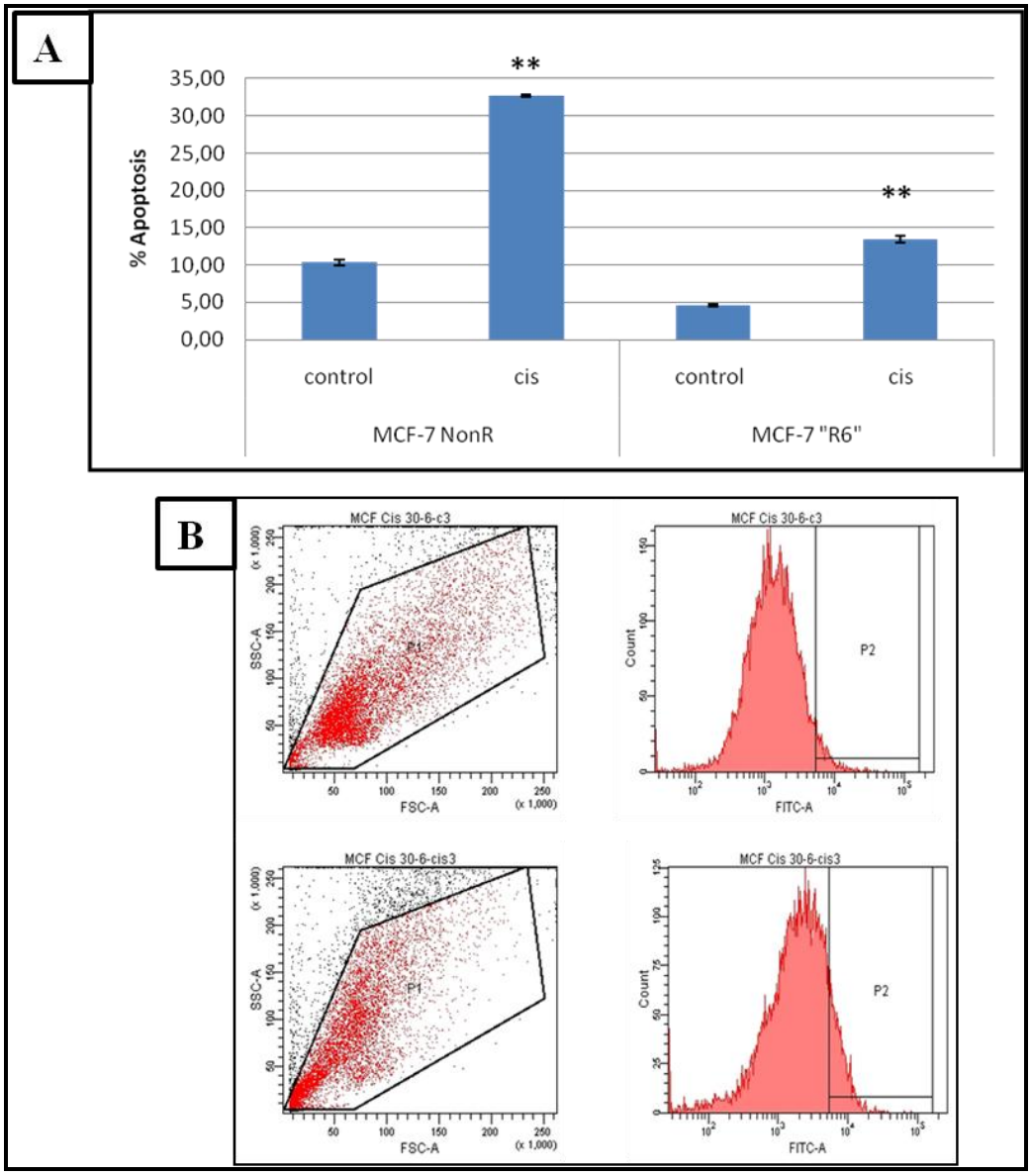


Figure 5. 3: A: Detailed graph of MCF-7 R6 data. B: Flow cytometry graphs of MCF-7 R6. The right side is shows a dot plot, in which each dot represents cells. P1 shows selected parental cell population. The left side graph is a histogram. It shows how intense P1 parental cells are stained and how much light they emit.

The peak's shift to the right means more cell population undergo cell death. The top graph shows the percentage of "R6" cells that die without cisplatin treatment and bottom graph shows the percentage of cells that undergo cell death after treating "R6" cells with 30μM cisplatin for 48 hours.

Table 5. 2: Triplicate of data obtained in cisplatin treatment of MCF-7 "R6" cell line.

		1	2	3	total	SD
Normal MCF-7	Control	10,3	10,8	10	10,37	0,40
	Cisplatin	32,9	32,6	32,7	32,73	0,15
MCF-7 "R6"	Control	4,5	4,5	4,7	4,57	0,12
	Cisplatin	13,4	13	13,9	13,43	0,45

A decline in the percent of control cells is also seen. Normal MCF-7 cells die at 13.43% but MCF-7 "R6" cells die at 4.57%. A nearly 20% increase in cell survival is seen. Control MCF-7 "R6" cells start to die at a low percent. The figure 5.2 shows the decline in all cell lines that continuously treated with cisplatin. Cisplatin makes cells secrete some materials that activate survival mechanism so cells overcome the death effect of cisplatin and begin to survive. This data shows that at the constitutional level of MCF-7 "R6" one or more survival pathway becomes active. Further experiments seek to identify the survival pathway.

This 20% decline shows that the cisplatin resistant cell population in the total population is much higher than the non-resistant MCF-7 population. Due to the resistant colony that come uppermost, cisplatin seems to kill fewer cells than normally does. At protein level, by looking at total proteins, we can see some fundamental differences.

5.1.5 Cell morphology

After a long period of treatments, cells begin to reshape their cytoskeleton and change their morphology. They began to resemble the other strain of breast cancer cell line, MDA-MB-231.

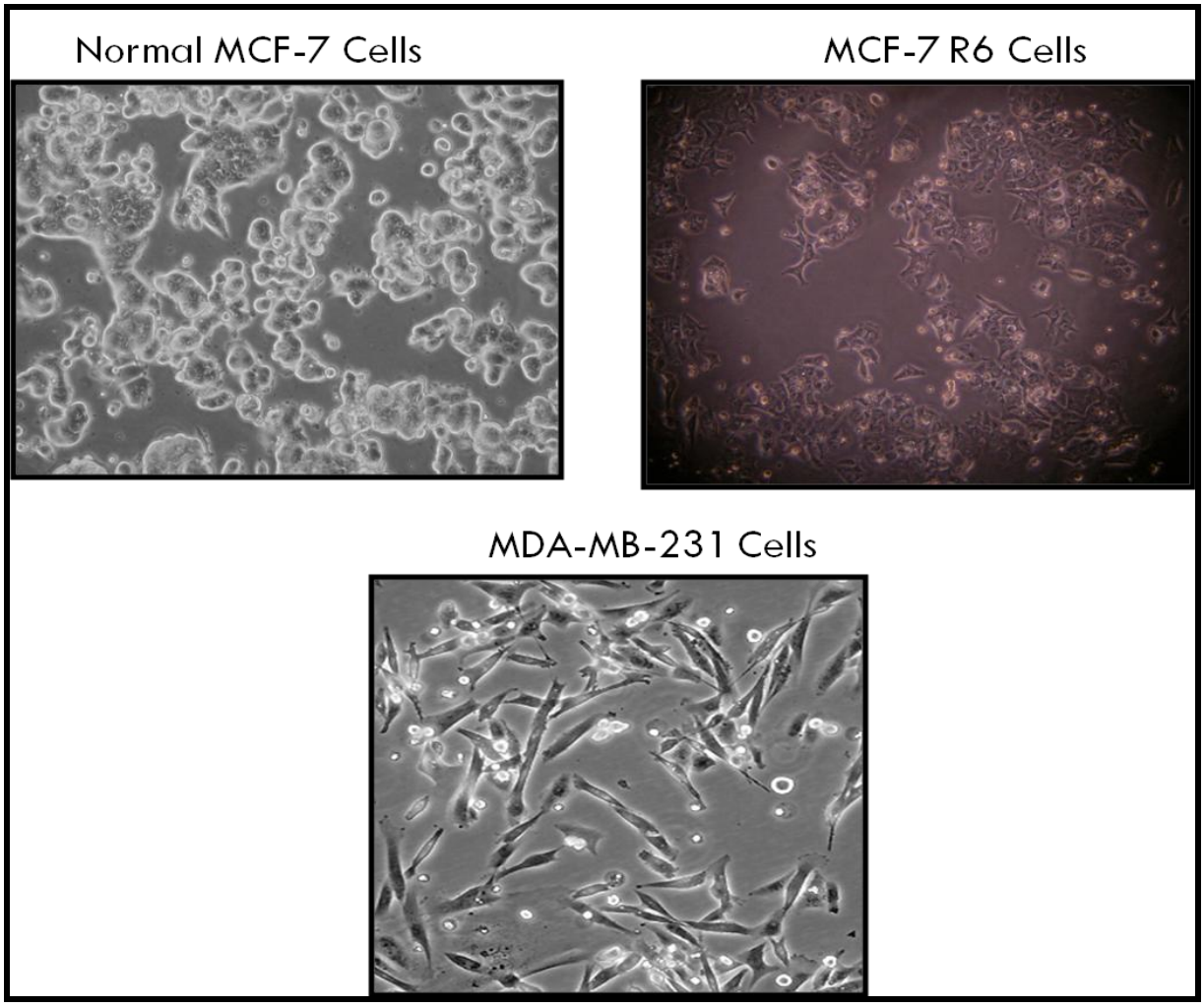


Figure 5. 4: Morphology of resistant cell. The upper left belongs to MCF-7 cells, the upper right belongs to MCF-7 "R6" cells and the bottom belongs to MDA-MB-231 [normal cell pictures are retrieved from atcc.org].

Normal MCF-7 cells have a circular shape and are capable of forming domes. They grow on top of themselves rather than expanding colonies to the bottom of the flask. As

the cell line progresses and cells retain more cisplatin into them, their shape begins to change and shift towards MDA-MB-231 cells. Just like MDA-MB-231 cells, resistant cells also begin to have extension rather than having a circular shape. They grow by expanding individually; few numbers of colonies are seen. They are not thin and elongated as MDA-MB-231 but compared to normal MCF-7 they have propagations. Normally MDA-MB-231 cells die at a rate of 10-15% after treatment with cisplatin. The resistant MCF-7 cell line also have a death rate in between, so metabolically the similarity between these 2 cell lines can be understood by looking at the morphology [Figure 5.4].

Also they start to behave in an extraordinary way. Normally, trypsinization of cell for passaging takes 5 minutes or less but in our case it began to take up to 10 minutes. So as the cell line progresses, trypsinization of MCF-7 cells started to be challenging. They tightly bind themselves to the bottom of the flask. Also the size of the cells began to be decrease.

5.1.6 Different drug trials on MCF-7 "R6" cells

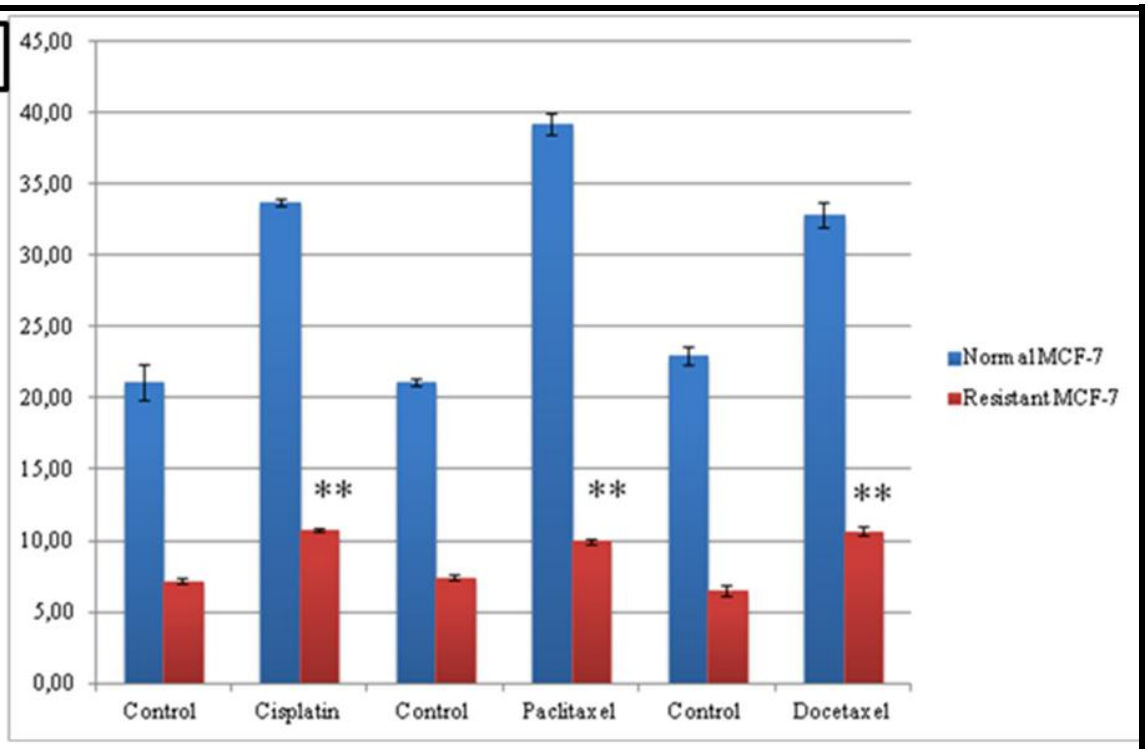
After obtaining successful results for the MCF-7 resistant cell line, we wanted to question if this cell line is also resistant to other anticancer agents; Docetaxel and Paclitaxel. These 2 agents belong to the same class of anticancer agents, taxol. Docetaxel and Paclitaxel inhibits microtubule dissociation and are used as a microtubule stabilizer.

After thawing the MCF-7 "R6" cell line, we waited about 15 days for the cells to reach high cell number. After passaging them, we seeded cells into 6 well plate at 300.000 cells/well. After waiting one day to make them attach to the surface, we treated them with 30μM cisplatin, 50nM Docetaxel and 50nM Paclitaxel for 48 hours. The gate of flow cytometry was adjusted according to the default cisplatin percent which is between 30-35%. All calculations were done based on control MCF-7 cells.

In general, after different drug treatments, resistant MCF-7 cell death was lower than normal MCF-7 cells in each treatment case [Figure 5.5]. Also MCF-7 "R6" cells did not seem to be affected by different drug treatments. At most 4% of MCF-7 "R6" cells die by the treatments. But in normal MCF-7 cells, Paclitaxel and Docetaxel caused

cell death at a rate similar to cisplatin. In normal MCF-7 cells, drugs kill between 9.9 - 18.1% of the cells whereas in MCF-7 "R6" cells this rate decreases to 2.2- 4.1%.

A



B

Normal MCF-7	Average	MCF-7 "R6"	Average
Control	21,13	Control	7,17
Cisplatin	33,73	Cisplatin	10,73
Control	21,13	Control	7,43
Paclitaxel	39,17	Paclitaxel	9,97
Control	23,00	Control	6,50
Docetaxel	32,87	Docetaxel	10,67

Figure 5. 5: A: Graph of different drug treatment results. B: Data showing average data of triplicates obtained from flow cytometry. Treatments were done for 48 hours.

Therefore this result explains how cisplatin resistant cells over-activated the survival mechanism which prevented them from undergoing apoptosis. In this way, cisplatin

resistant cells become death resistant and start to act like aggressive tumors. Hyperplasia is also seen. Corresponding doses of Docetaxel (50nM) and Paclitaxel (50nM) which were tested before in our lab were used.

5.1.7 Serum treatment of normal MCF-7 cells

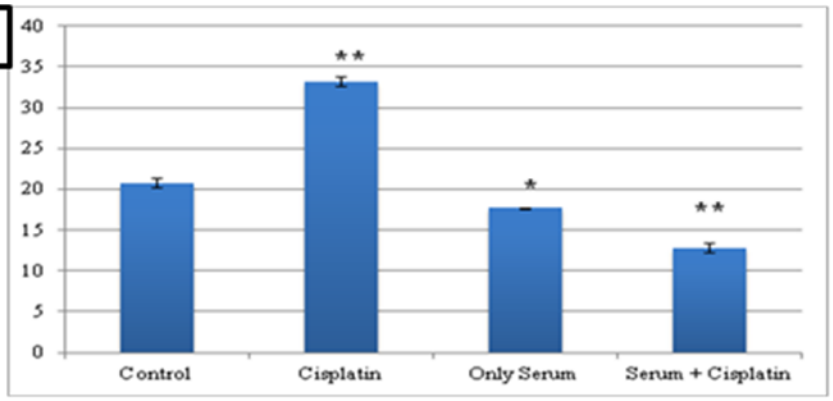
A further study was undertaken on MCF-7 "R6" cells to understand if extracellular ligand secreted from resistant cell made MCF-7 cells survive against cisplatin. To test this hypothesis, the medium above the resistant cells were taken and kept at -80^{o}C.

The medium was taken after 4 days because we aimed to have abundant amount of ligand and protein which were responsible for cell survival and exist in the medium. In this way we can see a dramatic effect caused by abundant ligand. After thawing the serum, we divided it into two. Half of it was filtered with a 0.22μM filter and half of it was not filtered. Then these serums were applied for 2 days to the normal MCF-7 cells to examine apoptosis rate of cisplatin and other drugs.

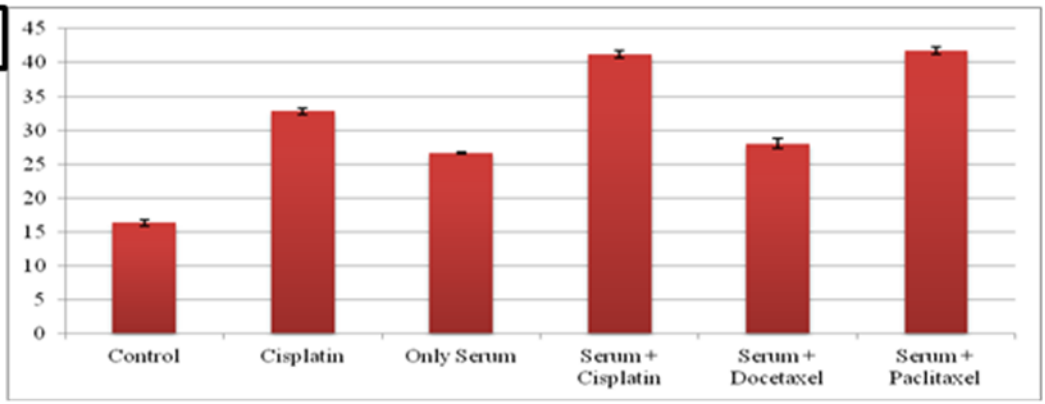
As seen in Figure 5.6A, the filtered serum treatment of normal MCF-7 cells enabled them to survive at a high rate similar to MCF-7/R6 cells. The death rate caused by cisplatin treatment of MCF-7 cells declined from 33.2% to 12.8%. Also the death rate of control cell also declined from 20% to 17%. These results are very similar to resistant cell line results. This shows that some elements present in the serum of MCF-7 "R6" cell line have a vital role in the survival mechanism.

Non filtered serum did not work well on MCF-7 as seen in Figure 5.6B because the death rate of cisplatin increased from 32.8% to 41.2%. Also other drugs were tried but compared to their control, the death rate always increased except for the Docetaxel treatment. Non filtered serum seems not to have affected the survival mechanism. A possible reason can be that the apoptotic ligand may have a size bigger than the filter so high amount of the ligand is left in the filter and in this way could not activate the cell death mechanism.

A



B



C

Filtered	Total	Un filtered	Total
Control	20,80	Control	16,33
Cisplatin	33,23	Cisplatin	32,80
Only Serum	17,67	Only Serum	26,63
Serum + Cisplatin	12,80	Serum + Cisplatin	41,20
		Serum + Docetaxel	28,03
		Serum + Paclitaxel	41,73

Figure 5. 6: Treatment of normal MCF-7 A: with filtered MCF-7 "R6" cell serum B: with non-filtered serum. C: Shows average data of triplicates obtained from MCF-7 "R6" serum treatments.

5.2 Microarray Results

5.2.1 RNA isolation:

After generating a resistant cell line, in order to have a general view about gene expression and compare it with normal MCF-7; the RNA of both were isolated for microarray experiment. RNA isolation was conducted with TRIZOL®.

5.2.2 RNA quality:

Prior to shipping an RNA integrity test must be performed in order to be sure if the RNAs of the samples are intact or not. RNA quality was checked with Denaturing 1% agarose gel, which was run in 1X MOPS buffer. 2 bands were aimed to seen. One band stands for large ribosomal RNA subunit which is 28S; the other band stands for small ribosomal RNA subunit which is 18S [Figure 5.7]. In this gel figure, we see a sharp band which means there is no RNA degradation in the 1 month period. If there was a smear, this would mean RNA had started to be ubiquinitated and some of it would be degraded. The samples were integrated. Also from this figure, we can estimate that the concentration of N1 and N2 RNA is higher than R1, R2 and R3 RNA. Also we should see 28S band intensity must be two times more concentrated than 18S band, which was seen.

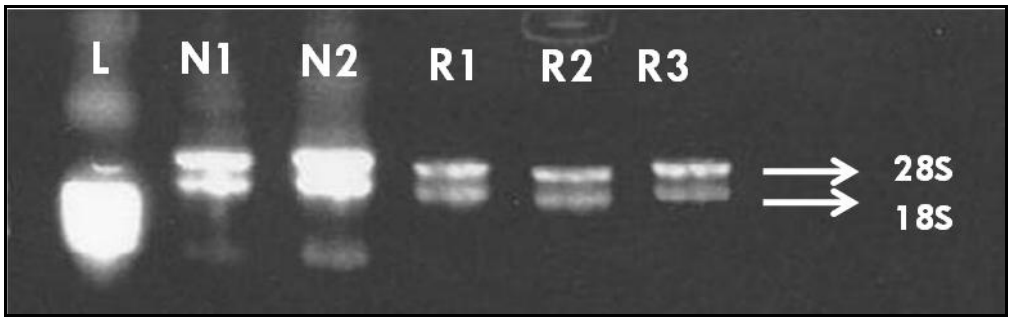


Figure 5. 7: RNA quality assay. N stands for Normal MCF-7 RNA and R stands for MCF-7 "R6" cell line RNA. We had triplicate RNA of each group. The ladder was expired so it was degraded and seen as a smear band at L lane.

5.2.3 RNA Concentration

Desired RNA concentration for microarray experiment was 200 ng/µl. We got RNA concentrated than that. After RNA dilution to equal concentration that is 200 ng/µl and volume is 20 µl; RNAs were shipped to Cleveland genomics center, OHIO, USA.

Table 5. 3: RNA concentration. Samples are measured in 3 biological replicates and in ng/µl.

Sample(ng/µl)	1	2	3
Normal MCF	424.87	541.91	542.0
MCF-7 "R6"	142.08	49.52	207.49

5.2.4 Microarray chip

Illumina Human WG-6 BeadChip array was used for gene expression experiments. It contains 48,000 bead types and takes 6 samples simultaneously. The array reader identifies each DNA oligomers using an autonomic registration algorithm [100] and a molecular address [101]. DNA sequences attached to the bead are 75 base pairs in length: 50 base pairs for target hybridization and 25 base pairs for decoding purposes.

From the image of the array using image processing techniques, numerical values are obtained and written in an excel file by BeadArray ^{TM} software. We downloaded this excel table from a universal domain. These data were not normalized but background subtraction was already made by US collaborators.

5.2.5 Data analysis

After downloading the excel file, genes that had negative values were eliminated because negative values mean that the background emits more light than the sample, that would probably sourced by either experimental error or maybe gene probe did not bind properly to the target.

Therefore we eliminated this gene manually. By manually we mean that we calculated the mean values of triplicates and then sorted them using an excel sorting filter and deleting rows with a negative value.

5.2.6 MATLAB

MATLAB 2009b was used for statistical analysis. I wrote a code that only picks the significant genes from the gene pile and writes them into a separate text file. Code strings are shown in Figure 5.8.

All data was normalized to the GAPDH gene which is generally used in RT PCR normalization. The expression level of this gene is nearly same for all cells. We did this for each value of normal MCF-7 and MCF-7 "R6"separately.

Then we executed the code in Figure 5.8. In the first step, the code finds NaN values in the excel spreadsheet; If there are any NaN values, it changes NaN into a mean value which is specifically calculated for the row. We need remove NaN values because they are non-numerical values and prevent calculation and analysis.

```
   fid = fopen('normalized data.txt', 'r+');
   s                          =                          size(data,1);
ALLDATA=data(:,1:6);
   NAN=findstr('NaN', s);
   D = zeros(s, 6);
   for i = 1:s
       D(i,:) = ALLDATA(i,:);
   end
   C = D(1:s, 1:3); R = D(1:s, 4:6);
   for i=1:s
       MeanC(i,1)=mean(C(i,1:3));
       SigmaC(i,1)=std(C(i,1:3));
       MeanR(i,1)=mean(R(i,1:3));
       SigmaR(i,1)=std(R(i,1:3));
       for j=1:3
           OutlierC(i,j)= abs(C(i,j)-MeanC(i,1)) > 3*SigmaC(i,1);
           OutlierR(i,j)= abs(R(i,j)-MeanR(i,1)) > 3*SigmaR(i,1);
       end
   end
   fid = fopen('significants.txt', 'wt');
   count_t = 0;
   count_r = 0;
   for i=1:s
       if(jbtest(C(i,1:3))==0)
           test_result(i,1) = ttest2(C(i,:), R(i,:));
           count_t = count_t + 1;
       else
           [p,h]             =             ranksum(C(i,:),R(i,:));
test_result(i,1) = h;
           count_r = count_r + 1;
       end
       if(test_result(i,1) == 1)

fprintf(fid,'%s\t%s\t%8.3f\t%8.3f\t%8.3f\t%8.3f\t%8.3f\t%8.3f\t'
,...

char(textdata(i+1,1)),char(textdata(i+1,2)),D(i,1),D(i,2),D(i,3)
,D(i,4),D(i,5),D(i,6));
       end
       fprintf(fid, '\n');
   end
```

Figure 5. 8: the code string.

In the following step, the code eliminates outliers if the absolute of the average minus gene value is more than three sigma, it is considered an outlier and program does not write this gene into matrix so this gene is eliminated. The program scans and calculates these values for each row.

In the next step, the program checks if the gene values are normally distributed. For checking distribution, we used a *jbtest* command. This commands usage is optional. There are many codes that can be used in analysis. If the data is distributed normally, a *jbtest* gives a value 0 and then *ttest2* command is executed automatically. The *ttest2* performs paired t-test. This code was picked because we have 2 groups which contain the same number of repetition and the same type of cell. If the gene passes both jb-test and t-test, then it is written into the significants.txt file. If data is not distributed normally, jbtest gives a value 1, then it performs rank sum test. Default *p-value* of t-test is $p \leq 0.05$. So genes in the Significant.txt file were trustable at least 95%. The program writes all information about the genes and values of each row together to a txt file.

In total, there were 48803 genes, after executing the code, 5639 genes were left. 11.6% of the data remained at $p \leq 0.05$ and 88.4% was eliminated. We also performed benforoni correction (BC) and after BC, only 10 genes were left which means only 0.021% of the data remained.

Significant.txt file was used as a fundamental datafile for this study. The folds of Resistance/Normal (R/C) were also calculated from mean values. According to fold results western blot experiments were performed.

5.2.7 Microarray results as tables

If genes are classified according to functions generally known, most of the gene expression can be said to be drastically changed in MCF-7 “R6” cell RNA compared to normal MCF-7 cell RNA.

Table 5. 4: classification of genes according to functions

Entity	Number
Cell Death	28
Growth factor	16
CD	14
Cell cycle	6
DNA repair	7
Drug Resistance marker	7
NF-KB	12
MAPK	14

As a general aspect, gene expression of growth factors increased up to 4 fold in MCF-7 "R6" cells. The apoptotic protein level declined but some Bcl-2 member levels increased in resistant cells. The expression of known drug resistance markers started to be built up at a constutional level in cisplatin resistant cell. The gene expression level of DNA repair enzyme increased in MCF-7 "R6" cells. Cell cycle protein level is elevated in MCF-7 "R6" cells compared to normal MCF-7 cells. Also levels of WNT, NF-κB and MAPK pathway entities increased in MCF-7 "R6" cells.

5.2.8 After Benforoni correction

The Benforoni correction is calculated from the following formula;

$$\text{Benforoni Correction} = \frac{p - value}{number - of\ - genes}$$

It only aims to select genes that have *p-value* less than $\leq \frac{0.05}{48803}$ which is equal to ≤ 0.0000102. The genes that are listed below have that p-value so their variance is very low and similar to each other.

Table 5. 5: list of the most significant genes after Benforoni correction

Fold (R/C)		Gene Definition
0.74	PINK1	Homo sapiens PTEN induced putative kinase 1 (PINK1), nuclear gene encoding mitochondrial protein, mRNA.
1.30	TPCN2	Homo sapiens two pore segment channel 2 (TPCN2), mRNA.
0.57	RWDD2A	Homo sapiens RWD domain containing 2A (RWDD2A), mRNA.
2.25	P2RY2	Homo sapiens purinergic receptor P2Y, G-protein coupled, 2 (P2RY2), transcript variant 2, mRNA.
0.44	CCNG1	Homo sapiens cyclin G1 (CCNG1), transcript variant 2, mRNA.
1.42	GSK3B	Homo sapiens glycogen synthase kinase 3 beta (GSK3B), mRNA.
1.88	YRDC	Homo sapiens yrdC domain containing (E. coli) (YRDC), mRNA.
1.67	FLAD1	Homo sapiens FAD1 flavin adenine dinucleotide synthetase homolog (S. cerevisiae) (FLAD1), transcript variant 2, mRNA.
1.40	KEAP1	Homo sapiens kelch-like ECH-associated protein 1 (KEAP1), transcript variant 2, mRNA.
10.3	ACTA2	Homo sapiens actin, alpha 2, smooth muscle, aorta (ACTA2), mRNA.

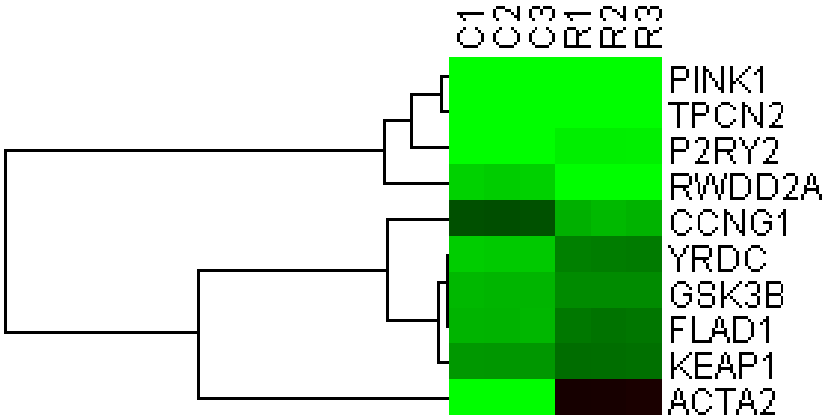


Figure 5. 9: classification of 10 genes by using Euclid distance (Cluster 3). Black color refers to increased gene expression and green means decreased gene expression level.

PINK1 gene expresses serine threonine kinase and localizes to mitochondria [102-104]. In cell culture studies the overexpression of PINK1 protects cell against apoptotic stimuli and stress whereas siRNA mediated depletion sensitizes cell to apoptotic threats [105]. In this study, PINK1 level is decreased 0.7 fold in resistant cells compared to normal cells and this contradicts with previous studies.

TPCN2 localizes in ER and lysosome. It is a two pore cation channel which forms lysosomal NAADP-sensitive Ca^{+2} releases. In resistant cells, level of this gene raised to 1.4 fold [108].

P2RY2 product belongs to the family of G-protein coupled receptors. This family has several receptor subtypes with different pharmacological selectivity, which intersect in some cases, for various adenosine and uridine nucleotides. This receptor is sensitive to both adenosine and uridine nucleotides [137, 138]. This gene is expressed 2.25 times more in cisplatin resistant cells breast cancer cells.

The CCNG1 gene level in resistant cells is 0.44 less than normal MCF-7 cells. Transcriptional activation of this gene is induced by p53 gene and p53 gene level is nearly half of normal MCF-7 cells therefore p53and this gene seems to be acting simultaneously in drug resistant cases [139].

The GSK3B gene is part of energy metabolism and Wnt pathway which is involved in cell differentiation, cell movement, polarity and proliferation [reviewed in ref 140]. GSK3B inhibits β-catenin to enter to cytoplasm and activates TCF/LEF transcription factors. One of the targets of TCF/LEF transcription factors are *c-jun*, *c-fos* and cyclin family of proteins [141]. Here, the level increased in resistant cells at 1.42 fold. But the Wnt pathway is overactivated in resistance cells up to 3 fold therefore this increase of GSK3B is related to non-canonical Wnt pathway probably.

FLAD1 catalyzes the adenylation of flavin mononucleotide (FMN) to form flavin adenine dinucleotide (FAD) coenzyme [retrieved from genecards.org]. the level of this gene ascended 1.67 fold in resistant cells.

KEAP1 regulates cytoprotective gene expression under stress conditions and is suppressed under unstressed conditions by inhibiting Nrf2. The level rise of KEAP1 at

1.4 fold explains how cells start to be insensitive to oxidative damage of cisplatin [109, 110].

ACTA2 is involved in cell motility, structure and integrity by polymerizing and depolymerizing of fibrils. Also it forms polar tracks within the cell and in this way helps motor proteins and vesicle transport system. Actin is linked to E-cadherin and beta-catenin at adherens junctions. This gives mechanical support and durability to cells. It may be the cause of cells to adhere surface much more than initial state. It is 10.3 times more in MCF-7 "R6" cell line. Cytoskeletal changes occurred to MCF-7 "R6" cell line in transition from cisplatin sensitive to resistance phase [retrieved from TOCRIS].

The functions of RWDD2A and YRDC genes are not known.

5.3 Selected genes

5.3.1 Drug resistance markers

There are already characterized drug resistant genes and they are characterized in other cell lines. In this section of the study, we want to examine if the drug resistant marker genes already known also play a role in breast cancer cisplatin resistant cells.

Table 5. 6: Drug resistance genes.

Fold (R/C)	Gene Name	Gene Definition
2.01	GSTP1	Homo sapiens glutathione S-transferase pi (GSTP1), mRNA.
1.97	MGST1	Homo sapiens microsomal glutathione S-transferase 1 (MGST1), transcript variant 1b, mRNA.
1.28	MT1F	Homo sapiens metallothionein 1F (MT1F), mRNA.
1.30	MT1H	Homo sapiens metallothionein 1H (MT1H), mRNA.
1.32	SLC31A1	Homo sapiens solute carrier family 31 (copper transporters), member 1 (SLC31A1), mRNA.
2.20	ABCB6	Homo sapiens ATP-binding cassette, sub-family B (MDR/TAP), member 6 (ABCB6), nuclear gene encoding mitochondrial protein, mRNA.
1.89	ABCB9	Homo sapiens ATP-binding cassette, sub-family B (MDR/TAP), member 9 (ABCB9), transcript variant 2, mRNA.

Table 5.6 shows that known genes are also involved in drug resistance but gene variants differ in some way. The GST protein has pi variant called GSTP1 gene which was expressed 2 times more in resistant cells than normal cells. Another type of GST, the MGST1 variant 1b, was nearly 2 times more expressed in MCF-7 "R6" cells. Metallothionein is also expressed more in oxidative stress formation and in resistant cells metallothionein 1F and 1H were expressed 1.3 times more.

The solute carrier family 31 is for exports copper and drug outside of the cell and occured 1.32 times more in resistant cells. In this way it can pump the drug out of the cell.

ATP binding cassettes are the largest and most ancient family of transporters. It utilizes energy to translocate several substances such as drug and the neutralized form of drug. Only ABCB6 and ABCB9 gene transcript variant 2 cassettes are expressed nearly 2 times more in MCF-7 "R6" cells.

5.3.2 Genes related to Cell Death

Apaf-1 has 2 transcript variants that do not differ in function. Apaf-1, BID, Caspase-6, CFLAR, CRADD, TUSC1, TUSC4, XAF1 and FOXO-1, FOXO-4 level declined in resistant cell. Pro-apoptotic members are: Apaf-1, BID, FOXO-1, FOXO-4, Caspase-6, CFLAR (key molecule between cell death and cell survival), and CRADD (apoptotic adaptor). TUSC1 is a tumor suppressor gene; as its level decreases, tumors become aggressive [provided by RefSeq].

The level of BCL, DAP, DAPK and ATG family proteins raised at 1.09- 1.99 fold. Some autophagy related genes seem to be increased such as ATG16, ATG3 and ATG4C.

Apaf-1 is a pro-apoptotic member of apoptosis proteins and XIAP is anti-apoptotic member. In the microarray data, the Apaf-1 expression level seems to be decreased to half in MCF-7 "R6" cells. At protein level, expression of Apaf-1 decreased to half therefore here gene expression level and protein level seems to be regulated in parallel.

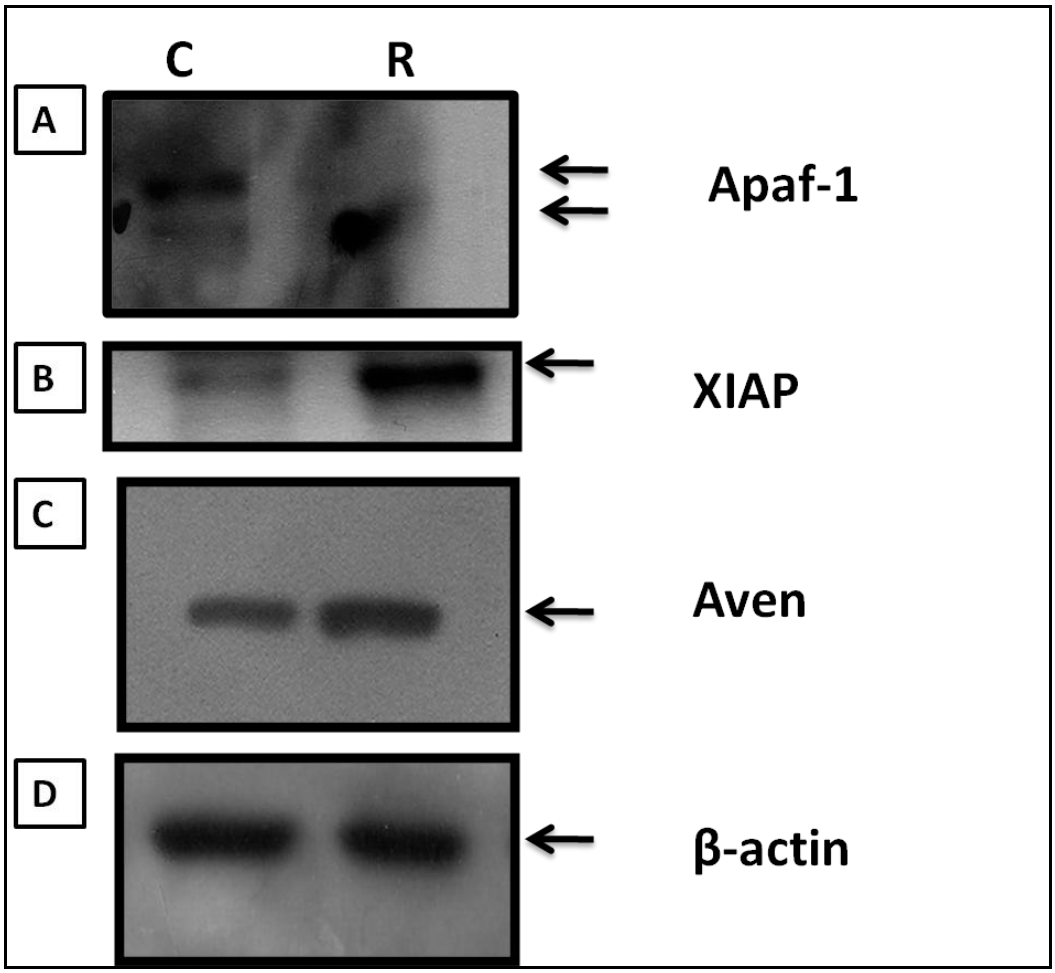


Figure 5. 10: Immunoblotting of Apaf-1, XIAP, Aven and β-actin.

Apaf-1 has 2 transcript and protein variants so these 2 bands that are 130 and 140kDa are seen in Figure 5.10. In the R column which corresponds to resistant cell line, the very faint band of Apaf-1 is seen. Apaf-1 level is decreased at 0.5 fold in both total proteins and gene expression level of MCF-7 "R6".

As seen in the Figure 5.10, XIAP is upregulated at the protein level whereas XIAP associated factor 1 (XAF1) transcript variant 2 gene expression level was downregulated at the RNA level. It shows that, gene expression and protein expression levels do not always have to be parallel.

Aven is a caspase activation inhibitor and binds to pro-apoptotic proteins such as Apaf-1, Bcl-xL. Aven level is upregulated in MCF-7 "R6" cell line. Therefore the resulting Apaf-1-Aven complex overcomes apoptosis.

Table 5. 7: Cell death related genes.

Fold		Gene Definition
0.50	APAF1	Homo sapiens apoptotic peptidase activating factor 1 (APAF1), transcript variant 1, mRNA.
0.89	BCL11B	Homo sapiens B-cell CLL/lymphoma 11B (zinc finger protein) (BCL11B), transcript variant 1, mRNA.
1.25	BCL2L2	Homo sapiens BCL2-like 2 (BCL2L2), mRNA.
0.84	BCL6	Homo sapiens B-cell CLL/lymphoma 6 (zinc finger protein 51) (BCL6), transcript variant 1, mRNA.
1.47	BCL7A	Homo sapiens B-cell CLL/lymphoma 7A (BCL7A), transcript variant 2, mRNA.
1.26	BCL7B	Homo sapiens B-cell CLL/lymphoma 7B (BCL7B), transcript variant 2, mRNA.
1.59	BCL7B	Homo sapiens B-cell CLL/lymphoma 7B (BCL7B), mRNA.
1.99	BCL9L	Homo sapiens B-cell CLL/lymphoma 9-like (BCL9L), mRNA.
0.87	BID	Homo sapiens BH3 interacting domain death agonist (BID), transcript variant 1, mRNA.
0.86	CASP6	Homo sapiens caspase 6, apoptosis-related cysteine peptidase (CASP6), transcript variant beta, mRNA.
0.83	CFLAR	Homo sapiens CASP8 and FADD-like apoptosis regulator (CFLAR), mRNA.
0.79	CRADD	Homo sapiens CASP2 and RIPK1 domain containing adaptor with death domain (CRADD), mRNA.
1.36	DAP	Homo sapiens death-associated protein (DAP), mRNA.
1.32	DAP3	H.S. death associated protein 3 (DAP3), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA.
1.82	DAPK2	Homo sapiens death-associated protein kinase 2 (DAPK2), mRNA.
0.33	FOXO1	Homo sapiens forkhead box O1 (FOXO1), mRNA.
0.44	FOXO4	Homo sapiens forkhead box O4 (FOXO4), mRNA.
0.73	TUSC1	Homo sapiens tumor suppressor candidate 1 (TUSC1), mRNA.
0.79	TUSC4	Homo sapiens tumor suppressor candidate 4 (TUSC4), mRNA.
0.27	XAF1	Homo sapiens XIAP associated factor 1 (XAF1), transcript variant 2, mRNA.
0.28	XAF1	Homo sapiens XIAP associated factor 1 (XAF1), transcript variant 2, mRNA.
1.09	ATG12	Homo sapiens ATG12 autophagy related 12 homolog (S. cerevisiae) (ATG12), mRNA.
1.29	ATG16L1	Homo sapiens ATG16 autophagy related 16-like 1 (S. cerevisiae) (ATG16L1), transcript variant 1, mRNA.
1.42	ATG16L1	Homo sapiens ATG16 autophagy related 16-like 1 (S. cerevisiae) (ATG16L1), transcript variant 2, mRNA.
1.13	ATG3	Homo sapiens ATG3 autophagy related 3 homolog (S. cerevisiae) (ATG3), mRNA.
1.43	ATG4A	Homo sapiens ATG4 autophagy related 4 homolog A (S. cerevisiae) (ATG4A), transcript variant 2, mRNA.
0.77	ATG4C	Homo sapiens ATG4 autophagy related 4 homolog C (S. cerevisiae) (ATG4C), transcript variant 7, mRNA.
0.78	MAP1L	Homo sapiens microtubule-associated protein 1 light chain 3 beta (MAP1LC3B), mRNA.

5.3.3 Foxo family

The gene expression level of Foxo-1 and Foxo-4 decreased 0.45 and 0.33 fold respectively in resistant cell. Foxo-1 and Foxo-4 are apoptotic proteins and their presence at a low-level is appropriate for cisplatin resistant cells [Figure 5.11].

A

R/C	Gene Name	Gene Explanation
0.45	FOXO4	Homo sapiens forkhead box O4 (FOXO4), mRNA.
0.33	FOXO1	Homo sapiens forkhead box O1 (FOXO1), mRNA.

B

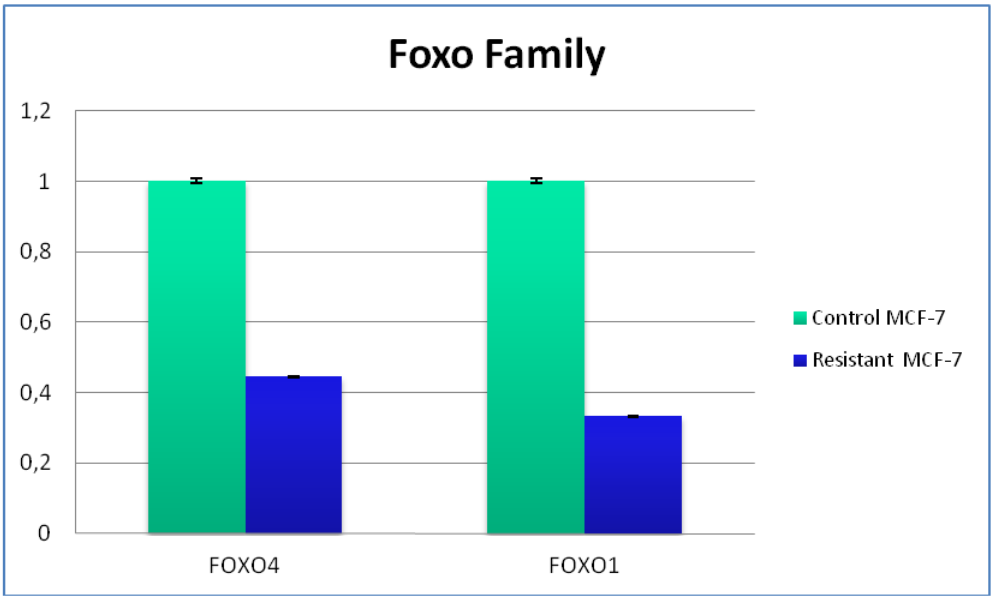


Figure 5. 11: A: Foxo family gene expression folds B: Graph of Foxo-1 and Foxo-4 (Microarray data).

The total protein level of Foxo-1, Foxo-3a and Foxo-4 was checked by immunoblotting. In the control cell, there is more Foxo-1, Foxo-3a and Foxo-4 than in MCF-7 "R6" cells. Phosphorylation of Foxo-1 at Ser 256 and at Thr-24 was checked as well. Foxo-1 is 78-82 kDa, Foxo-4 is 65 kDa and Foxo3 is 82 kDa. Phosphorylation of Foxo-1 from both residues was higher in the cisplatin-resistant MCF-7 cells than normal

MCF-7 cells. A very faint band can be seen in the total protein level of Foxo-1 in MCF-7 “R6” and P-Foxo-1in Normal MCF-7 cells [Figure 5.12].

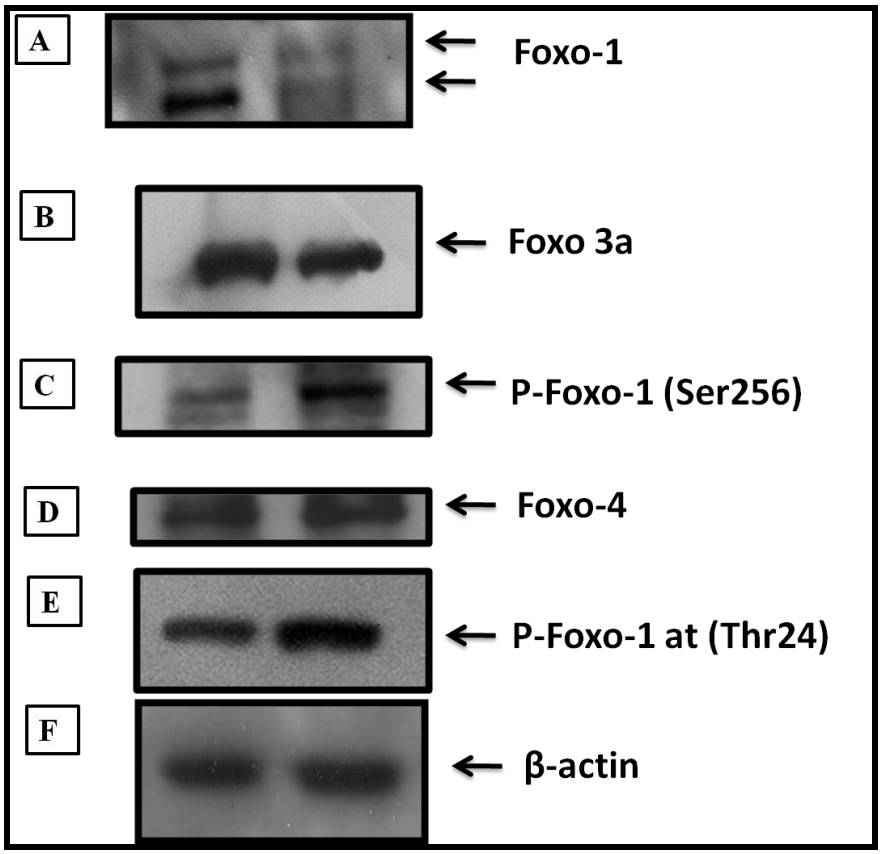


Figure 5. 12: Protein level of Foxo family proteins. A: Foxo-1, B: Foxo-3a, C: P-Foxo-1 at Ser-256 D: Foxo-4 E: P-Foxo-1 atThr-24 F: β-actin

EMSA for Foxo-3a is performed with normal MCF-7 cells and MCF-7 “R6” cells. In normal MCF-7 cells there is very low level of Foxo-3a activation in the nucleus. In the resistant cells, there is a high level of Foxo-3a transactivation [Figure 5.13]. Western blots showed very slight changes in the level of Foxo-3a but EMSA showed a drastic increase in the nucleus. This extreme difference may resulted from the resistance case. As the cell line progresses to being resistant, the total Foxo-3a protein level decreases to become very close to the basal level of the MDA-MB-231 cell line [Figure 5.14].

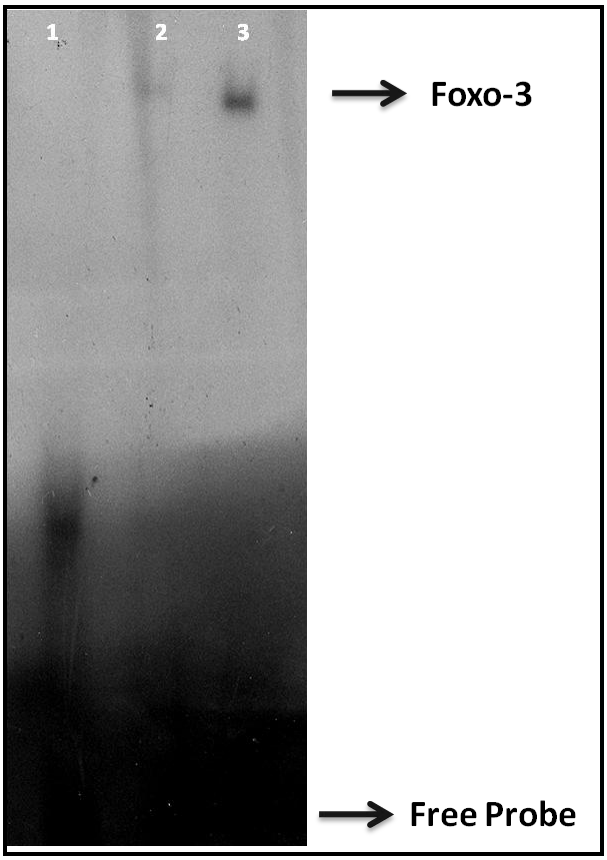


Figure 5. 13: EMSA results for Foxo 3a. The first lane contains the free probe, the second lane contains the nuclear isolate of normal MCF-7 cells and the third lane contains the MCF-7 "R6" cell line nuclear protein.

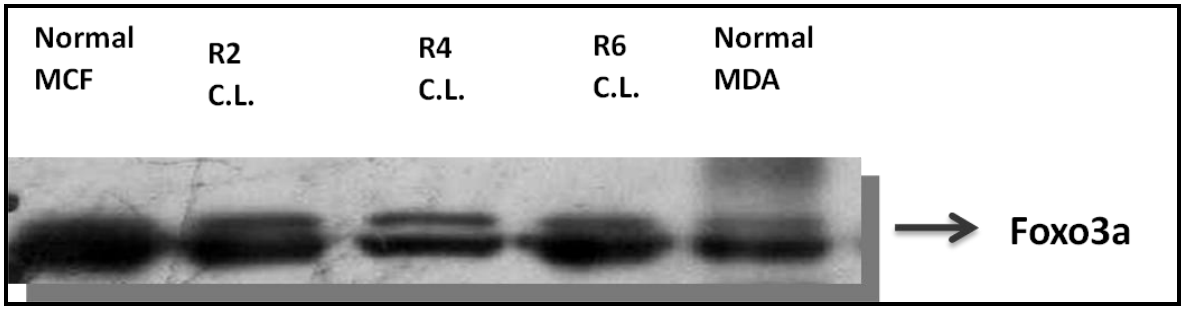


Figure 5. 14: Immunoblotting of Foxo3a in R2, R4, R6 and in normal MCF-7, MDA-MB-231 cell lines.

5.3.4 NF-κB

5.3.4.1 Immunoblotting and Microarray results

The CHUK gene encodes a member of the serine/threonine protein kinase family and this helps NF-κB be released into nucleus by help of MAP3K14. The levels of these two genes CHUK and MAP3K14 were 1.21 and 1.83 fold more in MCF-7 "R6" cells compared to normal MCF-7 cells. In parallel, NF-κB transcript variant 2 and variant 3 levels were 1.82 and 1.71 fold more in resistant cells. This implies that there is overactivation of non-canonical NF kappa B and it can be related to the CHUK gene.

All levels of the NF-κB inhibitors were down except NFKBID. Its expression level was 0.63 fold lower than normal MCF-7 cells. IκB kinase complex and IκB kinase epsilon levels were nearly 2 times more in MCF-7 "R6" cells.

NF kappa B pathway is transactivated nearly 2 times more in MCF-7 "R6" cells according to the gene expression level. This is confirmed by western blots in Figure 5.16.

Immunoblotting results also support the NF-kappa-B overactivation theory established according to the microarray results. In the western blot, a decreased level of IκB and an increased level of phoshorylated-IκB were seen as well as a high phosphorylated-IKK alpha/beta level at the basal state of the resistant cell. This shows that canonical NF-κB pathway become constitutively active in MCF-7 "R6" cells.

Table 5. 8: fold values of NF-κB entities.

R/C	Gene Name	Gene Explanation
1.21	CHUK	Homo sapiens conserved helix-loop-helix ubiquitous kinase (CHUK), mRNA.
1,83	MAP3K14	Homo sapiens mitogen-activated protein kinase kinase kinase 14 (MAP3K14), mRNA.
1.82	NFKB2	Homo sapiens nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100) (NFKB2), transcript variant 2, mRNA.
1.71	NFKB2	Homo sapiens nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100) (NFKB2), transcript variant 3, mRNA.
1.39	NFKBIL2	Homo sapiens nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 2 (NFKBIL2), mRNA.
0.63	NFKBID	Homo sapiens nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, delta (NFKBID), mRNA.
1.25	NFKBIZ	Homo sapiens nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta (NFKBIZ), transcript variant 2, mRNA.
1.91	NFKBIB	Homo sapiens nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta (NFKBIB), transcript variant 1, mRNA.
1.88	NFKBIB	Homo sapiens nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta (NFKBIB), transcript variant 1, mRNA.
1.91	IKBKAP	Homo sapiens inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein (IKBKAP), mRNA.
1.91	IKBKAP	Homo sapiens inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein (IKBKAP), mRNA.
1.38	IKBKE	Homo sapiens inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon (IKBKE), mRNA.

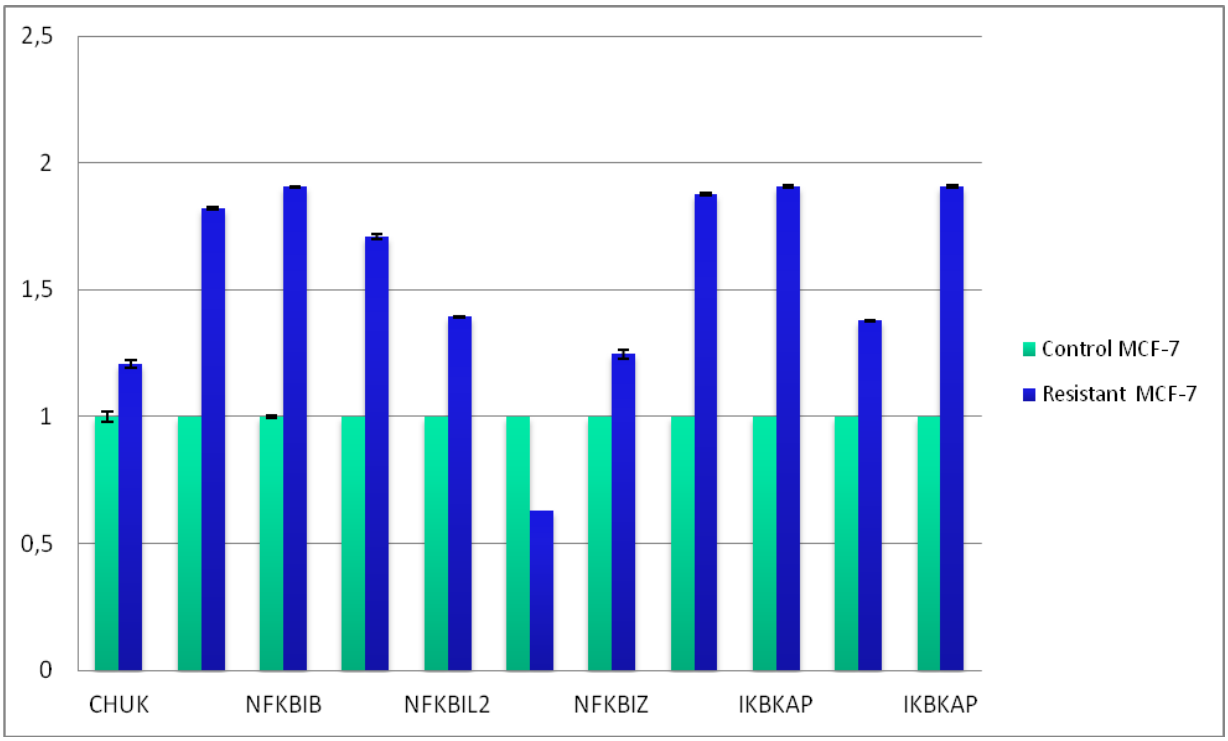


Figure 5. 15: Graph of folds showing NF-κB related gene expression.

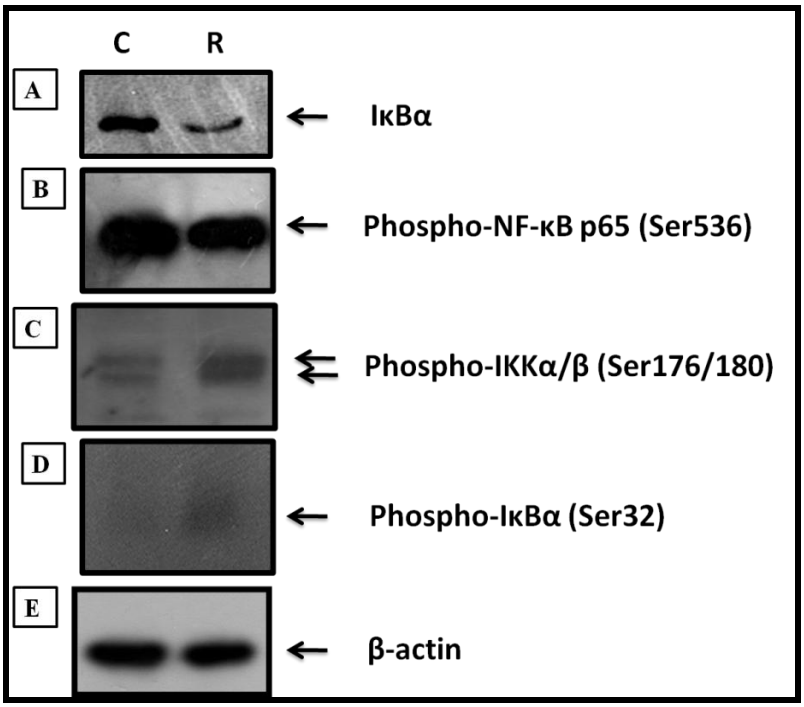


Figure 5. 16: Western blot of NF-κB entities in MCF-7/R6 cells. A: IκB-alpha, B: Phospho-NF-κB, C: Phospho-IKK alpha/Beta, D: Phospho-IκB-alpha, E: Beta Actin

IKK-ε is part of the IKK complex. The level of IKK-ε increased in MCF-7 "R6" cells. The basal IKK-ε level in MDA-MB-231 cells is very low compared to MCF-7 cells although β-actin level of MDA-MB-231 cells was slightly higher than MCF-7 cells. The gene expression level of IKK-ε was not in the significant list that because it had a *p value* higher than 0.05. Therefore, the gene expression and protein level were not compared.

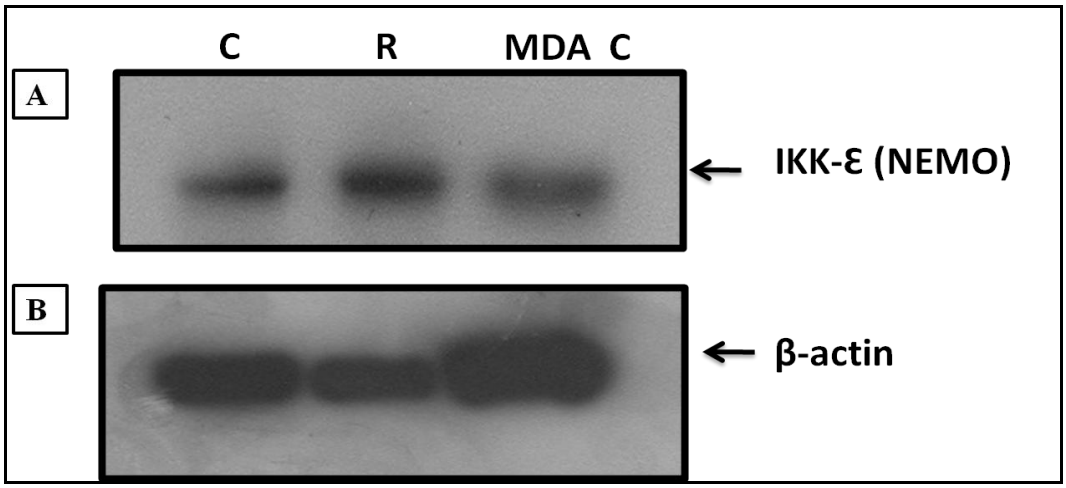


Figure 5. 17: Immunoblotting of A: IKK-Ɛ and B: beta actin. C: Normal MCF-7, R: MCF-7 "R6" and MDA C: MDA-MB-231 cells.

5.3.4.2 EMSA results for NF-kappa-B

EMSA is performed through protein isolation of both normal MCF-7 and MCF-7 "R6" cell lines. Cisplatin treatment was done to both cell lines for 15 minutes and 30 minutes, also 1hour and 2 hour. A control not treated with cisplatin is used. Radioactively labeled NF-kB probe is used.

At the basal state, there was no NF-κB activation in normal MCF-7 cells whereas there was strong activation of NF-κB in MCF-7 "R6" cells when C lanes were compared. When normal cells are treated with cisplatin (30μM), there is NF-κB activation in the 15 minute treatment, in 30 minute this effect is lost and in 1 hour NF-κB reactivates again. In the 2 hour treatment, NF-κB degraded again.

In MCF-7 "R6" cells, the basal state activation of NF-κB is seen. The cisplatin treatment does not change NF-kB activation drastically because it is already active at high ratio. In the 30 minute treatment, the NF-κB level reaches to maximum in the nucleus and then, its level is declined in the 1 hour and 2 hour treatment. However the level of NF-κB never decreases to basal state level.

This study searched for the subunits of the NF-κB that have a role in transactivation. In order to understand this Super-shift assay needs to be done. In addition to NF-κB also p50 or p65 bands were expected to be seen. In figure 5.19, the

same level of NF-κB is seen which is indicated by the bottom arrow. The p50 (second lane) or p65 (third lane) are pointed with the arrow above. In the second lane there is a band that shows super shift. A very faint band in the third lane is seen however when these 2 were compared, band in lane 2 was more intense than in lane 3. So the subunits of activated NF-kB contain p50 subunit more than p65 subunit but transcription factor complex contains p65 as well.

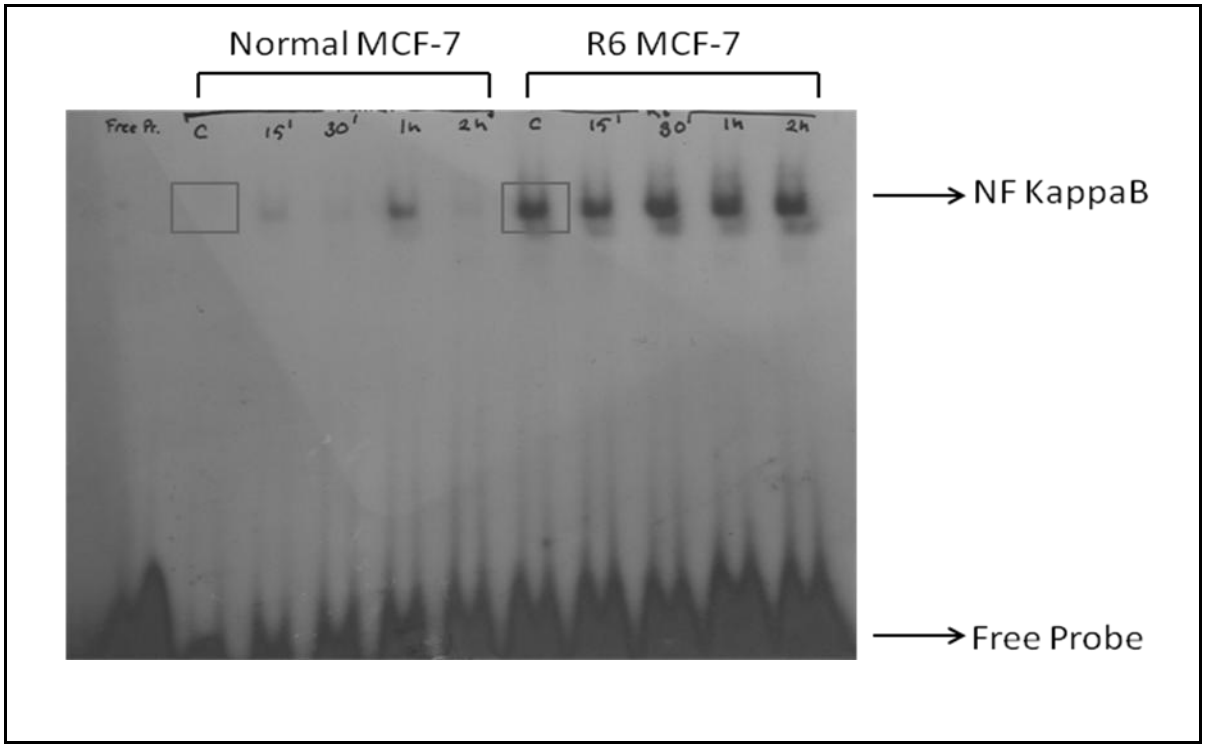


Figure 5. 18: Time dependent activation of nuclear NF-kB in both Normal MCF-7 and MCF-7 R6"

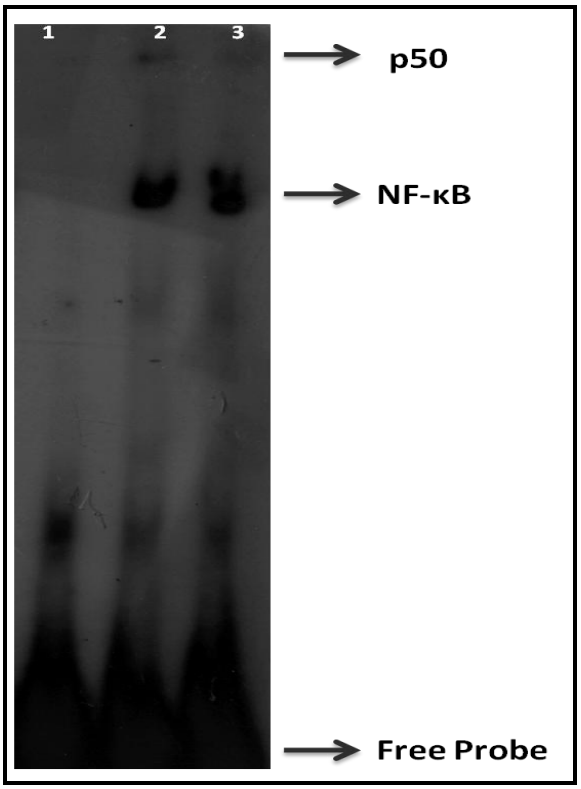


Figure 5. 19: Super-shift assay for p50 and p65 in nuclear proteins of MCF-7 "R6". The first lane contains free probe, second lane contains nuclear isolate of resistant cell and p50 antibody, the third lane contains nuclear isolate of resistant cell and p65 antibody.

5.3.5 MAP Kinases

A

R/C	Gene Name	Gene Name	Gene Explanation
0,18	JNK1	MAPK8	Homo sapiens mitogen-activated protein kinase 8 (MAPK8), transcript variant JNK1-a1, mRNA.
1,33	JNK2B	MAPK9	Homo sapiens mitogen-activated protein kinase 9 (MAPK9), transcript variant JNK2-a2, mRNA.
2,01	ERK6	MAPK12	Homo sapiens mitogen-activated protein kinase 12 (MAPK12), mRNA.
1,93	ERK3	MAPK4	Homo sapiens mitogen-activated protein kinase 4 (MAPK4), mRNA.
3,65	ERK3	MAPK4	Homo sapiens mitogen-activated protein kinase 4 (MAPK4), mRNA.
3,19	P38B	MAPK11	Homo sapiens mitogen-activated protein kinase 11 (MAPK11), mRNA.
1,46	MAP2K	MAP2K2	Homo sapiens mitogen-activated protein kinase kinase 2 (MAP2K2), mRNA.
0,78	MAP2K	MAP2K5	Homo sapiens mitogen-activated protein kinase kinase 5 (MAP2K5), transcript variant B, mRNA.
0,73	MAP2K	MAP2K5	Homo sapiens mitogen-activated protein kinase kinase 5 (MAP2K5), transcript variant A, mRNA.
0,37	MAP2K	MAP2K6	Homo sapiens mitogen-activated protein kinase kinase 6 (MAP2K6), mRNA.
0,46	MAP2K	MAP3K8	Homo sapiens mitogen-activated protein kinase kinase kinase 8 (MAP3K8), mRNA.
1,56	MAP3K	MAP3K9	Homo sapiens mitogen-activated protein kinase kinase kinase 9 (MAP3K9), mRNA.
1,83	MAP3K	MAP3K14	Homo sapiens mitogen-activated protein kinase kinase kinase 14 (MAP3K14), mRNA.
0,75	MAP4K	MAP4K2	Homo sapiens mitogen-activated protein kinase kinase kinase kinase 2 (MAP4K2), mRNA.

B

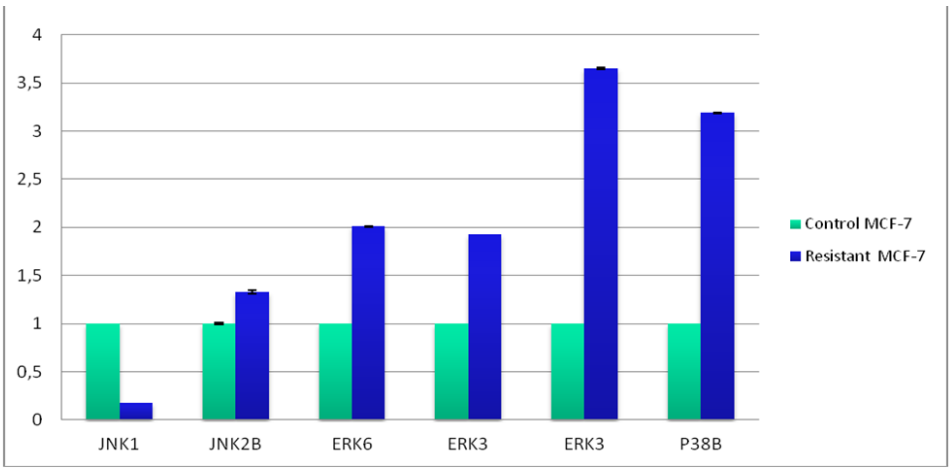


Figure 5. 20: Microarray data of MAP Kinases A: Fold values B: Graph of fold values.

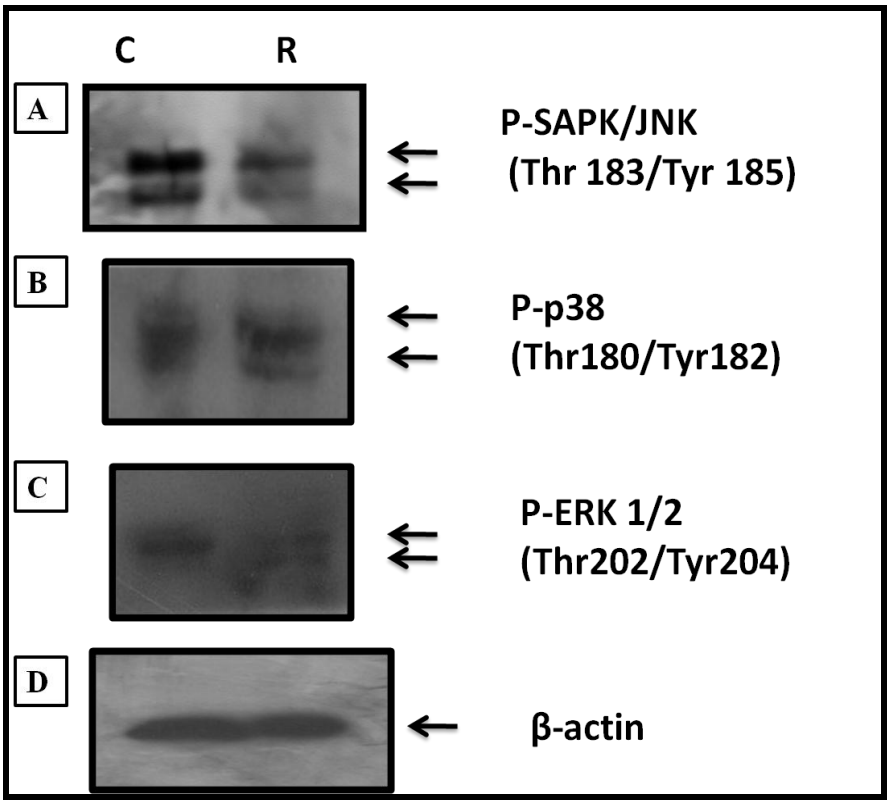


Figure 5. 21: Immunoblotting results of MAP kinases A: P-SAPK/JNK B: P-p38, C: P-ERK1/2 and D: Beta actin as a loading control.

There are 3 known types of MAP kinases: ERK, JNK and p38. When MAP kinase is phosphorylated from superior kinase in the cell, it becomes activated. The most superior kinase of MAPK is MAP4K. The gene expression level of MAP4K2 decreased at 0.75 fold in MCF-7 "R6" cells. MAP4K phosphorylates MAP3K. The gene expression level of MAP3K9 and MAP3K14 increased 1.56 and 1.83 fold respectively. MAP3K phosphorylates MAP2K. All MAP2K gene levels decreased except MAP2K2. MAP2K5, MAP2K6, MAP2K8 gene levels were 0.78-0.46 fold lower in MCF-7 "R6" cells. MAP2K phosphorylates the lowermost kinase, MAPK. MAPK gene expression levels were increased with the exception of JNK1. JNK1 in other words MAPK8 levels were 0.18 fold lower in resistant cells. In sum, ERK3 and ERK6 (MAPK4, MAPK12), JNK2 (MAPK9) and p38B (MAPK11) levels were nearly 3 times more in MCF-7 "R6" cells.

To sum up, MAP4K and MAP2K levels were lower whereas MAP3K and MAPK levels were higher in MCF-7 "R6" cells [Figure 5.20].

At protein levels only the phosphorylation state of JNK, ERK and p38 was examined. Phospho-JNK at Thr183/Tyr185and Phospho-ERK at Thr202/Tyr204 levels declined in resistant cells. On the other hand, phospho-p38 levels increased in resistant cells. So ERK and JNK activation became lower whereas p38 became more active in MCF-7 "R6" cells.

5.3.6 Some common proteins: p21 and p53

The TP53 gene level becomes lower as the cells become drug resistant. In Figure 5.22, fold graph shows that 0.21 fold lowness in p53gene in MCF-7 "R6" cells. Similar to the gene expression level, the intrinsic TP53 protein level is also low in chemotherapeutic resistant breast cancer cells. The p53 protein level becomes very similar to MDA-MB-231 which is intrinsically a cisplatin resistant cell.

A

R/C	Gene Name	Gene Explanation
0,21	TP53TG3	Homo sapiens TP53 target 3 (TP53TG3), mRNA.
1,55	RASA3	Homo sapiens RAS p21 protein activator 3 (RASA3), mRNA.

B

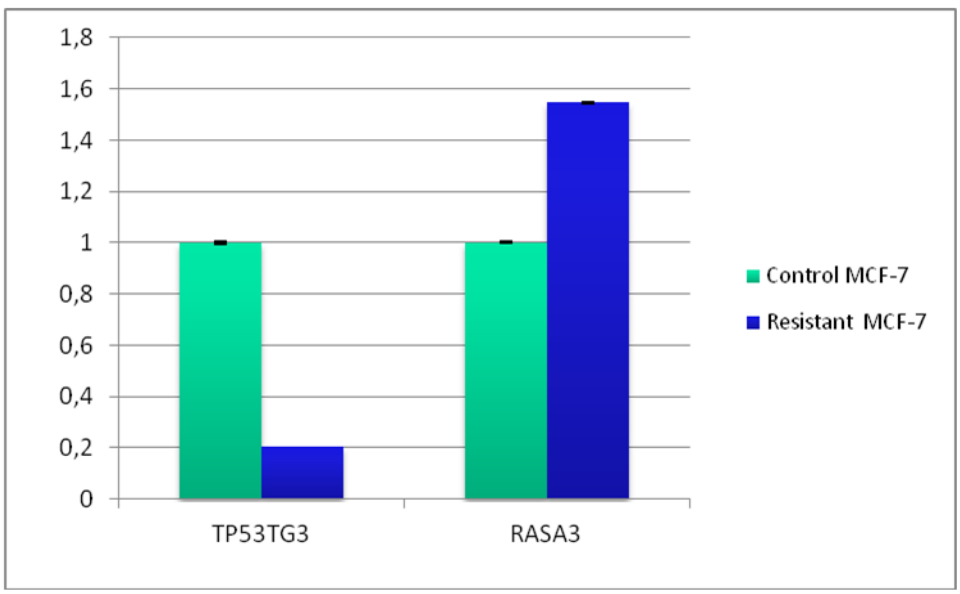


Figure 5. 22: p53 and p21 gene expression folds as A: table B: graph.

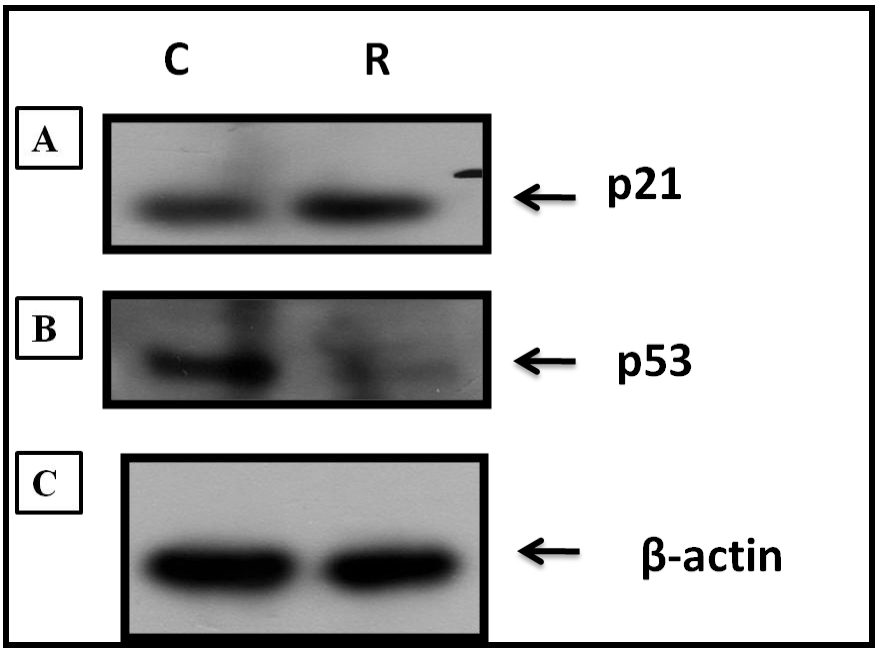


Figure 5. 23: Immunoblotting results of A: p21, B: p53, C: β-actin as a loading control.

P21 is a cyclin-dependent kinase inhibitor. It regulates the cell cycle at the S phase. The expression of p21 is sometimes controlled by p53, sometimes the regulation of p21 is independent from p53. The p21 level is increased in both gene expression and protein level. A 1.55 fold increment in the gene expression level is seen in the p21 gene. Figure 5.23suggests that p21 is regulated independently from p53 because once p21 is increased, p53 gene level and protein level decreased.

5.3.7 NFAT

NFAT signaling studies are primarily done in immune cells. NFAT is a kind of sensor that reports the Ca^{++} level rise within the cell. NFAT1 is ubiquinated by MDM2 which is a downstream target of GSK3B and Akt. There are many post-transcriptional modifications seen in NFAT.

In this part of the project, total level of NFAT transcription factor was studied. NFATc3 gene expression was double that of MCF-7 "R6" cells. If increased level of

gene expression level continues, it is translated into protein therefore protein level of NFATc3 was checked by immunoblotting. The basal level of NFATc3 is nearly equal and slightly more in MCF-7 "R6" cells when compared to MDA-MB-231 cells. The NFATc3 protein level is less in normal MCF-7 cells when compared to resistant MCF-7 cells and MDA-MB-231cells. The gene expression level is parallel with the protein level in the NFATc3 assessment.

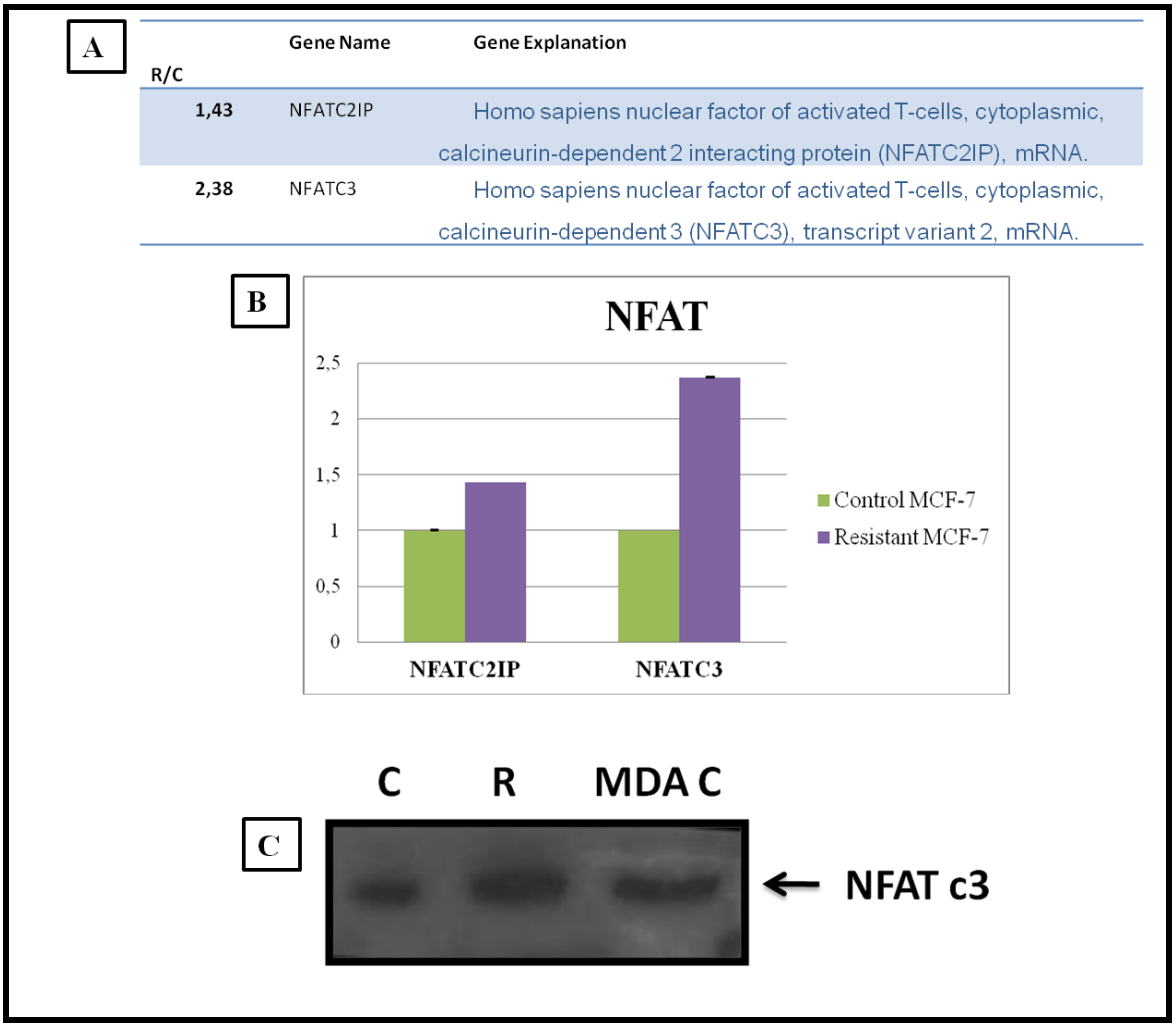


Figure 5. 24: A: Data table of NFAT gene expression level, B: Graph of NFAT gene expression levels, C: NFATc3 protein level.

5.3.8 Estrogen Receptor

The estrogen receptor is a growth receptor which needs estrogen as a ligand. The estrogen Receptor (ER) and NF-κB expression levels are in contrast to each other reported in the literature [55]. Breast cancer cells growth is firstly dependent on the estrogen receptor level. At this phase growth of breast cancer cell can be inhibited by anti-estrogens such as tamoxifen. However after some time, cells become insensitive to this agent and estrogen, therefore, they develop into estrogen resistant cells. Our data shows that breast cancer anti-estrogen resistance 3 gene (BCAR3) levels is heightened at 2.1 fold in MCF-7 "R6" cells. This rise means that cisplatin resistant cell start to grow without needing estrogen and its receptor. And they become ER resistant cells [Figure 5.25].

In previous results, the hyper-activation of NF-κB is seen [Figure 5.19]. Our data is consistent with the literature. ER levels are lower at 0.28-0.79 fold in MCF-7 "R6" cells. RelA expression is increased at 1.61 fold in resistant cells. Similar to MDA-MB-231 cells and MCF-7 "R6" cells by means of RelA and ER, expression is seen. MDA-MB-231 cells are ER-/- and the level of ER gene expression in MCF-7 "R6" became very similar to MDA-MB-231 cells. In previous studies, The RelA gene level is higher in MDA-MB-231 than MCF-7 cells is reported. And in this part of the project, level of RelA was higher in MCF-7 "R6" than in normal MCF-7 cells is seen.

The Prolactin receptor level increased in MCF-7 "R6" cells. The overexpression of prolactin results in p53 gene downregulation which is seen in our data previously.

A

R/C	Gene Name	Gene Explanation
2,1	BCAR3	Homo sapiens breast cancer anti-estrogen resistance 3 (BCAR3), mRNA.
1,49	ESRRA	Homo sapiens estrogen-related receptor alpha (ESRRA), mRNA.
0,79	RERG	Homo sapiens RAS-like, estrogen-regulated, growth inhibitor (RERG), mRNA.
0,6	EBAG9	Homo sapiens estrogen receptor binding site associated, antigen, 9 (EBAG9), transcript variant 2, mRNA.
0,54	ESR1	Homo sapiens estrogen receptor 1 (ESR1), mRNA.
0,28	GPER	Homo sapiens G protein-coupled estrogen receptor 1 (GPER), transcript variant 3, mRNA.
1,4	PRLR	Homo sapiens prolactin receptor (PRLR), mRNA.
1,61	RELA	Homo sapiens v-rel reticuloendotheliosis viral oncogene homolog A (avian) (RELA), mRNA.

B

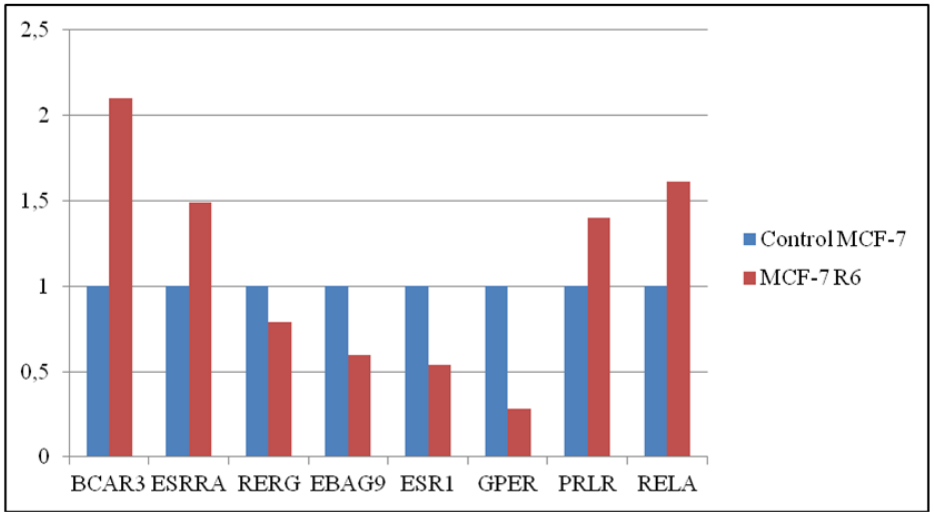


Figure 5. 25: Gene expression level of estrogen receptors. A: table of ER gene values, B: Graph of fold obtained from table.

5.4 Establishment of cisplatin-resistant MDA-MB-231 cell line

MDA-MB-231 cells are intrinsically a cisplatin resistant cell line when compared to MCF-7 cells. Flow cytometry data of normal MDA-MB-231 shows that; without cisplatin treatment, control cells undergo apoptosis at 5-10%, and after 48 hours treatment of cisplatin, normal treated cells undergo apoptosis at 20-25%.

The same "unique method1 for establishing MCF-7/R6 cell line is applied to MDA-MB-231 cells. They are also treated with 1μM cisplatin for 4 days and 30μM cisplatin for 4 hours. In the MDA-MB-231 "R2" cell line; there is a dramatic fall in apoptosis percent as seen in Figure 5.26. A 16% decrease in cell death is obtained. The detailed graph of MDA-MB-231 cell lines that are periodically treated with cisplatin is seen in Figure 26. Total protein is isolated from each cell line. This treatment is continued up to 10 treatments over 1 year.

Fluctuations in the apoptosis percent of MDA-MB-231/ R-5-R11 were seen, graphs of other 7 cell lines are not put here because they were not consistent. However, after "R2" cell line, cells became sensitized to cisplatin. The cell death ratios climbed 50%. Hence, intrinsic cisplatin resistant cells became sensitive to cisplatin with this method. Pretreatment with cisplatin affect cell viability and cells become resistant to drug at first but after the same procedure was applied periodically, cells become sensitive [Figure 5.26, R3 and R4 columns].

5.4.1 Cell morphology

Cells become smaller as they are treated with more cisplatin. MDA-MB-231/R2 cells became to be lifted up with trypsin easily, actually MDA-MB-231 cells are always lifted up more easily when it is compared to MCF-7 cells. But after "R2" cell line, this process became easier.

A

MDA-MB-231 (% Apoptosis)		
	Control	Cisplatin
R1	5,4	23,07
R2	2,5	7,7
R3	13,1	21,17
R4	28,1	35,5

B

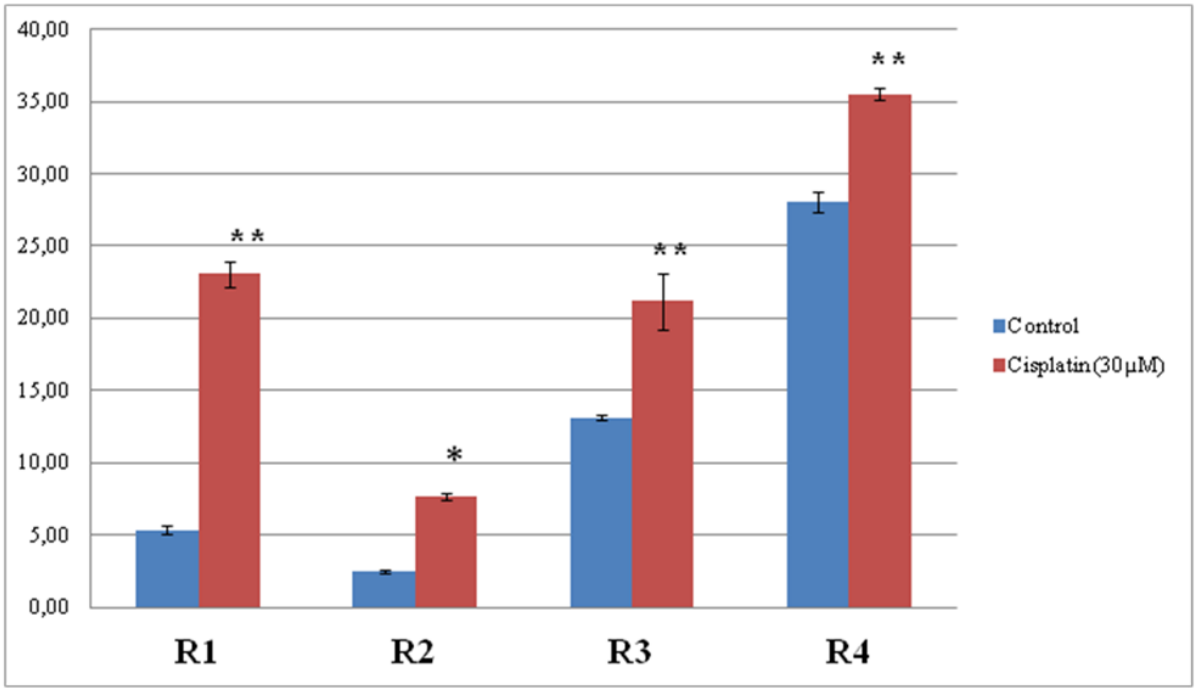


Figure 5. 26: Flow cytometry data as A: table B: graph of 4 cisplatin treated MDA-MB-231 cell line.

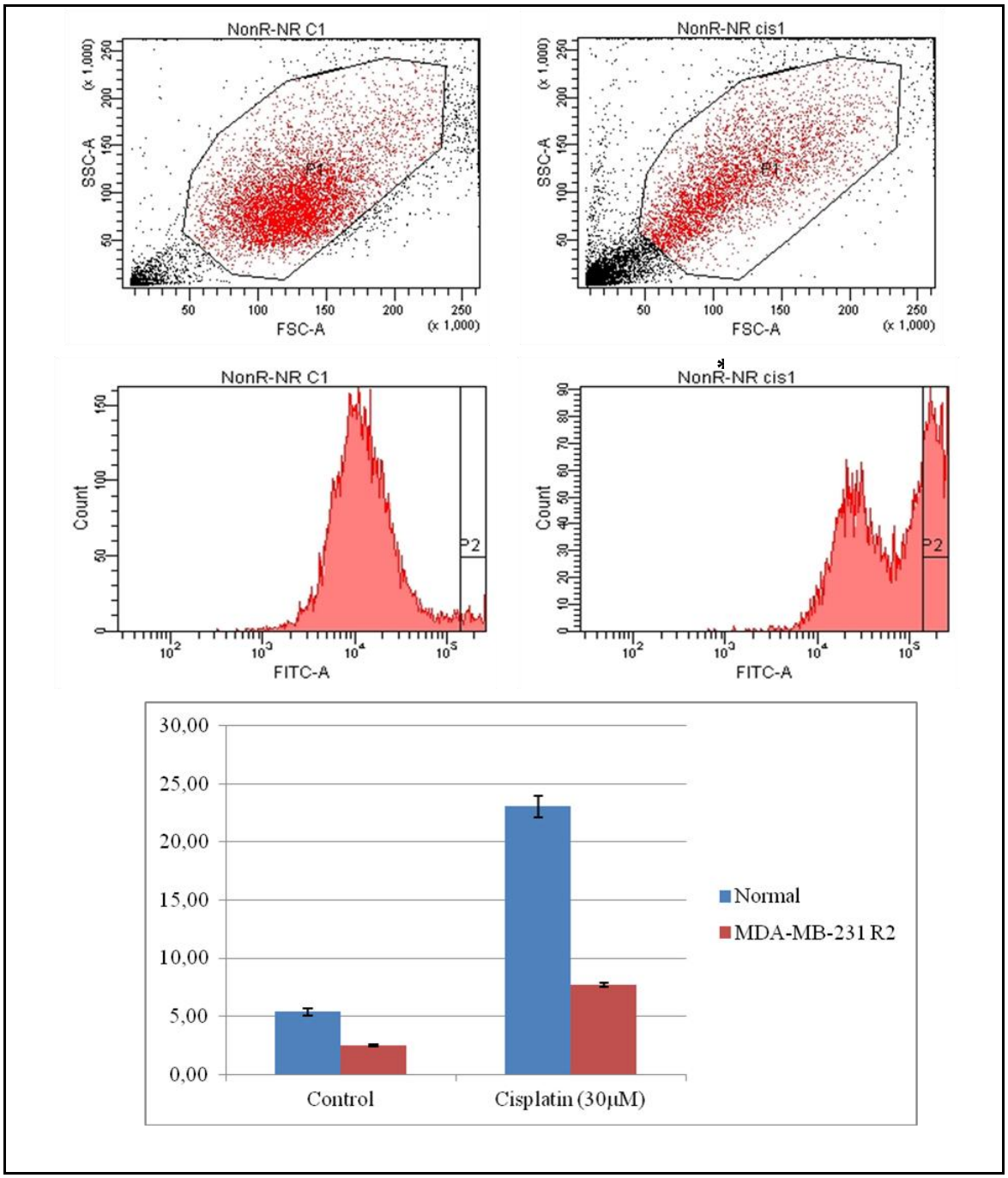


Figure 5. 27: Detailed flow cytometry graph of MDA-MB-231 "R2" cell line. Each flow cytometry experiment is done in triplicate.

5.4.2 MAP kinases

P38 and JNK are both stress and drug induced MAP kinases. P38 became to be constutively active when N and R2 lanes are compared at 0hour [Figure 5.28A: lane 1 and 2]. In the MCF-7 cells, p-p38 levels were constutively active [Figure 28A and lane 9]. Starting from the 0 hour, the early activation of p38 can be seen at the 1h, 3h and 6h treatments in MDA-MB-231 "R2" cells. P38 is activated after 0h and this is incrementally continued 6hours of treatment incrementally in both normal MDA-MB-231 and MDA-MB-231 "R2". However activation signals for MDA-MB-231 "R2" cells are always stronger when lanes next to each other were compared. The p-p38 arrow indicates 43kDa.

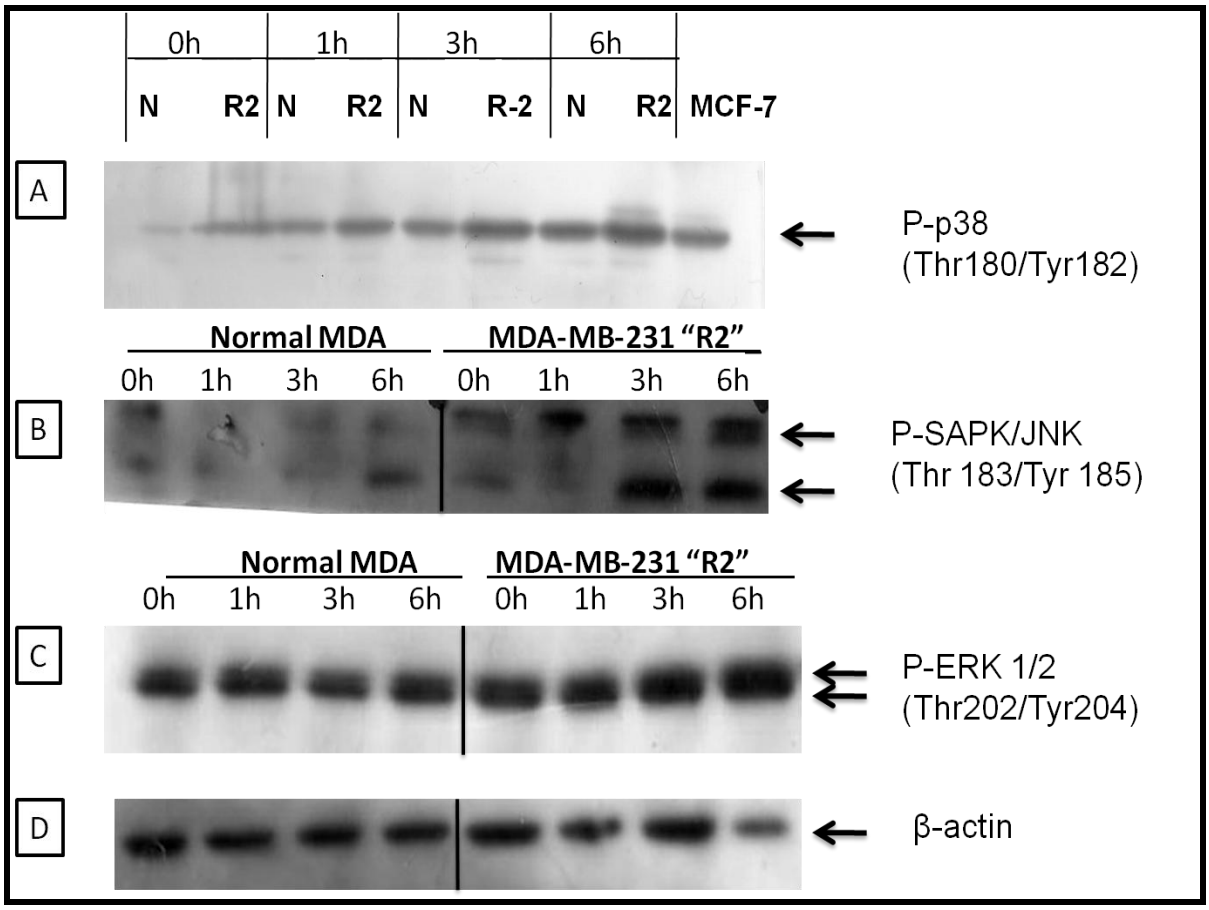


Figure 5. 28: Immunoblotting results of MAP kinases A: p-p38, B: P-SAPK, C: P-ERK and D: β-actin as a loading control.

Phospho-JNK level for both normal and MDA-MB-231/R2 cells were nearly same at the 0 hour but it is slightly more in MDA-MB-231/R2 cells. After 1 hour of treatment the 46 kDa (Figure 5.28B, upper arrow) band become intense and after 3hour treatment, 54 kDa (Figure 28B, below arrow) band become intense and after 6 hour treatment both band intensity became stronger and JNK became activated at a high level in MDA-MB-231 cells "R2". On the other hand, in normal MDA-MB-231 cells, after 1 and 3 hours cisplatin treatment, no dramatic change occurred but after 6 hours of treatment, 54 kDa band become intense. In MDA-MB-231/R2 cells the 46 kDa band began to appear first whereas in normal MDA-MB-231 cells, the 54 kDa band started to appear first.

Phospho-ERK1/2 protein level for both normal MDA-MB-231 cells and MDA-MB-231 cells/R2 cells was same at the 0h cisplatin treatment. After 1hour to 6 hours of treatment of normal MDA-MB-231 cells, the ERK level increased slightly on the other hand, the ERK1/2 level increased more strongly from 1hour to 6 hour treatment in MDA-MB-231/R2 cells. In both cell lines the 44 kDa band became stronger. The top arrow indicates 42 kDa and the lower arrow indicates 44 kDa. β-actin was used as a loading control [Figure 5.28D].

5.4.3 NF –kappa-B

The upper arrow indicates Phospho- IKK-α, 85 kDa and the bottom arrow indicates Phospho –IKK-β, 87 kDa [Figure 5.29A]. Phospho-IKK-α level of normal MDA-MB-231 cells neither increased nor decreased in normal MDA-MB-231 cells in treated timepoints. However the Phospho-IKK-α level increased in MDA-MB-231 "R2" cells. The phospho –IKK-β level increased after 1 to 6 hours of cisplatin treatment in normal MDA-MB-231 cells. In contrast, Phospho–IKK-β level decreased in MDA-MB-231 "R2" cells. At the 0 hour, Phospho-IKKα/β levels were lower in MDA-MB-231 "R2" cells.

The arrow indicates the 40 kDa protein [Figure 5.29], Phospho-IκB-α. Phospho-IκB-α is phosphorylated by-IKKα/β. The phospho-IκB-α level decreased in both normal MDA-MB-231 and MDA-MB-231/R2 cells. However, starting signal was more intense in normal MDA-MB-231 cells than MDA-MB-231/R2 cells [Figure 5.29B lane 1 and

5]. Once IκB-α is phosphorylated, it is targeted for degradation and NF-kappa-B is released therefore increased level of phosphorylated IκB-α means more NF-kappa-B release. Therefore in MDA-MB-231/R2 cells decreased level of NF-kappa-B signaling can be seen. MDA-MB-231 cells have constutive NF-kappa-B activation and as the cells become cisplatin resistant this activation level is lowered.

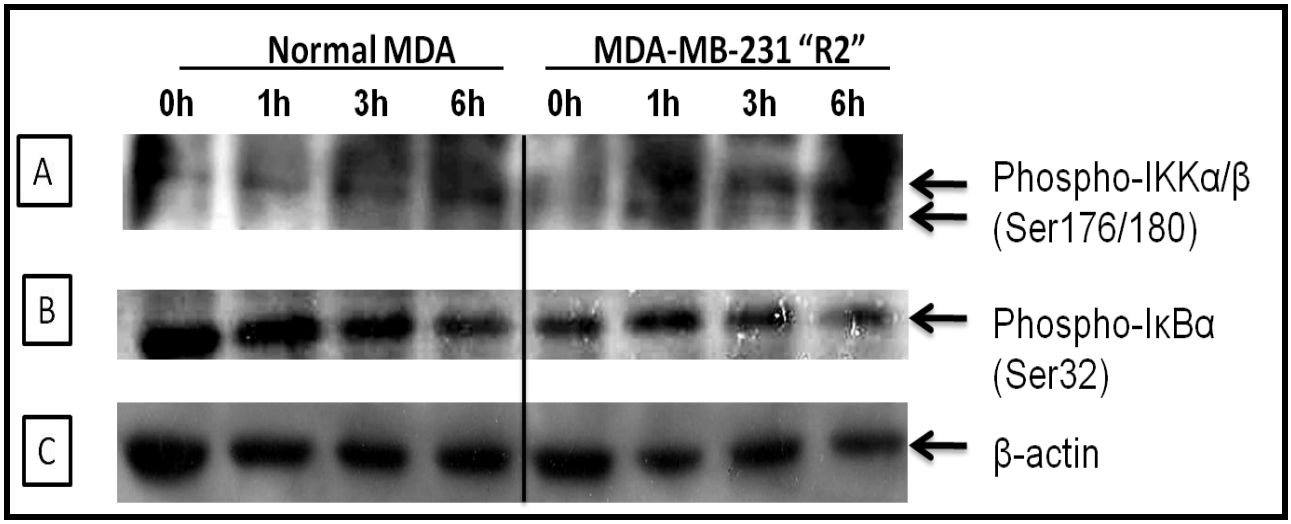


Figure 5. 29: NF kappa B entities. A: Phospho-IKKα/β (Ser176/180), B: Phospho-IκBα (Ser32), C: β-Actin.

5.4.4 Foxo3a and Bcl-2

Active forkhead members act as tumor suppressors and are involved in tumorigenesis. Increased expression of the Foxo family causes cell death. In this study, Foxo3a expression decreased in MDA-MB-231 "R2" cells slightly [Figure 5.30A]. But after 1 to 6 hours of cisplatin treatment the level of Foxo3a in both cell lines did not change. The upper arrow indicates 97 kDa; arrow below corresponds to 82 kDa. Both band intensities did not change over time.

Bcl-2 is an anti-apoptotic protein that protects cell against cell death stimuli. The level of Bcl-2 is constant over time in both normal MDA-MB-231 and MDA-MB-231 "R2" cells [Figure 5.30B]. However starting level of Bcl-2 was higher in MDA-MB-

231 “R2” cells. These increments can reason that; cells are protecting itself from cisplatin caused cell death. Arrow in Figure 5.29B indicates 26 kDa.

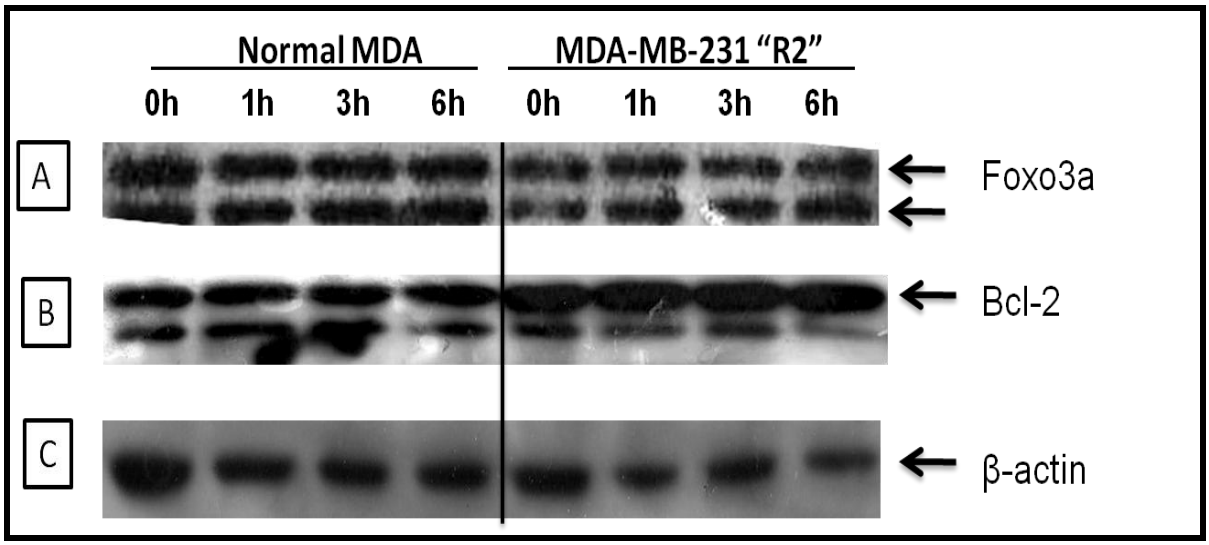


Figure 5. 30: Immunoblotting of different proteins. A: Foxo3a, B: Bcl-2 and C: β-actin as a loading control.

6. DISCUSSION AND CONCLUSION

Cisplatin is a chemotherapy agent used in the treatment of a variety of neoplasms and versatile cancers. Cisplatin has propensity to generate DNA damage, suppress DNA synthesis and RNA transcription, affect the cell cycle progression, and provoke apoptosis in cancer cells by impeding with the resistance mechanism [155]. It becomes activated inside the cell by substituting two *cis*-chloro "leaving" groups with water and subsequently engages to DNA with a covalent bond, results DNA adducts [125]. The oxidative and apoptotic effects of cisplatin are attributed to several properties of this prominent reactive intermediate. In some cases, after cisplatin-DNA complex formation, cells are arrested at G2 phase [124]. Pathway linking Pt-DNA adducts to apoptosis stays unrevealed.

In recent years, cisplatin resistance (CR) is found to be existed as a natural mechanism against drug in cells. CR can be either intrinsic resistance or can be acquired resistance. Acquired resistance can be gained during cycles of application of the drug along the therapy by some effected organs with cancer such as ovarian, lung, colorectal. CR has a significant contribution to the pathophysiology of cancer progression, especially ovarian, testicular and breast cancer development. Despite the fact that, cisplatin is the most frequently studied drug, little is known about the drug and resistant mechanism. It is an astonishing fact that: there is no comprehensive study done on generating acquired cisplatin resistant breast cancer cell lines: MCF-7 and MDA-MB-231 cells also detailed pathway analysis. Within this study, a newly established cisplatin resistant cell lines so called MCF-7/R6 and MDA-MB-231/R2 cells were investigated by means of genetic and proteomic level. Throughout the cell death analysis experiments, Annexin-V coupled with FITC dye was applied to the cells subsequently flow-cytometry method was used and resistant cell line was chosen accordingly. MCF-7 cells which have an apoptosis rate between 30-35% [10] started to undergo apoptosis

between 10-15% after being cisplatin resistant. And MDA-MB-231 cells which have an apoptosis rate between 20-25%, has started to have a new apoptosis rate, 7-10%.

Of the numerous methods applied for generating drug resistance cell lines, our unique method is particularly promising because of application of the 2 graded drug doses. In this method cells are treated with the 2 different drug doses; first dose is the subtoxic low concentration dose (1 μM) which is given to the cells for long period (4 day) and second dose is the toxic high concentration dose (30 μM) which is given to the cells for short period (4 hours). In the conventional methods: Treatments are done with the only one dose for constant period of time or concentration of the treatment starts with the low dose and then increases to the dose that is exceeding the clinically relevant dose. The method we used comes through to the result rapidly by means of resistance at relatively high rate.

Recovery time required for MCF-7/R cells did not give relevant information about resistance status. On the other hand, MCF-7 /R6 cell morphology which was shifting towards to morphology of intrinsic resistant MDA-MB-231 cells was elucidative. Hardness of trypsinization was also pointing cytoskeletal and attachment changes albeit. MCF-7/R6 cells were also cryo-resistant since after thawing of cells and treating cells with cisplatin, apoptosis rate did not differed compared to initial response.

There are several reports indicating that; some drug-specific resistant cell lines show cross-resistance to other drugs in addition to drug we are currently treating with. We wanted to question if this hypothesis was true for MCF/R cell line. To test this, we examined 2 different classes of anti-cancer agents which were Docetaxel and Paclitaxel on MCF-7/R6 for the same treatment period of cisplatin. Our results clearly indicated that; Cisplatin resistant MCF-7 cells were also resistant to Docetaxel and Paclitaxel at 5% and 16% increase at death rate respectively. Further investigations done in microarray showed that; Responsible multi-drug resistance genes (MDR/TAP) were identified in this study and they were: SLC31A1, ABCB6 and ABCB9. Gene transcription level increased at 2 fold in the resistant cell line at basal state. Consequently, transcription of these 3 gene level can be used as a drug resistance marker in clinics for MCF-7 cells.

Previous studies reported that; many resistant cell lines show altered drug uptake and processing in pathways. Glutathionine S-transferases (GSTs) are important enzymes that catalyze conjugation of glutothionine to drug in order to make compound more anionic and eventually to pump the drug out of the cell more easily by ATP dependent glutothionine S-conjugate export (GS-X) pump namely, MRP1 and MRP2 [126]. Our results demonstrated that; glutathione S-transferase pi (GSTP1) is the version of overly transcribed gene encoding drug exporter channel. In addition to that transcription of microsomal glutathione S-transferase 1 (MGST1), metallothionein 1F (MT1F) and metallothionein 1H (MT1H) increased nearly 2 fold in MCF-7/R6 cells. Consequently cisplatin is exported to outside of the breast cancer cell by elevating expression level of these genes. Microsomal glutathione S-transferase 1(MGST1) is a novel gene found in MCF-7 /R6 cells and transcribed 2 times more in this cell. Although much has been published on the drug resistant marker genes activated in drug resistant cells, the discovery of which version of drug resistant genes were transactivated in drug resistant breast cancer cells were firstly identified in this thesis.

Further evidence for cisplatin resistant cells were also resistant to the other drug anti-cancer agents were provided from the results obtained by pre-incubating the non-resistant MCF-7 cells with serum of resistant cells prior to the anti-cancer agent treatment. Serum of resistant cells was obtained after 4 days of passaging MCF-7/R6 cells. Serum was divided into 2 groups; one group was filtered with 0.22μm filter, and the another group was not filtered. Filtered serum was able to inhibit apoptosis by 20% decline at death rate of normal MCF-7 cells. Non-filtered serum caused more cell death except for Docetaxel treatment. Due to the activation of cell survival pathways, filtered serum increased cell survival. So the factor causing this effect can be a ligand found in the serum which activates cell survival signaling mechanisms such as NF-kappa-B. Later in this study, NF-kappa-B is understood to be constutively active by selecting p50 subunit dimerization.

In the following part of the study, microarray was conducted to have aspect of global gene transcription data. Illumina Human WG-6 BeadChip array was used for gene expression analysis. MATLAB 2009b was used for statistical analysis. Explanation of the written code was done in detail in the result section. This code found 10 marker genes among 48,803 genes. Genes are PINK1, TPCN2, P2RY2, CCNG1,

GSK3B, FLAD1, KEAP1, ACTA2, RWDD2A and YRDC. Latter 2 genes are not characterized well until now. But former 8 genes are related to; mitochondria metabolism, Ca^{++} metabolism, G protein coupled receptor, cell cycle, WNT pathway, FAD co-enzyme, cytoprotective, cytoskeleton respectively. These 10 genes intersect at some points. ACTA2 and GSK3B genes intersect at non-canonical WNT pathway. CCGN1 gene and WNT pathway intersect at p21 and p53 gene. TPCN2 and P2RY2 both encodes for channel and receptor that may be responsible for efflux of drug to outside of the cell. Both PINK1 and KEAP genes are overexpressed in drug resistance cells. FLAD1 gene is related to DNA and it intersects with p53 gene. So all 10 genes are related to each other and they may establish a new pathway that is directly related to cisplatin resistance in MCF-7 cells.

A very well documented transcription factor, NF-κB, has emerged as a key mediator of a wide variety of cellular processes which is involving; growth, differentiation, and survival [131]. Overexpression of IκB-α or IκB-β which are upstream molecule of NF-κB reduces the constitutive activity of the NF-κB/CAT reporter gene in the MDA-MB-231 cell line. Thus, reduced expression of either IκB-α or IκB-β is sufficient for constitutive activation of NF-κB [55]. In this part of the study, we tried to differentiate if NF-κB binding to DNA is constitutive or inducible and also we tried to find out canonical or non- canonical NF-κB pathway is activated. Transactivation properties of NF-κB in acquired drug resistant and in normal adenocarcinoma breast cancer cell lines (MCF-7) were also checked by treating cells with cisplatin. A direct positive correlation of constitutive NF-κB-DNA binding in MCF-7/R6 and early transactivation of NF-κB in acquisition of drug resistance properties of a breast cancer cell line is seen in EMSA. Canonical NF-κB pathway activation was documented in MCF-7/R6 cells by Immunoblotting experiment.

Before activation of NF-κB subunits, they can undergo either homo- or hetero-dimerization in the cytoplasm in this way it that binds to promoter regions which is decameric sequences of both cellular and viral genes [45-48, 72]. In detailed analysis, we have shown precisely that activated NF-κB contains mostly subunits of p50 subsequent to p65 which in turn induces p50-p50 or p50-p65 dimmer formation and subsequently activates transcription of cell survival target genes and in turn cell survival mechanism albeit. Recently, Zhou et al. suggested that the p50 transactivation in the

nucleus of ER positive breast cancer cells might be used as a prognostic marker in a subset of patients [132,133]. As part of the non-canonical NF-κB pathway activation, the MAP3K14-activated CHUK/IKKA homodimer phosphorylates NF-κB2/p100 associated with Rel B. It induces proteolytic processing and NF-κB-RelB-p52 complex formation. In our study, gene expression of both CHUK and MAP3K14 also NFKB2 levels increased in resistant cell.

Gene transcription level sometimes does not directly correlates to protein level. For instance, gene expression level of IκB-α and IκB-β is increased in MCF-7/R6 cells however at protein level both IκB started to be degraded. Eventually level of them decreased whereas Phospho- IκB level is increased because phosphorylation of IκB ends with degradation. In immunoblotting, low level of IκB and high level of P- IκB was seen in addition to that high P-IKK α /β level at basal state of resistance cell tells us canonical NF-κB pathway become constutively active in MCF-7/R6 cells. The same inverse correlation was seen in MAPK gene and protein expression level. JNK and ERK1/2 activation is decreased in MCF-7/R6 cells at protein level whereas JNK and ERK1/2 gene expression level increased in the same cell line.

Increasing of prolactin receptor level and declining of estrogen receptor level downregulates p53 gene [135]. And p53 gene works inversely with the p21 gene. Prolactin receptor also activates both Jak–Stat and MAPK pathway [136] which was the case also in this study. Increased expression of prolactin and decreased expression of ER is seen here and this results P53 gene downregulation and p21 gene is transactivation. In intrinsic cisplatin resistant cell which isMDA-MB-231, p53 is mutant and found to be very low level.

ER represses NF-κB pathway in normal MCF-7 cells and also Estrogen receptor (ESR1 gene) loss cause AP-1 activation but this finding remains controversial [55, 127-130]. As claimed by many authors, our data showed that ER (ESR1 and GPER), ER binding site (EBAG9) and Estrogen receptor regulated inhibitor (RERG) RNA level is declined to half compared to control. Supported by literature, this caused NF-κB activation being constutive. ER receptor is absent in MDA-MB-231 cells and there is an analogy seen in MCF-7/R6 cisplatin resistant cell. ER receptor expression declination

can confer that; one of the important resistance mechanism element candidates should be that.

Foxo-1 is phosphorylated by AKT1 which is an insulin stimulated protein. IGF1 rapidly induces phosphorylation of Ser-256, Thr-24, and Ser-319. When Foxo-1 is phosphorylated from Ser-256 residue, affinity to DNA decreases and this promotes the phosphorylation of Thr-24, and Ser-319, permitting phosphorylation of Ser-322 and Ser-325, probably by CK1, and this leads to nuclear exclusion and loss of function. Foxo-1 is phosphorylated upon DNA damage, probably by ATM or ATR [111-115]. When Foxo-1 is silenced with siRNA technology, apoptosis of fibroblast is reversed at 76% [116]. In our study, Foxo-1 level is decreased both at gene and protein level. Phosphorylation of Foxo-1 at Ser 256 and Thr-24 is seen obviously in cisplatin resistance phenotype. As Foxo-1 phosphorylated, total Foxo-1 level is decreased.

Pro-apoptotic members whose levels are down in MCF-7/R6 cells are; Apaf-1, BID, Caspase-6, CFLAR, CRADD, TUSC1, TUSC4, XAF1 and FOXO-1, FOXO-4. The level of BCL, DAP, DAPK and ATG are increased in MCF-7/R6. We also confirmed Apaf-1 declination and Aven and XIAP increment by Immunoblotting.

NFAT is a transcription factor that is activated after Ca^{+2} rise in the cell. Level of NFATc3 was checked by immunoblotting and increment was seen. TPCN2 and NFATc3 gene levels increased in MCF-7/R6 cells.

Generally, SAPK/JNK and p38 are key mediators of stress and inflammation responses evoked by a variety of physical, chemical and biological stress stimuli on the other hand ERK 1/2 cascade is mostly induced by growth factors [134]. In normal MDA-MB-231 cells, there is no constitutive activation of MAP kinases. Activation of MAP kinases in response to cisplatin is already known. But in MDA-MB-231 “R2” cell line, there is constitutive activation of all MAP kinases and also early activation of all 3 kinases; p38, ERK1/2 and JNK when cisplatin is given 0 hour to 6 hours. On the other hand, MAP kinases in MCF-7 behave individually. JNK and ERK1/2 activation was lowered but p38 activation was heightened in MCF-7/R6 cell line.

Also Foxo3a which is a tumor suppressor protein was downregulated and Bcl-2 which is anti-apoptotic protein was upregulated in MDA-MB-231 “R2” cell line.

Phospho-IKKα/β and in parallel to this Phospho-IκB-α level is downregulated after 0 hour to 6 hours cisplatin treatment in MDA-MB-231 “R2”cells.

7. FUTURE STUDIES

Since filtered serum of MCF-7/R6 cells gained normal cell a resistance status, filtered serum studies should be progressed and serum should be used on normal MCF-7 cells but this time, protein isolation and cross resistance should be checked.

Protein-protein interaction of 10 genes which resulted from microarray analysis should be study more; there may be new pathway links which will establish and they may be drug resistance marker genes for MCF-7 type breast cancer. One output can be; NFATc2 and TPCN2 may be correlated. Because they are both activated through Ca^{+2} metabolism.

Foxo-1, Foxo-3, Foxo-4 needs to be examined at protein level and for protein-protein interaction because their level changed significantly between normal and resistant MCF-7 cells.

NF-κB activation in MDA-MB-231/R2 needs to be examined in more detail. Because some increments and decline of some subunits was inconsistent, when they differentially thought. Outcome of examined subunits may result downregulation of canonical NF-κB pathway.

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APPENDIX A

CHEMICALS

(in alpabetical order)

Name of chemical	Supplier Company	Catalog number
2-Mercaptoethanol	Fluka, Switzerland	63700
Acrylamide/Bis-Acrylamide	Sigma, Germany	A3699
Agarose	AppliChem, Germany	
Ammonium persulfate	Sigma, Germany	A3768-25G
Bradford Solution	Biorad Inc., USA	500-0006
CM-H_2DCF-DA	Fluka, Switzerland	WA12820
EDTA	Riedel-de Haén, Germany	27248
Ethanol	Riedel-de Haén, Germany	32221
Glycine	Molekula, UK	M10795955
HCl	Merck, Germany	100314
Hepes	Molekula, UK	M55704197
Isopropanol	Merck, Germany	1009952500
KCl	Fluka, Switzerland	60129
KH_2PO_4	Riedel-de Haén, Germany	4243
Liquid nitrogen	Karbogaz, Turkey	
Methanol	Sigma-Aldrich	24229
Na_2HPO_4	Merck, Germany	1065791000
NaCl	Duchefa Biochemie	S05205000
NP-40	Sigma, Germany	I3021
Penicilin/Streptomycin	PAN, Germany	P06-07100
Phosphatase Inhibitor coctail tablet	Roche, Germany	4906837001
PMSF	Amresco®,USA	0754-25G
Protease Inhibitor coctail tablet	Roche, Germany	4693124001

Sodium Dodecyl Sulphate	Sigma, Germany	L4390
TEMED	Sigma, Germany	T7024-100ml
Tris	Molekula, UK	M11946779
Trypsin/EDTA	PAN, Germany	P10-0231SP
Tween 20	Molekula, UK	18945167

APPENDIX B

ANTIBODY

(in alpabetical order)

Nameo of Antibody	Supplier Company	Catalog number
Annexin V-FITC	Alexis Biochemicals	ALX-209-250-T100
Anti p50 ab		
Anti p65 ab		
Anti-Apaf-1 ab	Cell Signal Tech., USA	5088
Anti-Aven		
Anti-Bcl-2	Cell Signal Tech., USA	
Anti-Foxo1 ab	Cell Signal Tech., USA	
Anti-Foxo3a ab	Cell Signal Tech., USA	
Anti-Foxo4 ab	Cell Signal Tech., USA	
Anti-IKK-ε ab	Cell Signal Tech., USA	
Anti-IκB-α ab	Cell Signal Tech., USA	
Anti-mouse IgG HRP ab	Cell Signal Tech., USA	7076
Anti-NFATc3 ab		
Anti-p21 ab	Cell Signal Tech., USA	2946
Anti-p53 ab	Cell Signal Tech., USA	9282
Anti-p-ERK 1/2 ab	Cell Signal Tech., USA	4376
Anti-p-Foxo1 ab	Cell Signal Tech., USA	

Anti-p-Foxo1 ab #2	Cell Signal Tech., USA	
Anti-p-IKK-α/β ab	Cell Signal Tech., USA	
Anti-p-IκB-α ab	Cell Signal Tech., USA	
Anti-p-JNK ab	Cell Signal Tech., USA	9251
Anti-p-NFκB/p65 ab	Cell Signal Tech., USA	
Anti-p-p38 ab	Cell Signal Tech., USA	4631
Anti-rabbit IgG HRP ab	Cell Signal Tech., USA	7074
Anti-XIAP ab	Santa Cruz Biotechnology Inc., USA	
Anti-β-actin ab	Cell Signal Tech., USA	4967

APPENDIX C

MOLECULAR BIOLOGY AND CELL CULTURE MATERIALS

Name of material	**Supplier Company**	**Catalog Number**
ECL Advance Chemiluminescence	Amersham Biosciences, UK	RPN2135
Blocking agent	ECL Advanced ™ blocking agent	CPK1075
Hyperfilm ECL	Amersham Biosciences, UK	RPN2103K
Page Ruler Prestained Ladder	Fermentas, Germany	#SM1811
PVDF membrane	Roche, Germany	3010
Dulbecco's Modified Eagle Medium	Pan Biotech, Germany	
Fetal Bovine Serum	PAN, Germany	
Cisplatin	Sigma, Germany	
Developer Solution	Agfa, USA	
Bradford dye		
Whatman Papers		
Trypsin 0,25 %/EDTA 0,02 % in PBS with Phenol red		P10-019100
Trypsin 0,05 %/EDTA 0,02 % in PBS with Phenol red		P10-023100SP
2X RNA Loading Dye	Fermentas, Germany	#R0641

APPENDIX D

EQUIPMENTS

Name of material	Supplier Company
Autoclave:	Hirayama, Hi-Clave HV-110, JAPAN
Balance:	Sartorius, BP211D, GERMANY
Centrifuge:	Eppendorf, 5415D, GERMANY
	Eppendorf, 5415R, GERMANY
	Kendro Lab. Prod. Heraues Multifuge 3L, GERMANY
Deepfreeze:	-70^{0}C, Kendro Lab. Prod. Hearus Hfu486 Basic, GERMANY
	-20^{0}C, Bosch, TURKEY
Distilled Water:	Milipore, MiliQ Academic, FRANCE
Electrophoresis:	Biorad Inc., USA
Flow Cytometry:	BD Biosciences FACS Canto^{TM}, USA
Ice Machine:	Scotsman Inc., AF20, USA
Incubator:	
Laminar Flow:	Kendro Lab. Prod. Heraues, HeraSafe, HS12, GERMANY
Magnetic Stirrer:	VELP Scientifica, ARE Heating Magnetic Stirrer, ITALY
Microliter Pipette:	Eppendorf, Research
Microwave Oven:	Bosch, TURKEY
pH meter:	WTW, pH540 GLP MultiCal®, GERMANY
Power Supply:	Biorad, PowerPac 300, USA

	Wealtec, Elite 300, USA
Refrigerator:	Bosch, TURKEY
Shaker:	Stuart® Gyratory rocker, SSL3, USA
Thermocycler:	Eppendorf, Mastercycler Gradient, GERMANY
Water Bath:	Huber, Polystat cc1, GERMANY
Gel Dryer	E-C Apparatus Corp. EC355, USA
Hemacytometer	
Cell culture products	TRP, SWITZERLAND
Transfer apparatus	Biorad, PowerPac 300, USA
Gel documentatiton	UVITEC, UVIdoc Gel Documentation System,UK BIO-RAD, UV-Transilluminator 2000, USA