Title: Chemotherapy response and the role of the WWOX tumour suppressor

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CHEMOTHERAPY RESPONSE AND THE ROLE OF THE WWOX TUMOUR SUPPRESSOR

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By

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CHEMOTHERAPY RESPONSE AND THE ROLE OF THE WWOX TUMOUR SUPPRESSOR

ABSTRACT

Paclitaxel is a widely used chemotherapy drug for the treatment of multiple cancers. Paclitaxel works by inhibiting the disassembly of microtubules. Besides the role of paclitaxel in mitotic arrest, many studies suggest that paclitaxel could initiate apoptosis by activating ER-stress pathways. Cisplatin, another chemotherapy drug is also used for the treatment of solid tumours. It works by forming adducts with the structure of DNA leading to cell cycle arrest, DNA damage and apoptosis. These two chemotherapy drugs have different mechanisms of killing tumour cells, but they are often combined to give greater effect. WWOX is a tumour suppressor in many cancers. It has been shown that WWOX promotes apoptosis and suppresses in vivo tumorigenesis, but its function remains unclear. Here in my study I hypothesize that ER-stress is an important part of paclitaxel response in cancer cells, and the WWOX tumour suppressor is important in mediating paclitaxel induced ER-stress response. I also hypothesize that WWOX is important in mediating the apoptotic response to cisplatin. My results show that paclitaxel could not induce ER stress in multiple cancer cells lines, and WWOX did not affect the cytotoxic action of paclitaxel. I show some evidence that ER-stress may influence survival in ovarian cancer patients, but this is independent of the effect of WWOX expression. I show that WWOX is not important for cisplatin response since cisplatin suppresses the expression of WWOX. This effect may relate to its location at a common fragile site since cisplatin also down-regulates another fragile site-associated gene FHIT. However, I also show the apparent involvement of NF-kB2 and PAR2 transcription factors in the down regulation of WWOX. These findings increase our understanding of the unintended effects of chemotherapy treatments.

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At this time I think of my parents whose selfless sacrificial life and their great efforts and prayers enabled me to reach the present position in my life. I would like to thank my wife for her unconditional support and help.

Finally I would like to dedicate all my work to my Late Father, who was a great role model for me in my life. The greatest gift that my father gave me was, he believed in me. I will always miss his guidance and support in my life.

List of abbreviations

5-FU - Fluorouracil

ASK1- Apoptotic signalling kinase 1

ATF6 - Activating transcription factor 6

ATP - Adenosine triphonspahte

ATR - Ataxia-telangiectasia and Rad3 Related

Bcl - B-cell lymphoma

BUB - Budding uninhibited by benzimidazoles

cDNA - Complementary deoxyribonucleic acid

CFS - Common Fragile site

DMF - Dimethylformamide

DMSO - Dimethyl sulfoxide

EGFP - Enhanced green fluorescent protein

eIF2α - Eukaryotic translation initiation factor 2 on the alpha subunit

ERAD - Endoplasmic reticulum associated

ERK - Extracellular signal regulated kinase

ER Stress - Endoplasmic reticulum-stress

FBS - Fetal bovine serum

FDA - Food and drug administration

FHIT - Fragile histidine triad

FIGO - The international federation of gynaecology and obstetrics

GRP-78 - Glucose regulated protein

IHC - Immunohistochemistry

IRE1 - Inositol requiring enzyme 1

JNK - Jun amino terminal kinases

kDa - Kilo Dalton

KO - Knockout

LOH - Loss of heterozygosity

MAP4 - Microtubule associated protein 4

MAPK - Mitogen activated protein kinases

MDM2 - MOUSE DOUBLE MUTANT 2

MFSD2A - Major facilitator superfamily domain containing protein 2

mRNA - Messenger RNA

ND - No drug

NF-Kb - Nuclear factor kappa B

NLS - Nuclear localisation sequence

OS - overall survival

PBS-T - Phosphate buffer saline-tween

PERK - Protein kinase like ER kinase

PFS - Progression free survival

QRT-PCR - Quantitative real time polymerase chain reaction

SDR - Short-chain dehydrogenase/reductase

SEM - Standard error mean

SRB - Sulforhodamine B

TBP - TATA binding protein

TCA - Trichloro acetic acid

TCGA - The cancer genome atlas

TNF - Tumour necrosis factor

TRAF2 - TNF receptor-associated protein

TVS - Transvaginal ultrasonography

UPR - Unfolded protein response

UV - Ultraviolet

VCP - Valosin containing protein

WT - Wild type

WWOX - WW Domain Containing Oxidoreductase

XBP-1 - X-box binding protein

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Chapter 1 Introduction

1.1 FRA16D and WWOX

Common fragile sites (CFS) are large regions of genomic instability which are present in all individuals. These instable regions are distributed throughout the genome (Arlt et al., 2003; Smith et al., 1998). The second most frequently expressed common fragile site in human genome is FRA16D [16q23.2] (Sutherland and Richards, 1995). Many types of cancer show deletion of this genomic region (Hansen et al., 1998; Balsara et al., 2001). Paige et al., (2000) first cloned this chromosomal region FRA16D and reported that there is a 700-kb physical map of a region of 16q23.2 which is homozygously deleted in multiple cancers and spans the CFS FRA16D. Characterization of FRA16D and its involvement in multiple myelomas was reported by Krumel et al., (2000). They showed FRA16D exhibited breakages within a region of 2.0 Mbs. It was also demonstrated 16q23.2 region spanned five multiple myeloma translocation breakpoints. The genomic region 16q23.3-24.1 (1.0Mb) was named as WWOX by Badnarek et al., (2000). The structure of WWOX shows it has an open reading frame 1245 bp long and encodes a 414-amino acid protein. WWOX consist of two N-terminal WW domains (first domain consists of 17-49 amino acids and second domain has 58-90 amino acids), a C terminal short chain dehydrogenase reductase (SDR) domain and a nuclear localization sequence (NLS) between two WW domains (figure 1.1). Of the two N-terminal WW domains, the first domain is required for classical WW-PPXY interaction. The SDR domain associated proteins are needed for oxidation or reduction of lipids hormones, sugars and alcohols (Chang et al., 2007).

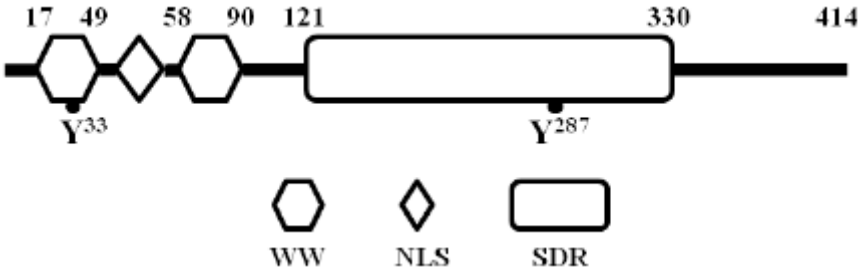


Figure 1.1: Domains and phosphorylation of human WWOX (WW domain containing oxidoreductase) protein. WW, WW-domains; NLS, nuclear localisation signal; SDR, short chain dehydrogenase domain; Y, tyrosine residues at amino acid positions 33 and 287 (Li et al., 2014)

1.1.1 WWOX and spliced variants

Human WWOX mRNA has at least eight transcripts (figure 1.2) (Chang et al., 2003) but currently it is unknown if all these 8 transcripts can be translated into proteins. The presence of wild type WWOX 46-kDa protein is well known (Badnarek et al., 2000). There are 9 exons in human WWOX gene which encode a full length WWOX. The other seven transcripts are formed by alternate splicing indicating the isoforms of WWOX protein. The N terminal WW domain is encoded by exons 1-4, whereas SDR domain is encoded by exons 4-8. Tyr33 (y33) and Tyr287 (Y287) are the two known phosphorylation sites. For hormones or substrate binding there is a conserved catalytic NSYK motif (N232, S281, Y293 and K297) on SDR domain. Within the second WW domain between amino acid 65-68, there is a conserved caspase-recognition motif QETD (Chang et al., 2001).

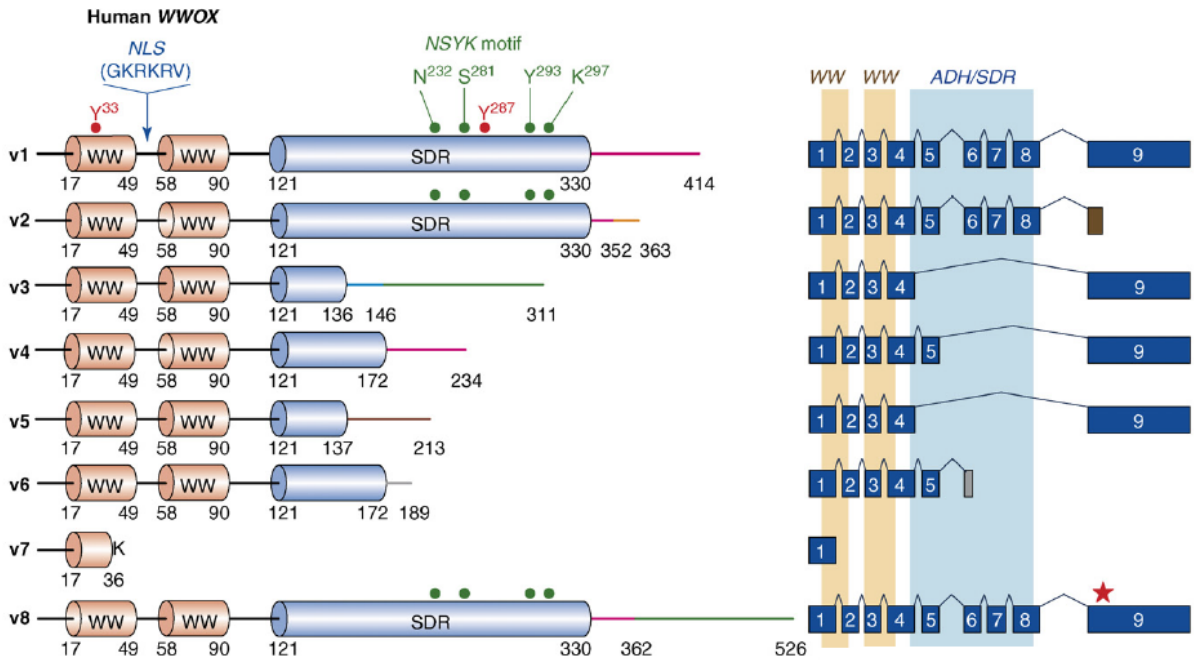


Figure 1.2: Human WWOX (WW domain containing oxidoreductase) gene and its sliced variants. WW domains are shown in gold and the highly conserved short chain dehydrogenase (SDR)/alcohol dehydrogenase domains are shown in blue colour. A nuclear localization sequence (NLS) between two WW domains. Tyr33 (y33) and Tyr287 (Y287) are the two known phosphorylation sites. For hormones or substrate binding there is a conserved catalytic NSYK motif (N232, S281, Y293 and K297) on SDR domain.v1 wild type WWOX gene (46kDa), v2 (41kDa) shows partial deletion of exon 9 and a different c terminus (orange), v3 (35kDa) deletion of exon 5-8, v4 (26kDa) deletion of exon 6-8, v5 (24kDa) exon 5-9 deletion, v6 (22kDa) has an alternative exon 6, v7 (4kDa) exon 2-9 deletion, v8 (59kDa) TG-deletion at exon 9 (red star) (Chang et al., 2007).

1.1.2 Subcellular and tissue distribution of WWOX

Some studies suggest WWOX is localized in mitochondria while other suggests it is localized in other parts of the cell. The ectopic expression of murine Wwox has been observed in mitochondria by tagging it with EGFP (green fluorescence protein). Monkey kidney fibroblasts (COS7) and lung cells (H1299) were tagged with ECFP (cyan fluorescence protein) has shown localization of Wwox in mitochondria. Rat liver cells also showed the presence of endogenous Wwox in purified mitochondria (Chang, 2002; Chang et al., 2001). In a study of normal human breast cells (MCF-10F) by Badnarek et al., (2001), WWOX

expression was observed in Golgi apparatus. The reason for the different subcellular localisation in these studies is unclear, but may be related to differences in cell lines, species, transfection method, or fusion proteins used. In another study by Watanabe et al., (2003) WWOX was observed in non-cancerous epithelial cells, mammary gland cells, prostate epithelium and follicular cells. Mammary gland cells showed localization of WWOX in nucleus, whereas epithelium cells showed WWOX is localized in cytoplasm. The switching of WWOX between nucleus and cytoplasm was observed in human fibroblast KMS-6 where in less confluence cells WWOX was observed in mitochondria and when cell reached full confluence, WWOX was observed in nucleus.

Chang et al., (2005) observed the effects of hormones on the translocation of WWOX in COS7 fibroblasts and MCF7 breast cells. COS7 cells showed nuclear translocation of WWOX due to estrogen or androgen, whereas MCF7 cells showed WWOX in peri-nuclear area and did not responded to androgen or estrogen induced translocation of WWOX. In the same study, similar observation was observed during breast cancer progression where no change in nuclear localization of WWOX was observed at hypertrophic and cancerous stages. However, prostate cancer showed 1.5-fold increase in nuclear translocation of WWOX at hypertrophic and cancerous stages (Chang et al., 2005).

Chen and colleagues while studying the role of WWOX in light-induced retinal degradation of normal mature rat cells reported the presence of a large number of Wwox positive immunogold particles near nuclear membrane and rough endoplasmic reticulum, whereas little or no Wwox was detected in Golgi apparatus or mitochondria of normal cells. Dead photoreceptors by apoptosis showed Tyr33 phosphorylated Wwox (p-wox1) was detected in large numbers of immunogold particles in damaged mitochondria and condensed nuclei of degenerating photoreceptors which shows activation of wox1 and its translocation to these organelles. Little or no Wwox was detected in Golgi apparatus of dead cells (Chen et al., 2005).

Immunohistochemical (IHC) analysis show WWOX protein is ubiquitously expressed in human tissues but high levels are expressed in epithelial cells and exocrine and endocrine organs such as testis, mammary and prostate glands. Strong expression of WWOX has also been observed in neuronal bodies and cerebellum. A summary of WWOX positive organs and cells is shown table 1.1. Organs negative for WWOX protein expression are shown in table 1.2. Figure 1.3 shows the detection of WWOX by western blotting. Variation in WWOX expression levels can clearly be observed.

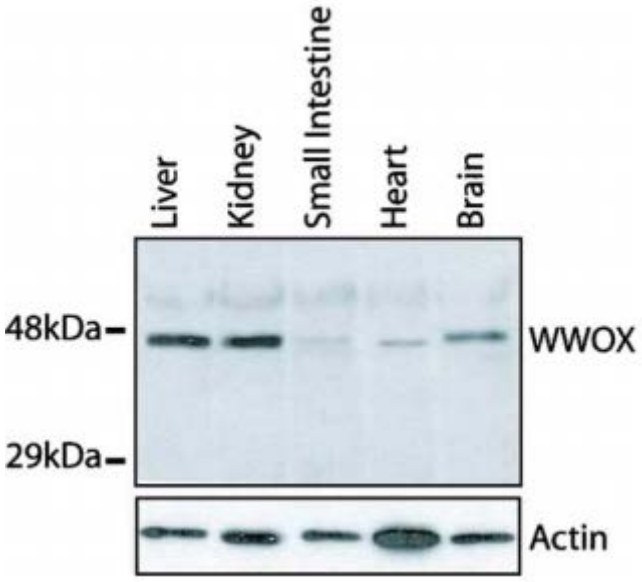


Figure 1.3: Western blot analysis of WWOX protein in human tissues. Full length WWOX protein is 46kDa. Actin shows equal loading (Nunez et al., 2006).

Table 1.1: Summary of organs showing the distribution of WWOX positive staining according to tissue and cell type (Nunez et al., 2006)

Organs	WWOX staining cell/tissue specificity	WWOX intensity
Breast	Resting ductal luminal & myoepithelial cells	++/+++
	Secretion	++
	Epithelial cells lactation changes	+++
Ovary	Ovarian surface epithelial cells (OSE)	+++
	Inclusion cyst	+++
	Walthard nest	++
	Stromal luteinized cells	++ patchy
	Stromal cells	–
Fallopian tube	Endoluminal epithelial cells	+++
	Tubal stromal cells	–
Placenta	Immature Villi, Cytotrophoblast	++
	Hofbauer cells	++
	Mature Villi, Syncytiotrophoblast	–
Uterus	Proliferative & secretory phase endometrial glands	+++
	Endometrial stromal cells	–
	Myometrium	–
Uterine	Mucus secreting cells of endocervix	++/+
Cervix	Subcolumnar endocervical cells	+++
	Squamous epithelium from exocervix	–
Bronchus	Mucus secreting cells	+++
Omentum	Cubic mesothelial cells	+
	Adipose tissue	–
Pituitary gland	Basophilic cells nests/cords	+++/++
	Acidophilic cells	++
	Chromophobic cells	+/ –
	Pituitary stalk glial cells and Herring bodies	++
Adrenal	Cortex, zona glomerulosa, zona fasciculate and zona reticularis	+++
	Medulla, Pheocromocytes adrenal medulla	+++
Liver	Hepatocytes	+++
	Intrahepatic biliar ducts	++
	Connective tissue septum	–
	Blood vessels walls	–
Gall bladder	Epithelial cells lining mucosa	++
Thyroid	Cuboideal follicular cells	++
	Flat follicular cells	–
	Para follicular cell (C cells)	++
Parathyroid	Inactive chief cells cords/sheets/pseudofollicules	+++ variable
	Oxyphil cells nests/cords	+++
Pancreas	Epithelial cells in acini	+++
	Ducts	++
	Langerhans nests	++
Salivary gland	Serous acinar epithelial cells	+++
	Mucous acinar cells	–
	Luminal ductal and myoepithelial cells	++
	Epithelial cells in interlobular ducts	++
Esophagus	Squamous epithelium	–
	Submucosal esophageal glands	–
Stomach (fundal mucosa)	Parietal cells in the isthmus and neck of the gland	++
	Chief cells in the base of the gland	++
	Tall mucus secreting epithelial cells in surface and pit	–
Small bowel	Duodenum enterocytes	++
	Jejunum and ileum enterocytes	–
	Paneth cells, granular supranuclear	++
	Enteroendocrine cells, granular infranuclear	+++
	Goblet cells	–
Colon	Goblet cells in crypts	–
	Enteroendocrine cells	+++
Cerebellum (Folia)	Purkinje cells layer	+++/++
	Molecular layer	++
	Internal granular cells neurons	+++

Organs	WWOX staining cell/tissue specificity	WWOX intensity
Cerebrum	Frontal, occipital, limbic cortex and nucleus caudate:	
	Soma and dendrites of large neurons	+++
	Soma and processes of astrocytes	+++
	Neuropil of gray matter	++
	Parietal cortex, temporal cortex and substantia nigra	+/ –
Pons and medulla	Neurons	++
Peripheral nerve	Schwann cells	–
Urinary bladder	Transitional epithelium	+++/++
Kidney	Proximal collector ducts (CD) and distal convolute duct (DCD)	+++
	Proximal convoluted duct (PCD)	++
	Parietal layer of Bowman capsule	–
	Intraglomerular mesangial cells	–
	Apherent and epherent arterioles in glomeruli (vascular tuft)	–
Prostate	Luminal cubic cells in active glands	++
	Flat cells of glands with cystic atrophy	+
	Basal cells	+++
	Prostatic urethra transitional epithelium	+++
Testis	Leydig cells in interstitium	+++
	Spermatogonias and sertoli cells in seminiferous tubules	++/+
Epididymus, rete testis and vas deferens	Basal cells and luminal epithelial cells	+++
	Basal membrane	–
	Smooth muscle in vas deferens	–
Skin	Keratinocytes	++ patchy
	Hair follicle	++
	Sebocytes	+++
	Sweat glands myoepithelial cells	++

Manual and ACIS® final scale: negative (–) = < 59, mild (+) = 60–68, moderate (++) = 69–79, strong (+++) = > 80

Table 1.2: Summary of organs negative for WWOX protein expression (Nunez et al., 2006)

Organs	WWOX staining cell/tissue specificity	WWOX intensity
Thymus	Cortical thymocytes	–
	Lymphocytes	–
Lymph node	Cortex and medulla	–
Spleen	Red pulp and White pulp	–
Lung	Alveolar lining epithelial cells	–
	Interalveolar septum	–
Aorta	Intima, muscular and adventitia	–
Soft tissue	Adipose tissue and connective tissue	–

1.1.3 WWOX: Tyr33 phosphorylation, death signalling and tumour suppression

Tumour suppressor and pro-apoptotic function of WWOX has been shown by *in vivo* or *in vitro* analysis. Over expression of WWOX induces apoptosis by cytotoxic action of TNF (Chang et al., 2001). WWOX undergoes phosphorylation at Tyr33 under stress response or apoptotic stimuli, forming a complex with JNK and p53. WWOX-p53 complex synergistically induces apoptosis by translocation to mitochondria and nuclei. However, JNK may be involved in inhibition of WWOX-induced apoptosis (Chang et al., 2003; Chang, 2002). Expression of WWOX was supressed by antisense RNA and protected apoptotic cell death from TNF, staurosphire, UV light and ectopic p53 in vitro (Chang et al., 2005). Breast cancer cells MDA-MB-435 and T47D showed inhibition of growth in soft agar by ectopic WWOX expression. WWOX expression strongly inhibited tumorigenicity of MDA-MB-435 in nude mice. Deletions of exon 5-8 or 6-8 of WWOX transcripts were observed in various carcinomas but not in normal tissues (Badnarek et al., 2001). *In vivo* tumorigenesis by loss of WWOX was demonstrated by Mahanjan et al., (2005). Activation of tyrosine kinase Ack1 in prostate cancer cells (LNCap) showed minimal growth *in vitro* but increased tumorigenesis in nude mice which was partially due to negative regulation of WWOX (Mahanjan et al., 2005). Transfection of stable Wwox in L929 cells enhanced the cytotoxicity of TNF (Chang et al., 2001). Overexpression of both domains i.e., WW and SDR induces cell death which confirms both are functional (Change et al., 2001; Chang et al., 2003). The enhanced apoptosis due to overexpression of WWOX is partially due to upregulation of p53 (pro-apoptotic) and down regulation of anti-apoptotic Bcl2 and Bcl-xL in mouse fibroblast L929 (Change et al., 2001; Chang et al., 2003). Overexpression of transfected WWOX in prostate cancer cells (DU145) reduced colony growth and overexpression of WWOX by adenoviral suppressed cell growth by inducing caspase dependent apoptosis. Ectopic Ad-WWOX expression suppressed tumorigenicity in vivo (Qin et al., 2006). Contrary to this, the study of Chang et al., (2001) showed that overexpression of Wwox induced cell death in mouse NIH/3T3 fibroblasts which was not blocked by caspase and serine protease inhibitors. This

shows the role of caspase in WWOX mediated apoptosis is elusive as shown by another study of Fabbri et al., (2005) where re-expression of WWOX by adenovirus in WWOX negative lung cancer cells (A549, H460 and H1299) activated caspase-3 and PARP cleavage but not in WWOX positive U2020 cells. These studies suggest WWOX may or may not be involved in activation of apoptotic caspases.

Gourley et al., (2009) showed that targeted deletion of Wwox in mice increased tumorigenesis and stable transfection WWOX into human ovarian cancer PEO1 cells lacking WWOX expression abolished in vivo tumorigenicity, although no correlation was observed with in vitro growth. It was further showed that WWOX expression reduced membranous integrin α3 protein bit not integrin α3mRNA. This study postulated WWOX acts as ovarian cancer tumour suppressor by modulating the interaction between tumour cells and extracellular matrix and by inducing apoptosis in detached cells.

1.1.4 WWOX binding proteins

Both domains of the WWOX (WW and SDR) are involved in binding with other proteins. Proteins bind to WW domain of WWOX via PPxY motif. WW domain of WWOX binds to p73 (a p53 homolog) by PPPPY motif (Aqeilan et al., 2004a). In another study by the same group Aqeilan et al., (2004b) other WWOX interacting proteins were investigated. Activating protein-2ɣ (AP-2ɣ) has high binding affinity with the WW domain of WWOX. AP-2ɣ family members are important transcription factors in breast carcinogenesis (Williamson et al., 1996). The genomic locus 20q13.2 contains AP-2ɣ and show frequent amplification in breast carcinoma. The findings of Aqeilan et al., (2004b) showed that oncogenic activity of AP-2ɣ was inhibited by WWOX protein by sequestering it in the cytoplasm. Verb-a erythroblastic leukaemia viral oncogene homolog 4 (ERBB4) is a transcription factor which also binds to first WW domain of WWOX (Aqeilan et al., 2005). Ezrin (a signal transducer at the cell membrane) (Jin et al., 2006) and small integral membrane protein of the lysosome/ late

endosome (SIMPLE) are binding protein which interact with first WW domain of WWOX (Ludes et al., 2004).

The recognition of proline rich motif in all above-mentioned proteins is enhanced by the Tyr33 phosphorylation in WWOX/Wwox, whereas any change in the phosphorylation of Tyr33 can inhibit the binding of these proteins with first WW domain (Aqeilan et al., 2004a; Aqeilan et al., 2004b; Aqeilan et al., 2005). Transcription of p73 and AP-2γ is blocked due to their restricted nuclear localization by ectopic WWOX (Aqeilan et al., 2004a; Aqeilan et al., 2004b). There is a competition between WWOX and YAP (WW domain-containing Yes-associated protein) for binding with ERBB4 which influence the transcriptional activity of ERBB4 (Aqeilan et al., 2005). Currently the involvement of phosphorylation of Tyr33 in binding of WWOX with PPxY motifs in ezrin and SIMPLE is not known (Jin et al., 2006; Ludes et al., 2004).

Although there is no identification of any PPxY motif in JNK, P53 and MDM2 (mouse double mutant 2), yet their interaction with Tyr33 phosphorylated WW domain has been observed and also if there is any alteration in phosphorylation of Tyr33 it inhibits the bindings of these proteins with WW domain. The binding of p53 with Tyr33 phosphorylated WWOX takes place by Ser46-Pro47 and an adjacent N-terminal proline rich region (amino acid 66-100). Without Ser46 and Try33 phosphorylation, p53 does not interact and bind with WWOX. However, no binding of ectopic WWOX with ectopic p53 was observed by Arelin et al., (2004, possibly because cells used in experiments were not under stress stimulus (Chang et al., 2005; Aqeilan et al., 2004; Chang et al., 2005; Chang et al., 2001). In a study by Sze et al., (2004) mapping analysis showed that SDR domain of Wwox binds to Tau and preventing Tau hyperphosphorylation. However, no information is available about the protein motifs which may have been involved in this Tau interaction with SDR domain.

1.1.5 Murine models of WWOX

Mouse models of tumour suppressors are useful to investigate the biomedical aspects of cancer genetics. Tumour suppression function of any gene can be proved by formation of neoplasias in knockout mouse models. Although many studies suggest the role of WWOX as tumour suppressor gene, but mouse knockout models can provide an insight of its pathological functions (Pekarsky et al., 2002).

Previously it has been shown by Aqeilan et al., (2007) that targeted deletion of Wwox gene increase spontaneously and chemically induced tumorigenesis. The same group also investigated the *in vivo* functions of WWOX for phenotypical abnormalities in Wwox-deficient ($Wwox^{-/-}$) mice. These knockout mice are significantly smaller in size, show slow growth, suffer from metabolic skeleton disorder and have a life span of 2-3 weeks. These mice show a delay in bone formation. The Wwox null (KO) pups looked almost identical to Wwox heterozygous (HET) pups and wild type (WT) pups at birth stage. However, after 3 days of birth Wwox null pups were easily recognized from HET or WT pups due to their smaller size. Apart from size, the weight of the organs was less as compared to WT mice after 14 days of birth. However, the brain, adrenal and pituitary gland of KO mice were heavier then WT. Histological examination showed no lesions were found on organs but confirmed atrophy. Further analysis of serum chemistry showed prominent hypoglycaemia, hypocalcaemia, hypocholesterolaemia, hypoproteinaemia. Expression of mRNA from kidney, brain, spleen, femur and pituitary by Affymetrix microarray showed varied expression of chromatin and cell cycle related proteins in KO mice compared with WT. KO mice are smaller in size, so metabolic bone disorder in these mice was also observed. Radiographic images of femur bone after 3 weeks of birth showed less density of bone in KO mouse compared with WR or HET. However skeletal pattern at birth showed no difference within all three types of mice. Effect of Wwox on delayed bone growth was also observed in these mice. RUNX2 is a bone marker. The results showed Wwox supressed expression of RUNX2 in osteoblasts. Breast cancers cells MDA-MB-242 lacking endogenous WWOX, were re-expressed with WWOX

and showed inhibition of RUNX2 and its target genes linked with metastasis. The findings of this study suggest Wwox is important for post-natal survival and bone metastasis (Aqeilan et al., 2008). The findings of current study were in agreement with Ludes et al., (2009) who reported Wwox null mice showed hypoglycaemia and hypocalcaemia. Bone metabolic disorders were also observed in KO mouse. In addition, to this impaired haematopoiesis, leukopenia and splenic atrophy was also observed in KO mice. However, spontaneous neoplasias were not observed. This study provided an insight about functioning of Wwox in mice.

In another study by Aqeilan et al., (2008), effect of Wwox deletion on steroidogenesis in mice was examined. Testicular abnormalities were observed in Wwox null mice. Effect of Wwox deletion during spermatogenesis and follicles development could not be observed because Wwox null pups died at juvenile age. Further the effect of Wwox on follicle stimulating hormone and luteinizing hormone was observed. The results showed significant down regulation of follicle stimulating hormone in Wwox null mice compared to WT. However, the effect on luteinizing hormone was not much, suggesting Wwox may affect functioning of gonads.

Ludes and colleagues (2007) also used a mouse model to study the role of Wwox gene expression in tumorigenesis. Homozygous gene-trap mice (*Wwox*$^{gt/gt}$) was used but it showed low levels were still detectable in some tissues, so these mice were considered Wwox hypomorphs. Deletion of Wwox proves very lethal at postnatal stage but these hypomorph mice were more viable. The results showed higher incidence of B-cell lymphomas and testicular atrophy in Wwox hypomorphs which suggest Wwox is a tumour suppressor gene (Ludes et al., 2007).

1.2 Ovarian Cancer

1.2.1 Epidemiology

Ovaries are part of the female reproductive system and ovarian cancer arises in the cells in and around ovary. It is the most lethal gynaecological cancer. Ovarian cancer can affect younger women but usually women over 50 years of age are diagnosed with this cancer. Each year in UK 7400 women are diagnosed with ovarian cancer and 53% of diagnoses cases are in women aged 65 or over. This is the 6th most common cancer in women in UK. The 5-year survival rate has improved in last few years and the current 5-year survival rate is 50% in UK. The 10-year survival rate is 35%. The data of 2014 show there were 4128 deaths due to ovarian cancer in UK (Cancer Research, UK). The increase in survival rate has been suggested due to hormonal contraceptive use, pregnancy, lactation and decreasing postmenopausal hormone use (Mosher and Jones, 2010; Collaborative Group on Epidemiological Studies of Ovarian Cancer, 2008).

1.2.2 Molecular pathology of ovarian cancers

1.2.2.1 Hereditary ovarian carcinomas

Women with family history of ovarian and breast cancer are at risk of developing the disease. About 10-15% ovarian cancer cases are due to genetic factors (Christie and Oehler, 2006). Women with the BRCA1 mutation have a risk of 40-50% of epithelial ovarian cancer and 20-30% with mutation of BRCA2. DNA repair mismatch gene such as MLH1 and MSH2 are linked with hereditary non-polyposis colon cancer (HNPCC) syndrome and are believed to be responsible for 10% cases of serous ovarian cancer due to inheritance of genes from family (Aarnio et al., 1999). Polymorphism in glutathione S transferase M1 (GSTM1) has also been linked with endometrioid or clear cell ovarian carcinomas. Some other polymorphisms such as steroid-5- alpha reductase, alpha polypeptide 2 and progesterone receptor genes are also thought to be responsible for increasing the risk of ovarian cancer (Beesley et al., 2007; Pearce et al., 2008).

1.2.2.2 Sporadic ovarian cancer

Ovarian carcinomas which are not due to hereditary reasons are thought to be sporadic in nature and 85% of ovarian cancer cases belong to this category (Christie and Oehler, 2006). Many oncogenes such as *KRAS, BRAF, PIK3CA AND CTNNB1* show mutations in various ovarian carcinomas. Mucinous carcinomas show 50% and low grade serous carcinomas show 35% KRAS mutations. Low grade serous carcinomas show 30% BRAF mutation. Surprisingly, both KRAS and BRAF mutations do not appear together in the same tumours and also do not appear in high grade serous ovarian carcinomas (Gemignan et al., 2003; Singer et al., 2003).

About 30% of endometrioid ovarian carcinoma shows beta catenin (*CTNNB1*) and Wnt mutations (Palacios J, Gamallo, 1998). About 20% of clear cell and endometrioid show mutations in phosphatidylinositol 3-kinase PI3K gene (*PIK3CA*) and it's not common in serous carcinomas (Campbell et al., 2004). 15% endometrioid ovarian carcinomas show mutation of PTEN gene and the presence of this tumour suppressor gene is not common is other types of ovarian carcinomas (Obata et al., 1998). Mutation of p53 gene has been found in 96% of high grade serous ovarian carcinomas (Quartuccio et al., 2014) while clear cell carcinomas show 8% of mutation of p53. Presence of p53 mutation has been seen in the inclusion cysts and in the patients with positive BRCA mutation (Lee et al., 2007; Medeiros et al., 2006) Microsatellite instability (MSI) affects mismatch match repair (MMR) pathway due to genetic or epigenetic factors and it appears in 12% of ovarian carcinomas (Pal et al., 2008). 10-50% of ovarian carcinomas also shows promoter hyper methylation of MLH1 gene (Gras et al., 2001).

1.2.2.3 Borderline epithelial ovarian cancer

Border line tumours (BOT also known as epithelial ovarian cancer of LMP, are primary ovarian lesions with characteristics showing malignancy but different to epithelial high grade ovarian cancers. They show no stromal invasion, good prognosis and have a five years' survival rate of more than 80% (Cadron et al., 2007; Barakat et al., 1995). The procedure of

staging BOT is like invasive ovarian cancer. Patients with border lines tumours can have pregnancy by undergoing organ preserving surgery if contralateral ovary or ovary remnant is tumour free. However, there is a risk of reoccurrence if organs are preserved but it does not affect overall survival. Removal of lymph nodes is not required if they look normal on palpation. Adjuvant chemotherapy for BOT so far is not beneficial (du Bois Ad et al., 2009; Sutton et al., 1991).

1.2.3 Traditional theories about origin of ovarian cancer

It is very important to understand the biology of a tumour for its diagnosis and treatment. It can only be achieved if the site and originating tissues of the malignant tumour are known. Historically it used to be thought that high grade serous ovarian carcinoma arose from ovarian surface epithelium and epithelial inclusion glands. Because of the lack of knowledge about a precursor in the ovary or peritoneum, it was thought that the pathogenesis of ovarian carcinoma was de novo (Vang et al., 2013). Traditionally three inter related theories have been proposed to explain the start of epithelial ovarian cancer. According to incessant ovulation hypothesis, repetitive postovulatory repair can cause cell proliferation and wounding at the ovarian epithelium which can result in genomic abnormalities such as formation of cysts which enhances the risk of carcinogenesis. The second hypothesis 'gonadotropin theory' suggests that during ovulation increased release of gonadotropin hormone by pituitary gland and constantly high concentration after menopause can affect ovarian epithelium cells causing abnormalities and increasing the risk of carcinogenesis. According to the third theory, inflammation and alteration of redox potential during ovulation and ovarian epithelium repair can pose a risk of epithelial ovarian cancer (Rodriguez et al., 2002).

1.2.4 The dualistic model of epithelial ovarian carcinogenesis

There are two types of ovarian carcinomas based on their clinical and molecular pathology. This model of dividing tumours into type I and type II is called the two pathway concept or the dualistic model of epithelial ovarian carcinogenesis. This model tried to resolve the complicated molecular genetic pathways responsible for the pathogenesis of primary ovarian carcinomas and to link these pathways with the histopathologic classification (Shih and Kurman, 2004). Table 1.3 show some major characteristics of type I and type II ovarian carcinomas.

Table 1.3: Characteristics of Type I and Type II ovarian carcinomas (Koshiyama et al., 2017)

	Type I	Type II
Behavior	Indolent	Aggressive
Genetic instability	Not very unstable	Very unstable
TP 53 mutation	Low	High
*BRCA*1/*BRCA*2 mutation	Low	High
Ki 67 proliferative index	10%–15%	50%–75%
Histological subtype	Endometrioid Clear cell Mucinous Low grade serous	High grade serous
Precursor	Benign cyst	s/o Tubal dysplasia (de novo starting)
Discover a precursor	Easy	Difficult
Incidence	Asia > Europe, USA	Europe, USA > Asia

1.2.4.1 Type I ovarian tumours

Ovarian tumours belonging to Type I are mostly slow growing indolent neoplasms. These are the tumours in which precursor lesions in the ovaries have been clearly explained (Koshiyama et al., 2014). Figure 1.3B shows the major clinicopathologic and molecular differences between type 1 and type II tumours.

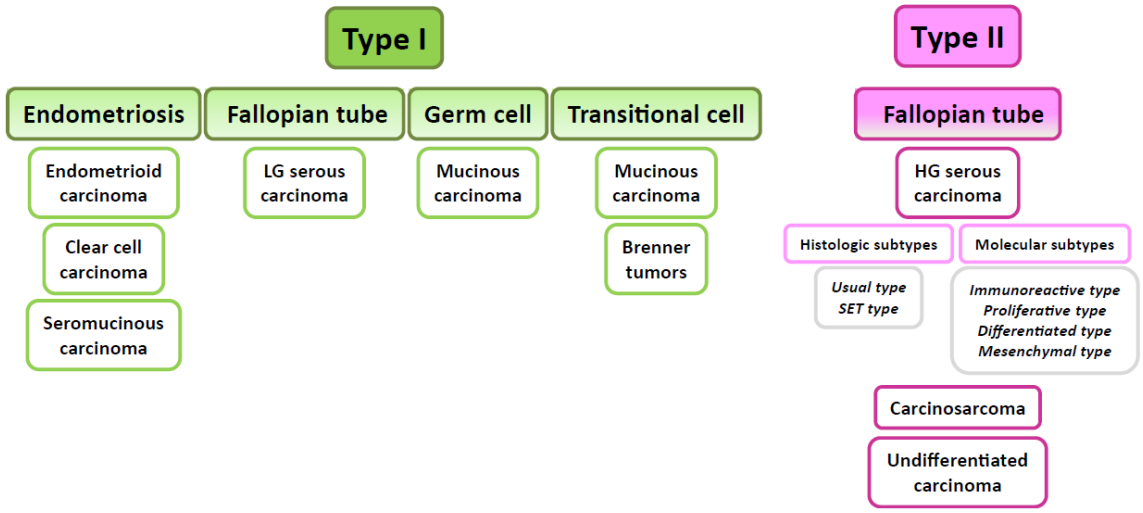


Figure 1.3B

A representation of dualistic model of ovarian carcinogenesis: Ovarian carcinoma belonging to type I arise from endometriosis, fallopian tube, germ cell and transitional cell. Ovarian carcinoma belonging to type II originate from fallopian tube. In type I endometrioid carcinoma, clear cell carcinoma and low grade serous carcinoma are the most types, whereas mucinous carcinoma and Bernner tumours are rare. Ovarian carcinoma belonging to type II are high grade serous carcinoma, carcinosarcoma and undifferentiated carcinoma (Shih and Kurman, 2016).

1.2.4.1.1 Endometrioid Carcinoma and Clear Cell Carcinoma

Endometrioid carcinomas are mostly very well differentiated but sometimes they can be moderately or poorly differentiated. It is considered that the poorly differentiated carcinoma is due to de-differentiation from low grade carcinomas. Clear cell carcinoma are not graded as compared to other type I tumours but generally they are considered as high grade tumours, contrary to other type I tumours. Sometimes components of both endometroid and clear cell carcinoma can be found together in ovarian tumours (Shih and Kurman, 2016).

Clinical, histopathological and genetic evidence suggest that endometriosis could be the precursor lesions responsible for the origin of endometrioid and clear cell ovarian carcinoma (Vaughan et al., 2011). Both Endometrioid (40%) and clear cell ovarian carcinoma (60%-90%) have association with endometrial cysts (Veras et al., 2009). Also, both the tumour and

neighbouring endometrioid epithelium show mutation in the genes encoding AT-rich interaction domain 1A (ARID1A) and phosphatidylinositol 3-kinase catalytic subunit (PI3KCA) (Wiegand et al., 2010 & Yamamoto et al., 2011). Endometrial tissue arising from cavum uteri form ectopic neoplasia, affecting 10-15% of reproductive age women. Retrograde menstruation (a process in which menstrual blood containing endometrial cells flows back into fallopian tubes and into the pelvic cavity instead of out of the body) is thought to be responsible for ectopic neoplasia (Hull et al., 2008). Fallopian tubes are the main linking connection between all these parts and this makes them very relevant during this process. Epidemiological analyses support the fact that the ligation of fallopian tube greatly reduces the risk for the development of endometrioid and clear cell ovarian cancer (Sieh et al., 2013). Also, ligation of fallopian tube restricts the retrograde transport of exfoliated endometrial cells, preventing them from implanting into ovarian or peritoneal surface, hence providing protection from these subtypes of ovarian cancer. However, ligation of fallopian tube does not provide enough protection against frequent serous ovarian carcinoma because cells from fimbriated end of fallopian tube can still penetrate ovaries or peritoneum (Dietl, 2014).

Ovarian clear cell carcinomas (OCCC) are not very common in Europe and only (4%) of ovarian carcinomas cases belong to this type (Gram et al., 2012). OCCC have been classified as type I but they can be considered as intermediate tumours (Yamaguchi et al., 2010). Studies of OCCC have revealed that many genes affected in OCCC are stress related genes and their upregulation is due to epigenetic mechanism. This suggests that OCCC is induced by stressful conditions in endometriotic cyst (Matsumura et al., 2010).

1.2.4.1.2 Mucinous Carcinoma

There are different theories regarding the origin of ovarian mucinous carcinomas. According to one theory a subset of mucinous carcinomas may develop along with ovarian teratomas but mucinous carcinomas themselves do not show any teratomatous elements (Czriker et al., 1954; Woodruff et al., 1960). Another theory suggests mucinous carcinomas originate

from endometriosis, Brenner tumours and metaplasia of surface epithelial inclusions (Horiuchi et al., 2003; Woodruff et al., 1960)., although some other findings suggest that their involvement is rare except for Mullerian endocervical mucinous or mixed border line tumours (Rutgers and Scully, 1988; Lin and Olivia, 2013). Mandai et al., (1998) have shown morphological transition from cystadenoma to mucinous borderline tumours (MBT) to intraepithelial carcinoma and invasive carcinoma. Cystadenomas, MBT and mucinous carcinoma also show an increased frequency of KRAS mutations at codons 12 and 13. These observations support the hypothesis of 'mucinous adenoma-carcinoma sequence and supporting the argument that mucinous cystadenomas and MBTs develop into mucinous carcinomas in an orderly manner (Ichikawa et al., 1994; Mok et al., 1993).

1.2.4.1.3 Low grade serous Carcinoma

These tumours are very rare but genetically stable and show less genetic mutations. They develop in an indolent manner from their precursors. Comparison of DNA content and alterations in the copy numbers closely relate low grade serous carcinoma with serous border line tumours (Pradhan et al., 2009; Kuo et al., 2009). Mutations of p53 are also not very common in low grade serous carcinomas (Singer et al., 2005). According to one theory the transformation of Mullerian metaplasia into ovarian epithelial give rise to low grade ovarian serous carcinomas (Lauchlan et al., 1972; Dubeau et al., 2008). The other theory suggests low grade serous carcinomas originate from embryological remains of proximal Mullerian ducts which are present in ovarian hilm. However, some recent theories suggest that ovarian low grade serous carcinomas and their implants, ovarian epithelial inclusion glands and endosalpingiosis may originate from fallopian tube (Vang et al., 2013). According to this recent theory the pathogenesis of invasive low grade serous carcinoma originates with serous cystadenoma/adenofibroma, which develops in slow stepwise sequence into atypical proliferative serous tumour (typical serous borderline tumour), non-invasive micropapillary (low grade) serous carcinoma (micropapillary serous border line tumour) and then invasive low grade serous carcinoma (Vang et al., 2013).

1.2.4.2 Type II ovarian tumours

These tumours are clinically aggressive in nature and they originate from tubal or ovarian surface epithelium.

1.2.4.2.1 High grade serous carcinomas

The ovarian carcinomas belonging to this category are very aggressive and show worst prognosis. It is thought that high grade serous carcinoma originates from fallopian tube (Vang et al., 2013). During the last few years advances in the histological analyses have shown that the candidate precursors are at the fimbriated ends of the fallopian tubes called as serous tubal intraepithelial carcinoma (STIC) (Vang et al., 2013). Molecular genetics and morphology show that STIC resembles serous ovarian cancer and it has been detected in >60% of BRCA-unrelated serous ovarian carcinomas. Based on this evidence STIC are now regarded as the earliest histological lesions in the pathogenesis of the most common ovarian cancer subtype (Dietl, 2014). It has been proposed that STIC implants onto the ovary which result in the development of invasive high grade serous carcinoma prompting tumour growth (Vang et al., 2013).

About 68% of ovarian cancer cases belong to this category and mostly they are diagnosed at advanced stage. Mutations of p53 have been observed in 96% of these cancers with a high Ki67 proliferation index (50%-75%) (Koshiyama et al., 1995; Salani et al., 2008; Quartuccio et al., 2014). In high grade serous carcinomas gene instability due to chromosomal arrangements is common and they also have mutations of BRAC1 and BRAC2 in 90% hereditary cases (Christie and Oehler, 2006). According to microarray analysis data set from The Cancer Genome Atlas (TCGA) project, high grade ovarian carcinomas can be divided into four categories based on their gene expression such as; mesenchymal, immunoreactive, proliferative and differentiated (Cancer Genome Atlas Research Network, 2011; Verhaak et al., 2013). A study by Murakami et al., (2016) show

that carcinomas belonging to subtype immunoreactive show best progression free survival and overall survival, while mesenchymal subtype show worst overall survival.

1.2.5 Stages of Ovarian cancer

The stage of ovarian cancer gives an idea how far it has grown and spread. There are four stages of ovarian cancer also known as FIGO system named after its author –the international Federation of Gynaecological Oncologists (Cancer Research, UK).

1.2.5.1 Stage 1

Ovarian cancer at stage I means it is limited only in the ovaries. Stage I is further divided into three sub-stages;

Stage 1a- Cancer is only inside 1 ovary

Stage 1b- Cancer completely inside both ovaries

Stage 1c- Apart from cancer in one or both ovaries, there is some cancer on the surface of the ovary. Presence of cancer cells in the fluid removed from abdomen during surgery.

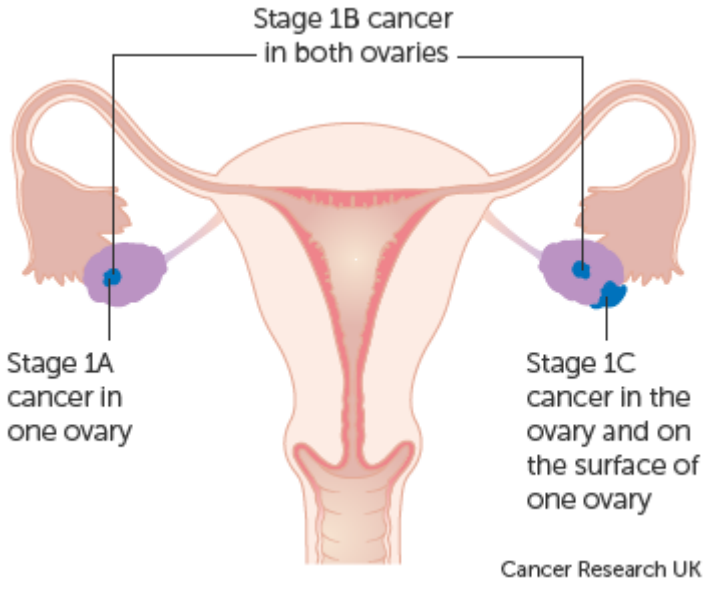


Figure 1.4: Stage I of ovarian cancer. The figure shows cancer is limited to ovaries

1.2.5.2 Stage II

In stage 2, cancer spreads outside the ovary or ovaries and also in the area of hip bones (the pelvis). Cancer cells can also be present in abdomen. Stage II cancer is also divided into three sub-stages;

Stage 2a- the spread of cancer into fallopian tube or the womb

Stage 2b- the spread of cancer into bladder or rectum

Stage 2c- the spread of cancer into other tissues of pelvis and also into the fluid inside abdomen

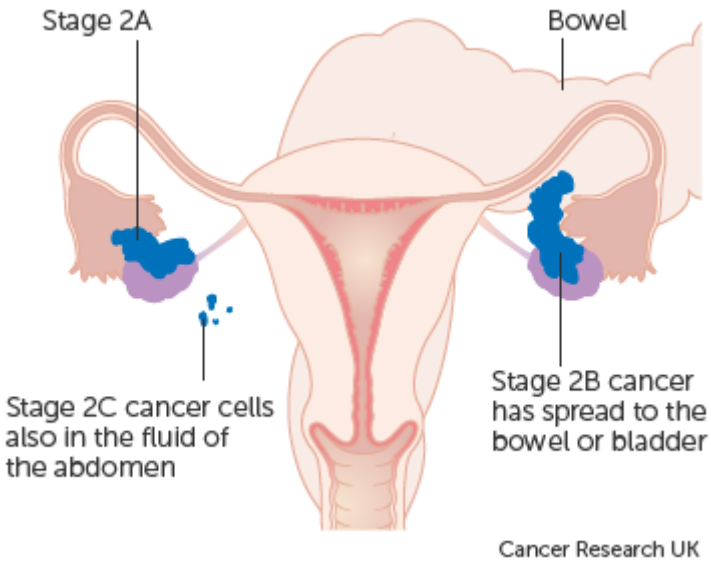


Figure 1.5: Stage II of ovarian cancer. The figure shows spread of cancer outside ovaries into the area of pelvis.

1.2.5.3 Stage III

In stage III cancer spread outside pelvis into the abdominal cavity. Cancer also spreads into lymph nodes, upper abdomen, and groin or behind the womb. Stage III cancer is also divided into three sub-stages;

Stage 3a- presence of cancer cells in tissues lining the abdomen

Stage 3b- growth of cancer up to 2cm or smaller in size within the lining of abdomen

Stage 3c- growth of cancer larger then 2cm and spread of cancer in lymph nodes in the upper abdomen, groin or behind the womb.

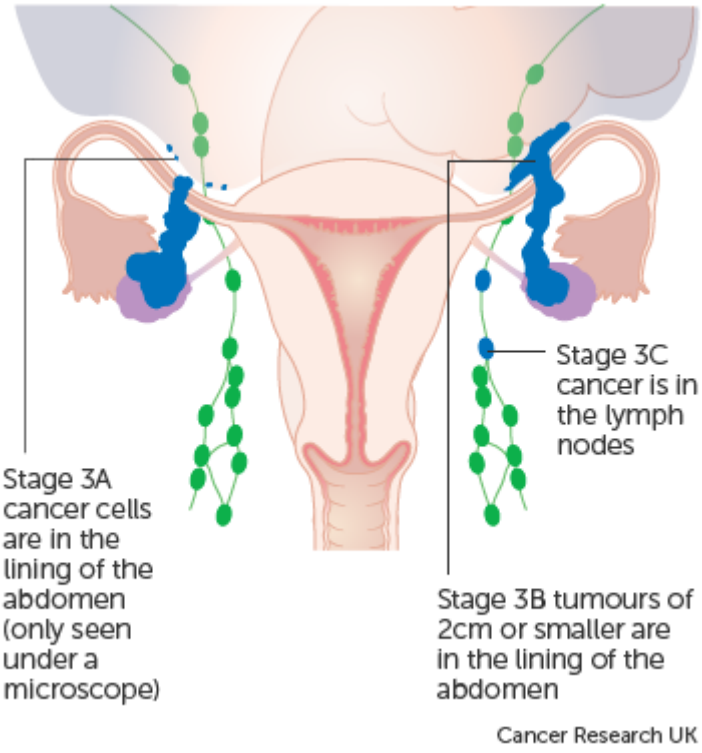


Figure 1.6: Stage III of ovarian cancer. The figure shows spread of cancer into the abdominal cavity.

1.2.5.4 Stage IV

In stage IV cancer spreads from ovaries to other body organs at some distance away such as liver or lungs. Stage IV cancer is also divided into 2 sub-stages;

Stage 4a- the cancer spreads into the fluid which builds up in the lining of lungs called pleura.

Stage 4b- the cancer spreads inside of liver or spleen, lymph nodes in the groin, outside abdomen or other organs such as lungs.

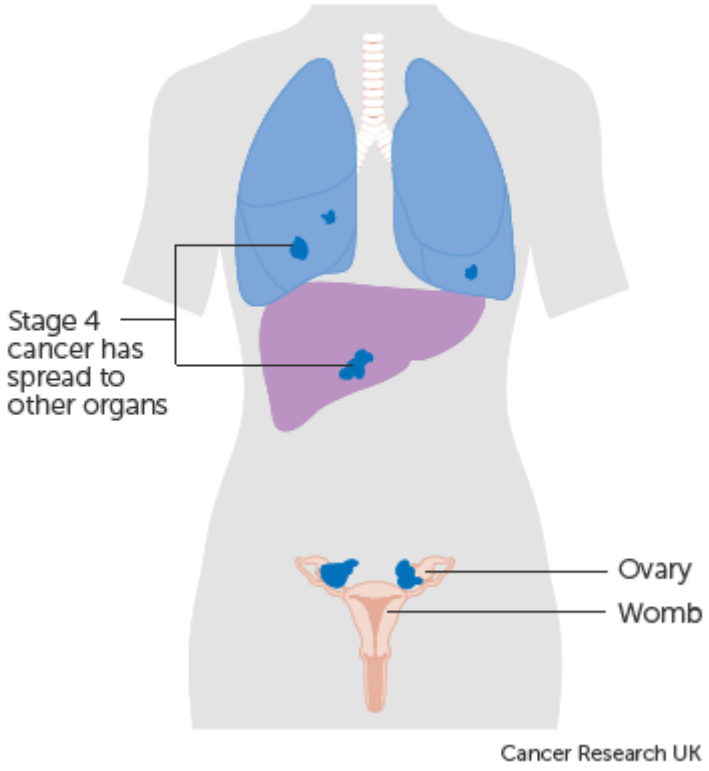


Figure 1.7: Stage IV of ovarian cancer. The figure shows spread of cancer to other body organs.

1.2.6 Screening of ovarian cancer

Early detection of cancer can significantly increase the chances of survival for the patients and specially if the cancer is metastatic and the lesions are detectable. Localization of cancer for longer periods also helps in cost effective screening (Badgwell and Bast, 2007). To get a positive prediction of 10% or higher for epithelial ovarian cancer, detection method should have sensitivity of more than 75% and 99.6% specificity. The concentration of serum 125 alone is not enough for screening because it's neither sensitive nor specific. Specificity of serum 125 is enhanced when combined with transvaginal ultrasonography (TVS) or by monitoring of its concentration over time or both. These findings were also validated by United Kingdom Collaborative Trial of Ovarian Cancer Screening in which 202638 postmenopausal women of age 50-74 years participated and showed sensitivity of yearly screening of CA125 combined with TVS as second line test but TVS alone was encouraging. When both serum 125 and TVS were combined it enhanced specificity (Menon et al., 2009).

Proteomics based biomarkers can also enhance the sensitivity and specificity of serum assays for early detection of ovarian tumours (Nossov et al., 2008).

Screening of germline mutations of *BRCA1* and *BRCA2* by using risky assessment programme BRCAPRO can also be used for early detection of ovarian cancer. In this programme patients own history and family history information is used for screening purposes (Berry et al., 2002; ACOG, 2009). Patients with the mutations of *BRCA1* and *BRCA2* undergo screening of CA125 and TVS at regular intervals between 30-35 years of age or 5-10 years earlier of the age in the family when ovarian cancer was first diagnosed (Burke et al., 1997; Daly et al., 2010). Women who are 40 years or older and have mutation of germline *BRCA1* and *BRCA2* are also advised to undergo bilateral salpingo-oophorectomy (BSO) to lower the risk of ovarian or breast cancer, although the risk of primary peritoneal carcinoma is still there even after BSO (Rebbeck et al., 2002; Burke et al., 1997).

1.2.7 Treatment of ovarian cancer

1.2.7.1 Primary Treatment

Surgery of ovarian cancer is called primary treatment and it consists of staging and cytoreduction (debulking). If the cancer is limited only to ovaries, then the surgery can be very curative. In surgery, comprehensive staging laparotomy combined with total abdominal hysterectomy and bilateral salpingo-oophorectomy is carried out (Giede et al., 2005; Earle et al., 2006; Du Bois et al., 2005). Young females with early stage invasive epithelial ovarian cancer or lesions which show abnormal cells but less risk of developing into cancer, can maintain their fertility by undergoing surgery called salpingo-oophorectomy. This procedure helps in preserving the uterus and contralateral ovary (Wright et al., 2009; Fader et al., 2007). Less invasive techniques can be used for stage I disease for example prophylactic oophorectomy. Ovarian cancer patients with disease at stage II, III and IV are recommended to undergo cytoreductive surgery (Stier et al., 1996; Eisenhauer et al., 2006). Removal of

gross disease should be done at its best. For cytologic examinations aspiration of ascites or peritoneal lavage should be done. If the disease has spread more than ovaries, then total hysterectomy and bilateral salpingo-oophorectomy need to be done. Removal of omentum and resection of suspicious large nodes should be done. Stage III patients should have bilateral pelvic and para-aortic lymph node dissection (Panici et al., 2005; Aletti et al., 2009). For all stages of ovarian cancer, the methods that may be considered are pelvic dissection, bowl resection, diaphragm or other peritoneal surface stripping, splenectomy, partial hepatectomy, cholecystectomy, partial gastrectomy or cystectomy, ureteroneocystostomy or distal pancreatectomy (Wimberger et al., 2007).

1.2.7.2 Chemotherapy

Majority of epithelial ovarian cancer patients undergo postoperative systemic chemotherapy. However, if the cancer is stage IA, IB or grade I tumours then there may be no need of chemotherapy and it has been observed only with surgery the survival rate is more than 90% for these patients. Epithelial ovarian, primary peritoneal and fallopian tubes cancer are recommended to undergo primary adjuvant therapy. Stage III patients (debulked) are recommended to undergo intraperitoneal chemotherapy according to randomized controlled trials (www.cancer.gov). Stage II patients can also undergo intraperitoneal chemotherapy, however randomized trials for them have not been published (Armstrong et al., 2006; Markman and Walker, 2006; Marth et al., 2007). Intraperitoneal therapy of cisplatin/paclitaxel enhanced the survival time by 16 months in stage III ovarian cancer patients as compared to standard intravenous therapy. However, the patients with poor performance status can be given intravenous paclitaxel/carboplatin (category I) (Giede et al., 2005; Ozols et al., 2003). The other alternative regimens for intravenous therapy are docetaxel/cisplatin or paclitaxel/cisplatin (both category I). Those patients who have high risk of neuropathy (diabetes patients) are recommended to be given docetaxel and carboplatin (Vasey et al., 2004; McGuire et al., 1996). The cycles of chemotherapy depend on the stage of the disease. Patients at early stage are recommended 3-6 cycles of chemotherapy and

advanced stage patients (II-IV) are recommended 6-8 cycles of chemotherapy (Bell et al., 2006).

1.2.7.3 Treatment of ovarian cancer by PARP inhibition

A promising advance in the treatment of ovarian cancer is the use of poly(ADP-ribose) polymerase (PARP) inhibitors. PARP inhibitors are drugs that interfere with PARP function and kill BRCA mutant cells (Lord and Ashworth, 2017). Epithelial ovarian cancer patients with known mutation (germ line or somatic) in BRCA have shown very promising preclinical data (phase II and phase III) for the clinical use of PARP. Effectiveness of PARP has also been observed in patients without BRCA mutation but possibly having other mutations due to molecular deficiencies in DNA repair (Markman, 2017 & Fong et al., 2010). FDA has approved three PARP inhibitors (olaparib, rucaparib and niraparib) for commercial use. Each of the three PARP inhibitors have different clinical indications and toxicity but so far there is no direct comparison data available of these inhibitors. The ongoing trails for the use of these inhibitors for managing treatment of ovarian cancer also include novel strategies of combining other antineoplastic agents such as antiangiogenic agents, and checkpoint inhibitors with PARP inhibitors (Markman, 2017).

1.2.7.4 Treatment of ovarian cancer by immunotherapy

Ovarian cancer can also be treated by immunotherapy. Presence of Immunogenic tumour associated antigens on ovarian cancer cells can produce a detectable immune response (Vlad et al., 2004). Although evidence suggests that the anti-tumour immunity is effective in ovarian cancer cells, the success ratio of immunotherapy is very modest at best. Immunotherapy for ovarian cancer patients was first used in 1987 by using intraperitoneal injections of anti-human fat globulin-1 antibodies (Epenetos et al., 1987). This was the first treatment where monoclonal antibodies were used for the treatment of cancer. Other antibodies used were the anti-CA125 antibody oregovomab, but it failed. More recent

developments, such as the generation of CAR-T cells targeting ovarian tumour antigens perhaps hold more promise (Zhu et al., 2017). Despite some reports of good clinical response of immunotherapy in ovarian cancer patients, there are no immunotherapy treatments approved by FDA. It has been suggested that immunotherapy could be more responsive if combined with cytotoxic agents, small molecules inhibitors or radiotherapy (Drerup et al., 2015)

1.2.8 WWOX expression in ovarian cancer

All normal ovarian epithelial cells express high amount of WWOX. In a study by Nunez et al., (2005) expression of WWOX was observed in ovarian carcinoma. Immunoblotting analysis showed consistently high levels of WWOX in normal ovarian samples, whereas undetectable or reduced levels of WWOX were observed in 37% of ovarian carcinoma. Immunohistochemistry analysis of normal human ovarian tissue showed very strong expression of WWOX in surface epithelial cells, whereas 30% of ovarian carcinoma (*n=444*) showed almost complete loss of WWOX expression. The other 70% of ovarian carcinoma samples showed moderate to strong WWOX protein. Reduced expression of WWOX showed a significant correlation with FIGO stage IV (p=0.007) and shorter overall survival (p=0.03). Correlation of WWOX and overall survival was observed by using cases from MDACC TMA (n=354). A statistical significant correlation was observed between WWOX and overall survival, whereas low WWOX protein expression correlated with shorter survival (p=0.03).

Association between WWOX and tumorigenicity was observed in a study by Gourley et al., (2009). In this study, ovarian cancer cells PEO1 were transfected with WWOX expressing plasmid and a vector. QRT-PCR and immunoblotting analysis showed that parental cells and vector transfected cells expressed no WWOX but WWOX transfected cells expressed various levels of WWOX. These cells were injected in nude mice and result showed that WWOX transfected cells formed no tumours, while parental PEO1 cells and vector

transfected cells formed large tumours. The results show WWOX abolished tumorigenicity *in vivo*, however it has no effect in *in vitro* proliferation when plated n plastic or soft agar.

Role of WWOX in apoptosis was also checked in adherent ovarian cancer cells. No significant difference was observed between parental PEO1, vector or WWOX transfected cells. Cells were then exposed to cisplatin (chemotherapy drug) which is used to treat ovarian cancer by causing cell death, but no evidence of apoptosis was found by WWOX. WWOX abolished tumorigenicity in vivo but not in adherent cells, so assessment of WWOX on apoptosis and proliferation was performed in vivo by injecting WWOX transfected and control cell in nude mice. Greater heterogenicity within tumours then between tumours was observed which showed tumour regression *in vivo* is not due to altered proliferation.

Further assessments were done to find out how WWOX suppressed *in vivo* tumorigenicity by examining the interaction between tumour cells and extracellular matrix (ECM). Restoration of WWOX in PEO1 cells and overexpression of WWOX in SKOV3 ovarian cancer cells showed reduction in migration and attachment with fibronectin, an ECM component involved in peritoneal metastasis. Contrary to this, knock down of WWOX in another ovarian cancer cells A2780 by siRNA enhanced attachment with fibronectin. There was no difference in rate of apoptosis in WWOX expression or WWOX non-expressing cells in adherent cultures. However, in detached cell cultures WWOX expression showed more cell death then WWOX non-expressing cells. It was further shown that WWOX expression showed reduction of membranous integrin A3 protein but not mRNA levels and adhesion of PEO1 cells to fibronectin is by integrin A3. This study shows the role of WWOX expression in abolishing tumorigenicity in vivo in ovarian cancer by reducing attachment to fibronectin via integrin α3.

1.2.9 Expression of FHIT in ovarian cancer

It has been reported by many studies that FHIT gene expression is altered in epithelial tumours. Ozaki et al., (2001) examined the role of FHIT in the development of ovarian cancer. 33 ovarian carcinomas, 2 borderline tumours and 10 benign adenomas were used in

the study to examine in alterations in FHIT using RT-PCR and sequencing. 15% of ovarian carcinoma and 1 of the 2 border lines tumours showed aberrant FHIT expression, whereas 15% of ovarian carcinoma showed loss of normal FHIT but border line or benign tumours did not show any loss of FHIT. LOH was observed at D3S1300 and D3S4103 (microsatellite markers) in 21% and 20% of ovarian carcinomas. Immunohistochemistry analysis showed of loss of FHIT expression in 14% of ovarian carcinoma; however, border line or benign tumours did not show loss of FHIT. Loss of FHIT expression was correlated with loss of normal FHIT transcript. This study suggests loss of normal FHIT transcription can inactivate FHIT expression resulting on genesis of ovarian cancer, mostly in high grade serous carcinomas.

Hendricks et al., (1997) have shown that expression of FHIT in human ovarian carcinomas. 14 ovarian, 8 cervical and 4 endometrial human cancer cell lines were selected of analysis of FHIT expression by RT-PCR. The results showed 63% cervical and 14% ovarian cell lines showed aberrant FHIT transcripts, whereas endometroid cell lines did not show any change in FHIT. Further analysis of DNA sequencing showed shorter mRNA transcripts with primarily missing exons 5, 6 and 7 were missing in aberrant bands along with some other exons. Further analysis of DNA by southern blotting revealed 4 of the 5 ovarian carcinomas showed altered expression of FHIT and among those 4, three ovarian carcinomas showed no normal FHIT transcripts. These findings suggest involvement of FHIT gene in cervical tumorigenesis and also FHIT is less important in ovarian and endometroid cancer development.

1.3 Colorectal Cancer

1.3.1 Epidemiology

Colon cancer also known as bowel cancer is one of the most common gastrointestinal malignancies. Larger and small intestine are part of digestive system. Colon cancers mostly

start in large intestine but sometimes it can start in small intestine as well. Bowel or colorectal cancer is one of the most common types of cancer diagnosed in UK after breast, prostate and lung cancer. Every year 40,000 new cases are diagnosed in UK alone and 1 in 20 people develop colorectal cancer later in their life (NHS Scotland, 2017). Although colorectal cancer can happen at any age but more than 9 out of 10 cases (94%) diagnosed in UK are people over the age of 50 years. Six out 10 (59%) cases diagnosed are in people over the age of 70. Approximately 268,000 people in UK have been diagnosed with colorectal cancer. Early detection of colorectal cancer is treatable and curable. 5 years' survival time depends upon the stage as people at stage 1 can survive more than 5 years. Relapse chances are less after 5 years. Every year about 16,000 people die from colorectal cancer which makes it second most common cause of cancer death. The death numbers are falling after 1970 because of better diagnosis and treatment (Bowel Cancer UK, 2017).

1.3.2 Pathophysiology

There are four tissue layers of colon which play an important role in determining the stage of colorectal cancer (figure 1.8). These four layers are serosal layer, thick muscle layers, submucosa and mucosal layer. The mucosal layer consists of epithelium containing mucus secreting factories called goblet cells. This is the inner most layer of colon which come in direct contact of the substance that pass through the gut and hence it's susceptible to damage. Crypts of Lieberkühn are housed in mucosal layer. Crypts are formed by epithelia of villi which extend into the lamina propria. Crypts contain cells which are involved in host defence and signalling. It also contains stem cells which replenish epithelial cells further up to the villi. The development of colon cancer takes place inside crypts of Lieberkühn (Leblond, 1981; Humphries and Wright, 2008). Formation of the new cells takes place in crypts while the old cells move into the lumen and shed. If there is any damage to the cells of the crypts it can cause inflammation and hyperproliferation which can result in formation of an adenoma. The mucosal layer and submucosa are separated by the basement membrane which is located directly against muscularis. The supply of blood and nutrients to gut is

provided by muscularis which is also accessible for transference of abnormal cells. The outer most layer of colon is serosal layer which is in touch with peritoneal structures.

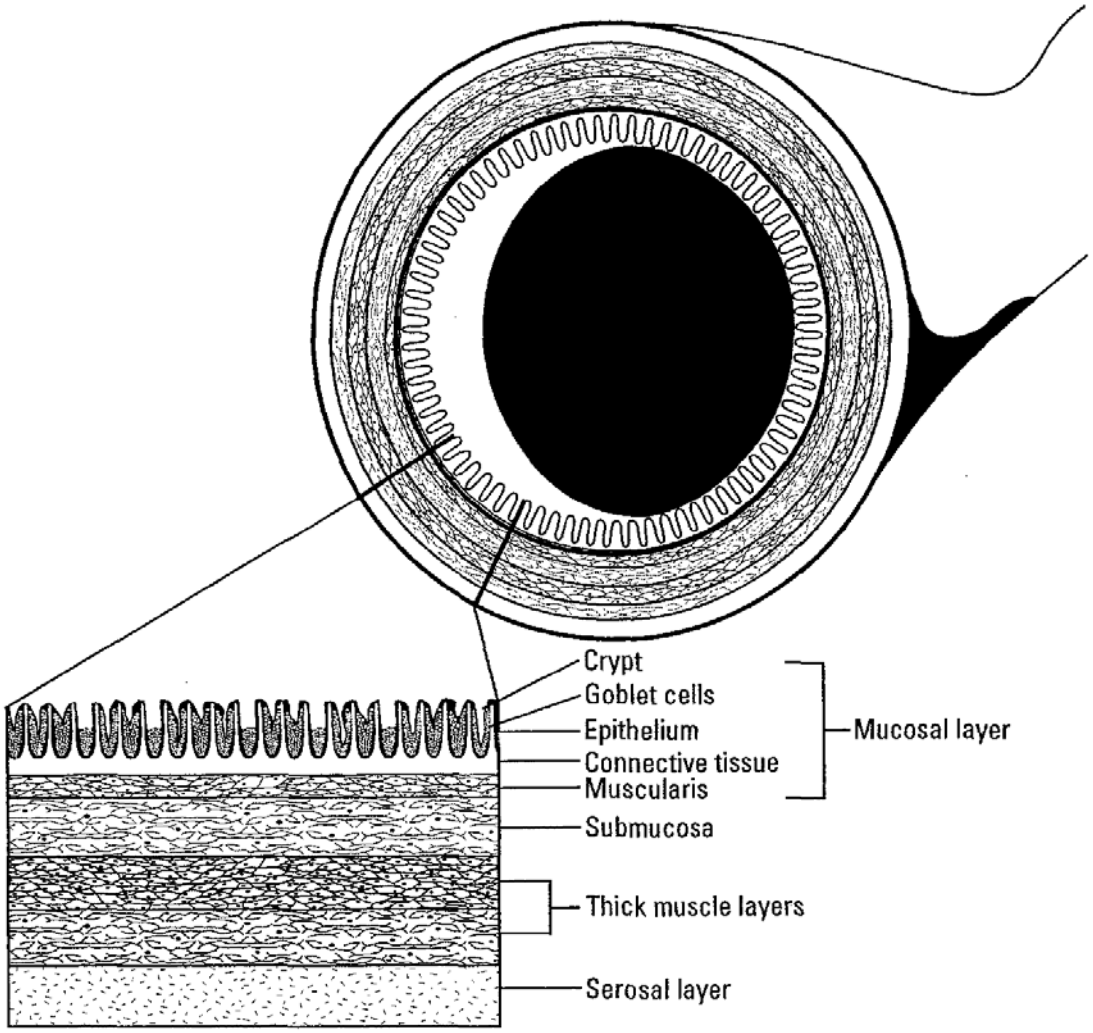


Figure 1.8: Anatomy of colon (Shelton, 2002). The layers of colon wall consist of three layers; mucosal layer, thick muscle layer and serosal layer.

Genetic mutations and molecular abnormalities can cause colorectal cancer, however the resultant changes that arise due to mutations and abnormalities are not easy to predict.

Some of the common molecular abnormalities responsible for colorectal cancer have been shown in figure 1.9 (Jessup et al., 1997; Glaser et al., 1999; Markowitz and Winawer, 1997).

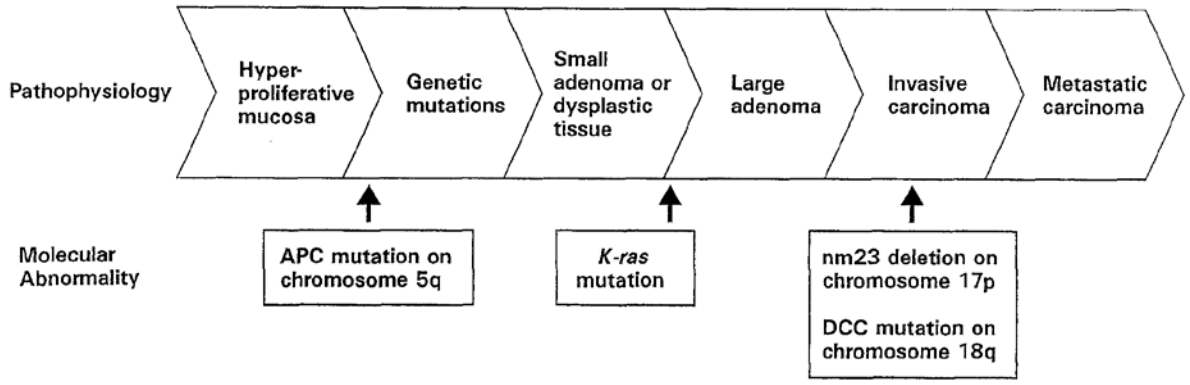


Figure 1.9: Pathogenesis of colorectal cancer (Burt, 1997). It shows molecular abnormalities responsible for colorectal cancer.

1.3.3 Types of colorectal cancer

Based on the histopathology of the malignant cells colorectal cancer is further divided into types.

1.3.3.1 Adenocarcinoma

Adenocarcinomas arise from the mucus secreting gland cells in the lining of the colon wall. This is the most common histologic type of colorectal cancer and more than 90% of colorectal cancer cases belong to this category (Pazdur et al., 1999). It typically starts as a growth of tissue in colon called polyp. Adenoma is a type of polyp that develops into carcinoma. Removal of polyp by colonoscopy can prevent development of cancer. There are two types of adenocarcinoma;

1.3.3.1.1 Mucinous tumours

These tumours are comprised of about 60% mucus which may cause cancer cells to spread quickly. These tumours are more aggressive then adenocarcinomas and represent 10-15% of colorectal adenocarcinomas. The treatments procedures are same for these two types as for adenocarcinomas (Cancer Research UK, 2017).

1.3.3.1.2 Singlet ring carcinomas

These carcinomas are aggressive and more difficult to treat. About 1% of adenocarcinomas are singlet ring cell carcinomas (Cancer Research UK, 2017).

1.3.3.2 Rare types of colorectal cancer

1.3.3.2.1 Squamous cell carcinomas

Squamous cells along with gland cells make the inner lining of the colon. These are unusual carcinomas and indicate chronic cellular changes due to repeated exposure to carcinogens (Sasaki et al., 1998).

1.3.3.2.2 Carcinoid tumours

Carcinoids are slow growing tumours which can arise in parts of digestive system (stomach, small intestine, colon, and rectum). These are called neuroendocrine tumours because they grow in hormone producing tissue. There are no early signs or symptoms but appear late in disease. The treatment includes surgery and medication (Cancer Research UK, 2017).

1.3.3.2.3 Sarcomas

The cancers of bone or muscles are called sarcomas. Sarcomas in the colon are called leiomyosarcomas. These sarcomas start in smooth muscles. The treatment for sarcomas is different to colorectal adenocarcinomas (Cancer Research UK, 2017).

1.3.3.2.4 Lymphomas

Following are the cancers of lymphatic system. Treatment of lymphomas is different to adenocarcinomas of colon.

1.3.4 Stages and grades of colorectal cancer

Prognosis of cancer is determined by its pathologic state. The stage of cancer gives an indication about the spread of cancer and how big the tumour is. Its gives an indication if the tumour has penetrated though the walls of intestine and if the cancer has reached into the lymph nodes. It also indicates about distant metastasis. If the tumour spreads through the muscularis into the submucosa, it is considered as invasive. Tumour can spread through the peritoneal wall or it can spread through the lymphatic or vascular system to the distant sites such as liver. Although less frequent but it can also spread into bones, kidneys and adrenal glands (Pazdur et al., 1999). There are three different systems used for staging of colorectal cancer; the American Joint Committee on Cancer tumour, nodes, metastasis (TNM), Dukes and the number stage system.

1.3.4.1 TNM Staging

TNM is the most commonly used system. TNM stands for tumour, node and metastasis.

1.3.4.1.1 Tumour (T)

Tumour stage describes the size of the tumour in the colon. There are four stages of tumour in colorectal cancer (figure 1.10). T1 (tumour only in the inner layer of colon), T2 (penetration of tumour into muscular layer of colon), T3 (penetration of tumour into outer lining of colon) and T4 (penetration of tumour through the outer lining of colon to the nearby organs) (Cancer Research UK, 2017)

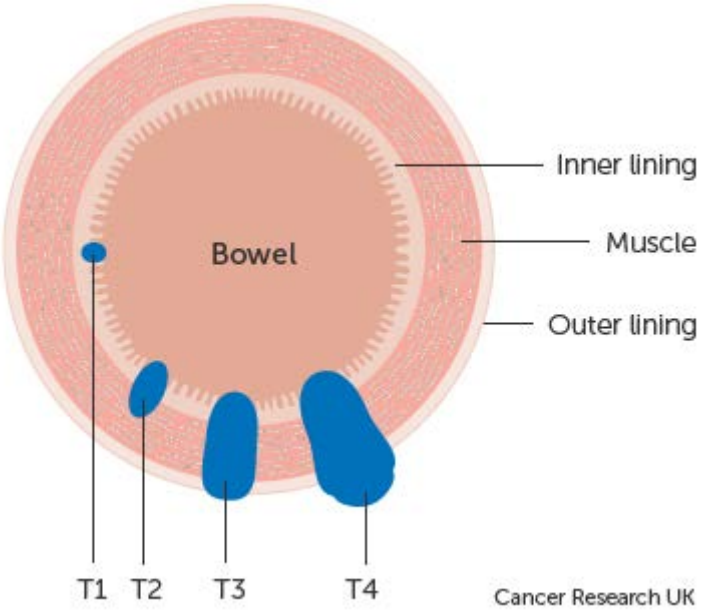


Figure 1.10: Tumour stages of colon (T1, T2, T3 and T4).

1.3.4.1.2 Node (N)

Node (N) describes the spread of cancer into the lymph nodes. There are three stages which explain the spread of cancer into the lymph nodes. N0 (no cancer into the lymph nodes), N1 (spread of cancer into 1-3 lymph nodes near colon), N2 (spread of cancer into 4 or more lymph nodes nearby colon) (Cancer Research UK, 2017).

1.3.4.1.3 Metastasis (M)

Metastasis (M) explains the spread of cancer to different parts of the body. There are two stages; M0 (spread of cancer to other organs), M1 (spread of cancer to other parts of body) (Cancer Research UK, 2017).

1.3.5 Grades

Grades of cancer show much cancer resembles with normal cells. It also helps in determining what type of treatment may be required. There are three grades of colorectal cancer; Grade 1 (low grade; look mostly to normal cells), Grade 2 (close to normal cells), Grade 3 (high grade; abnormal cells) (Cancer Research UK, 2017).

1.3.6 Risk Factors

There are some factors which are associated with risk of the development of colorectal cancer such as age above 40 years, family history, genetic mutations, inflammatory bowel disease or adenomatous colonic polyps, diet high in fats, cholesterol and low in fibre. Alcohol, smoking and sedentary lifestyle (Kelvin, 1998; Borum, 2001; Potter et al., 1996; Pazdur et al., 1999; Cancer facts and figures, 2002). A family history of inflammatory bowel disease such as Crohn's disease or ulcerative colitis is considered as one of the main reason for development of colorectal cancer (Burt and Peterson, 1996; Freeman, 2001). It has been hypothesized that genetic mutations along with tearing of mucosal wall due to inflammatory bowel disease can start malignant cells proliferation (Wong and Harrison, 2001). People suffering from bowel disease have a life time risk of colorectal cancer (2-18% ulcerative colitis; 0.7% Crohn's disease) (Freeman, 2001; Eaden et al., 2001). The duration and intensity of inflammation can cause detectable changes in mucosal layer of colon which can result in colorectal cancer (Wong and Harrison, 2001; Bond, 2001).

There are some types of polys that do not present any risk of colorectal cancer, but adenomatous colonic polyps are a growth inside the walls of colon which pose risk of colorectal cancer (Markowitz and Winawer, 1997; Jessup et al., 1997). These polyps are precancerous and are classified as villous, tubulovillous and tubular. There is a 2-5% risk of adenomatous polyp to become cancerous. Small sized polyps (0.5mm) are not risky but if the size is more than 2mm then there are 46% chances of malignancy over a time of 10 years (Kelvin, 1998; Markowitz and Winawer, 1997).

Genetic mutations are another factor which can enhance the risk of developing colorectal cancer. 90-95% of colorectal cancers are sporadic or common cancers, however there is no know genetic link for them (Ellis et al., 2000). Malignancies due to unknown risks are called 'de novo carcinomas'. Only 5-6% of colorectal cancer cases show genetic mutations and two of the most common genetic mutations are familial adenomatous polyposis and hereditary

nonpolyposis colorectal cancer (Jessup et al., 1997; Glaser and Grogan, 1999). Both types pose high risk of colorectal cancer and 50% of all colorectal cancers belong to these two types. The cause for familial adenomatous polyposis is an inherited autosomal dominant trait which is linked with virtually 100% incidence of colorectal cancer. 1-5% of colorectal cancers are hereditary nonpolyposis colorectal cancer which occurs in people with average age of 42 years. They are also known as Lynch syndrome which is autosomal dominant condition. Some other possible genetic mutations are adenomatous polyposis coli genes, the K-ras oncogene, p53 gene and DNA mismatch repair genes (Jessup et al., 1997; Glaser and Grogan, 1999).

1.3.7 Signs and Symptoms

The symptoms of colorectal cancer depend on the location of tumour as shown in figure 1.11

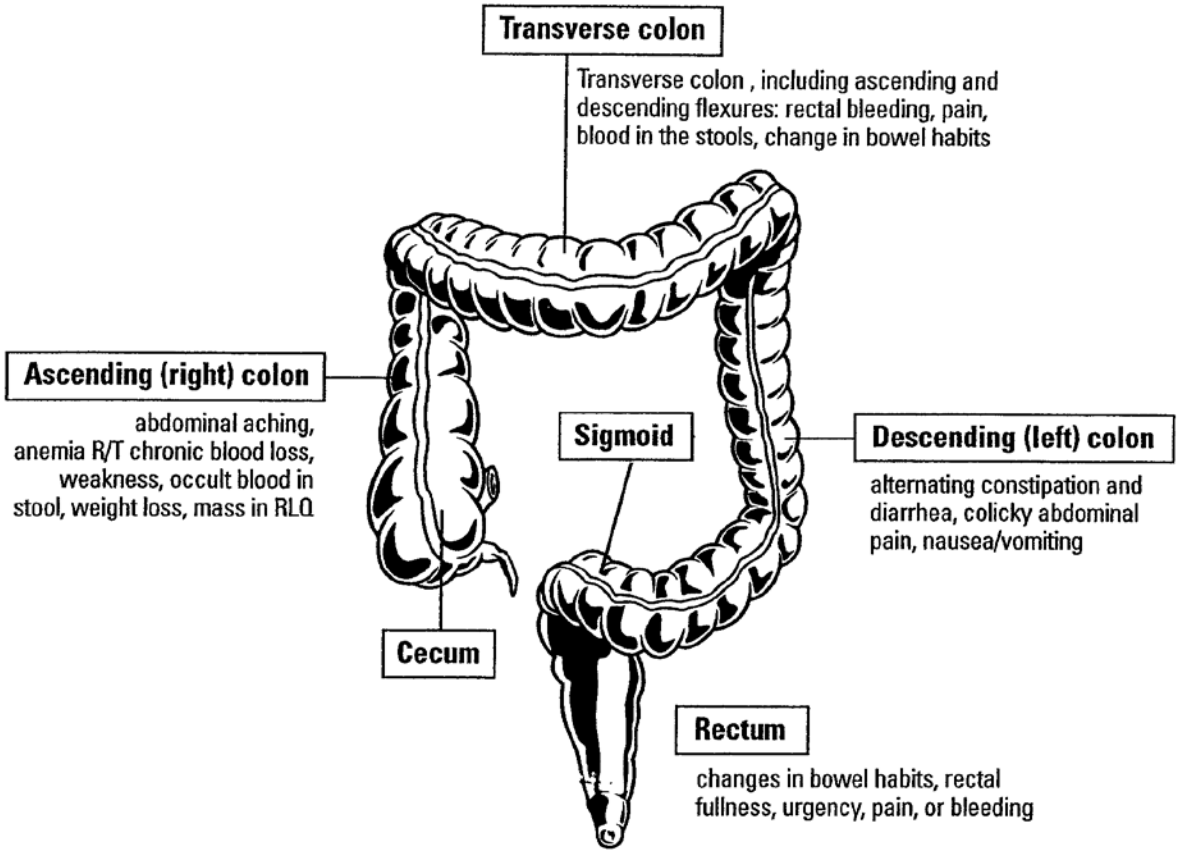


Figure 1.11: Different sections of colon showing colorectal cancer signs and symptoms (Ellis et al., 2000; Meyers, 1998; Skibber et al., 2001)

Tumours in the ascending colon usually large and bulky and show symptoms of vague, pain, anaemia. Sometimes these tumours are misunderstood as reasons for some other disorders and are recognised when tumour is in advanced stage. If the cancer is in transverse colon it shows symptoms like rectal bleeding, pain and blood in stools. Tumours in the descending colon show symptoms like alternating constipation, diarrhoea, colicky abdominal pain, nausea and vomiting. Tumours in rectum show symptoms like pain or bleeding, rectal fullness, changes in bowel habits and urgency. Symptoms for advanced colorectal cancer are severe pain, anorexia and weight loss (Ellis et al., 2000; Meyers, 1998; Skibber et al., 2001).

1.3.8 Screening of colorectal cancer

Early detection of colorectal cancer has proved to be very helpful in improving the survival outcomes. Colorectal cancer in the anus is detected by digital rectal exam. They appear as tumours of about 7cm in length near the anal verge (Ellis et al., 2000). There are different requirements for screening of colorectal cancer in UK. In England men and women aged 60-74 are advised to undergo faecal occult blood test every two years (Bowel Cancer UK, 2017). Sigmoidoscopy is also used to screen polyps in distal colon and if polyps are found, patients need colonoscopy to avoid the risk of adenomatous colonic polyps in other parts of bowel (Bond, 2000). Baseline colonoscopy is recommended to moderate to high risk patients every 3-5 years to check the presence of disease. Genetic testing is also recommended to individuals with a strong family history of colorectal cancer, polyps or other types of cancers such as ovarian, stomach etc. (Burke et al., 1997; Bond, 2000).

1.3.9 General treatment strategies

The treatment strategies for colorectal has changed over last couple of decades. For many years' surgery and radiation therapy were used as main treatment methods of colorectal cancer treatment, until it was found 5-fluorouracil (5-FU) based regimens are very effective when administered as adjuvant therapy. Various biological modulators such as leucovorin

and levamisole are used for the administration of 5-FU. Different schedules such as continuous infusion or pulse are used for the administration of 5-FU to improve its response rate (Bond, 2000; Benson et al., 2000). Recent advancement in the knowledge of genetic abnormalities linked with colorectal cancer has helped in the development of novel therapies which target genetic abnormalities associated with colorectal cancer (McLeod et al., 2000).

1.3.9.1 Conventional therapy

Surgery is considered as the most common treatment of colorectal cancer; however, it may not be applicable for some patients because of the stage of disease (Scoggins et al., 1999). Due to advancement in knowledge of response between primary and metastatic disease, some clinicians prefer to use systemic therapies before surgery. In cases where the penetration of cancer is deep in the colon wall, patients are recommended to undergo chemotherapy or chemotherapy plus radiation (Bond, 2000; Cancer facts and figure, 2002). The surgical methods are selected based on the location and blood supply of tumour and the pattern of lymph nodes. Each surgical procedure has specific indications and morbidities as shown in table 1.4 (Ellis et al., 2000; Hurd and Gutman, 1995).

Table 1.4: Surgical procedure, indication and morbidities of colorectal cancer (Ellis et al., 2000; Hurd and Gutman, 1995)

Surgical Procedure	Indications	Morbidities
Right hemicolectomy	Cecal, ascending, hepatic flexure lesions	Injury to duodenum or ureter, bile acid deficiency
Right radical hemicolectomy	Transverse colon or hepatic flexure lesions plus those removed in right hemicolectomy	Injury to duodenum or ureter, bile acid deficiency, anastomotic dehiscence, diarrhea
Transverse colectomy	Mid transverse colon lesions	Anastomotic dehiscence
Left hemicolectomy	Left colon lesions	Anastomotic dehiscence
Low anterior resection	Sigmoid colon and proximal rectum lesions	Anastomotic dehiscence, bowel ischemia
Subtotal colectomy	Multiple synchronous and distal transverse colon lesions	Diarrhea, perineal excoriation, anastomotic dehiscence

1.3.9.2 Adjuvant therapy

After surgery, patients with stage II or stage III colon cancer are recommended to undergo adjuvant chemotherapy to avoid recurrence of the tumour from the residual cells left after the surgery. Stage II or III rectal cancer patients are recommended to undergo chemotherapy plus radiation therapy. Adjuvant therapy comprising 5-fluorouracil and leucovorin is used as standard for early stage or advanced stage colorectal cancer patients (NCCN, 2000; Francini et al., 1994; Labianca et al., 1995).

1.3.9.3 Treatment of disease at advanced stage

5-fluorouracil and leucovorin are used as first line therapy for treatment of metastatic colorectal cancer. If the first line therapy fails, then Irinotecan is used as second line therapy. Irinotecan (CPT-11) is an antineoplastic agent which was approved in 1996 to be used for treatment of colorectal cancer. Few studies showed irinotecan showed a response rate of 13% and a median survival time of 9 months in metastatic colorectal cancer where 5-FU therapy failed to stop the recurrence or progression (Cunningham et al., 1998; Von Hoff D et al., 1997). Two studies showed the effect of irinotecan combined with 5-FU and leucovorin on untreated metastatic colorectal cancer (Saltz et al., 2000; Douillard et al., 2000) and the result showed the response rate was 39%-49%, median survival time was 14.8-17.4 months and progression time was 7 months. These results showed improved efficacy of the regimen compared with irinotecan alone, so this combined therapy was as first line therapy for treatment of colorectal cancer (Vanhoefer et al., 2001; Saltz, 1998; Peeters and Haller, 1999).

These chemotherapy drugs show some toxicities such as nausea, diarrhoea, mucositis and neutropenia (Berg and Lilienfeld, 2000; Royce et al., 2000; Berg, 1998) which sometimes require a change in the therapy administration such as dose reduction to reduce the effects of toxicity. Another major issue with colorectal cancer patients is drug resistance because it's a challenge to find an alternative therapy (Jansman et al., 2000). Patients who either do not tolerate standard 5-FU regimens or are elderly, they can be treated with some other

approved drugs such as oral fluorinated pyrimidines (capecitabine) (Hoff et al., 2001), oxaliplatin (Pelley, 2001) or some other biological targeted treatments (Donehower, 2000). IMC-C225 (Erbitux; Im-Clone Systems Incorporated, New York, NY, and Bristol-Myers Squibb Company, New York, NY) is a monoclonal antibody which targets the epidermal growth factor, is used in combination with irinotecan for the treatment of advanced colorectal cancer patients. This treatment has shown promising results in patients who do not have many options in terms of therapy resistance (Saltz et al., 2001).

1.3.10 Association of *WWOX* with colorectal cancer

It has been reported by various studies that WWOX expression is either lost or reduced in variety of human cancer such as breast, lung, liver, stomach and pancreas (Aqeilan et al., 2007). A study by Alsop et al., (2008) examined deletions of WWOX in HCT116 colorectal cancer cells. This study showed homozygous deletions of 25 to 50kb in WWOX in HCT116 cells which appeared due to loss of exons 6-8 by heterozygous deletions, some of which overlapped to show homozygous loss. The deletion of exon 6-8 showed deletions of transcripts which were allele specific and has been observed in some other tumours. The conclusion was targeted deletions at FRA16D were observed in HCT116 cells which resulted in removal of WWOX gene fragments. Another study by Żelazowski et al., (2011) reported the role of deletions of WWOX gene and its expression in colorectal cancer cells. In this expression of WWOX was observed in various cancer cell lines such as HCT116, SW480, SW620 and HT-29. HCT116 colon cancer cells showed 70% expression level. HT-29 colorectal adenocarcinoma cells showed very low expression level of WWOX. SW480 and SW620 primary tumour cells showed relative WWOX expression which suggests WWOX has some role progression of colorectal cancer. The relative WWOX expression levels of all four colorectal cancer cell lines as shown in (table 1.5) were compared against MCF-7 breast cancer cells which according to study of Płuciennik et al., (2006), breast cancer patients showed significantly higher WWOX expression and higher progression free survival compared with relative low WWOX levels.

Table 1.5: Expression of WWOX in cancer cells

Cell lines	Average *WWOX* relative expression
HCT116	1.075
HT29	0.080
SW480	0.230
SW620	0.175
MCF-7	1.497

Further in the study of Żelazowski et al., (2011) analysis of *WWOX* promoter methylation and loss of heterozygosity (LOH) at two *WWOX loci* were observed. The results showed no evidence of hypermethylation in *WWOX* promoter region and no LOH. Also, in this study correlation between WWOX expression and disease-free survival was analysed by Kaplan Meier test. Patients with high *WWOX* expression in tumours showed higher disease-free survival, however there was no significant relationship between among patient's groups stratified on the basis of clinicopathological features. Over all this study showed role of *WWOX* expression in tumour suppression in colon, however molecular mechanisms behind this were unidentified.

1.3.11 Association of *FHIT* with colorectal cancer

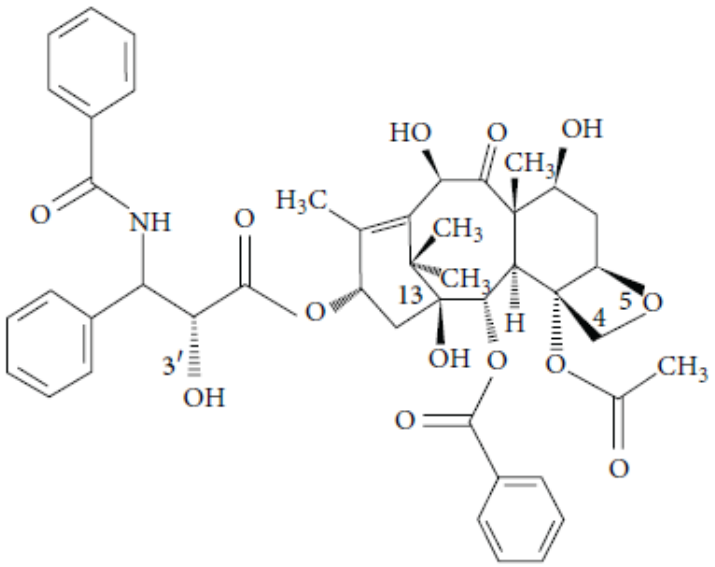
Involvement of FHIT gene has been reported by some studies in oncogenesis and progression of colorectal cancers (Hihi et al., 1997; Hao et al., 2000). A study by Ohta et al., (1996) showed 50% of digestive tract carcinomas (oesophageal, stomach and colon) showed aberrations in transcription of FHIT gene. In another study by Mori et al., (2001) expression of FHIT in colorectal cancer tissues was examined. Immunohistochemical analysis showed half of the colorectal cancer cases (n=62) were positive for FHIT protein. Significant correlation between loss of FHIT protein and progression of carcinoma and lymph node metastasis was observed. 50% of sporadic colorectal cancer cases showed loss of

FHIT protein which was significantly more frequent in advanced stage cases. Andachi et al., (2002) reported loss of FHIT expression in 52 advanced colorectal carcinomas by immunohistochemical method. The results showed loss of FHIT in 18 (34%) of the 52 cases. Loss of FHIT expression in colorectal carcinomas and premalignant lesions was observed in a study by Hao et al., (2000). Immunohistochemical analysis showed FHIT was strongly expressed in all normal colonic epithelium. FHIT expression was absent is 44% (n=84) of carcinomas. 12 out of 13 metastatic lesions showed reduced FHIT expression. The trend of reduced FHIT expression was more in tumours with decreased differentiation and in tumours with metastases. These finding suggest an important role of FHIT in the development and progression of colorectal carcinomas.

1.4 Paclitaxel treatment and ER stress response in cancer cells

1.4.1 Paclitaxel

Paclitaxel (Taxol) is a cytotoxic agent (Figure 1.12) first discovered in the bark of pacific yew tree (*Taxus brevifolia*) in 1962 during a plant screening programme conducted by U.S department of agriculture and National cancer institute (NCI). Paclitaxel is used as first line therapy for the treatment for various types of cancers such as ovarian cancer (McGuire et al., 1996). Most commonly a dose of 200-250mg m^{-2} (depending upon the patients' status) is given by infusion at intervals of 3 hours and 24 hours. The vehicle used for delivery of Paclitaxel consist of 1:1 mixture of Cremophor EL (polyethoxylated castor oil) and ethanol which is either mixed with 5 % dextrose solution or 15-20% saline (Singla et al., 2002). Plasma protein take up 90% of the drug and 10% is excreted in urine (Rizzo et al., 1990; Wiernk et al., 1987 and Rowinsky et al., 1990).



Empirical formula: $C_{47}H_{51}NO_{14}$
Molecular weight: 853.9 g/mol
Average mass: 853.9 Da
Systemic name: (2α, 5β, 7β, 10β, 13α)-4,10-Diacetoxy-13-{[(2R, 3S)-3-(benzoylamino)-2-hydroxy-3-phenylpropanoyl]oxy}-1,7-dihydroxy-9-oxo-5,20-epoxytax-11-en-2-yl benzoate

Figure 1.12: Chemical structure of paclitaxel. It consists of a texane ring with a four membered oxetane side ring at CA and C5 and an active homochiral ester side chain at C13 which binds to microtubule in a guanosine triphosphate independent manner to induce toxicity (Kampan et al., 2015)

1.4.1.1 Paclitaxel interference with microtubules

Paclitaxel has shown its antitumor properties in a number of human malignancies. Initial testing of taxol showed that it was effective in mouse tumour models and P388 leukaemia (Fuchs and Johnson 1978). Human clinical trials of ovarian cancer patients at advanced stage showed that 30% patients showed good response to taxol treatment (McGuire et al., 1989), and breast cancer patients showed that 56% responded to taxol (Holmes et al., 1991). Taxol promotes the assembly of microtubules. Pure tubulin subunits which are important for the polymerisation of α and β heterodimers into microtubules in vitro are lowered in their concentration by paclitaxel; also, their disassembly due to cold or Ca^{2}+ treatment is prevented by paclitaxel (Schiff et al., 1979).

The formation of microtubules under the influence of paclitaxel makes them very strong and stable but dysfunctional resulting in cell death due to disruption of normal tubules functions needed for cell division. Different tumour models in animals and cell cultures show paclitaxel induced mitotic arrest (Fuchs and Johnson, 1978; Schiff and Horwitz, 1980; Milas et al.,

1995; Jordan and Wilson, 2004; Orth et al., 2008). Some studies suggest that cells upon treatment with paclitaxel are arrested in metaphase and show nearly normal spindles. A few cases reported misalignment of the chromosomes at the cell equator (Jordan et al., 1993, 1996; Waters et al., 1996), and some studies reported multipolar spindles (Chen and Horwitz, 2002; Hornick et al., 2008), but so far there is no evidence which show that these effects are clinically relevant for anti-tumour activity of paclitaxel.

Many research papers have shown the link between paclitaxel and apoptosis (Bhalla et al., 1993; Havrilesky et al., 1995; Chang et al., 1996; Li et al., 1994; Yen et al., 1996; Gan et al., 1996). Lieu et al., show that low concentrations of paclitaxel could initiate apoptosis without undergoing mitotic arrest and depending upon the cell cycle various apoptotic mechanisms could be triggered by paclitaxel (Lieu et al., 1998). Furthermore, mitotic arrest due to paclitaxel may not always trigger apoptosis (Huang et al., 2002), and one study reported that at low concentration of paclitaxel apoptosis and at high concentration necrosis can take place (Yeung et al., 1999). Interestingly another study reported that both necrosis and apoptosis can take place in human endothelial cells due to paclitaxel (Mailloux et al., 2001). In my study, I focused only on the apoptotic pathways triggered due to paclitaxel.

1.4.1.2 Paclitaxel induced apoptosis due to cell cycle arrest

How binding of paclitaxel with microtubules and stabilizing them leads to initiation of downstream mechanisms causing apoptosis is not fully understood but recent research in the signalling molecules activated by paclitaxel and activation of transcription of various genes show that there are multiple mechanism which could be responsible for apoptosis (Wang et al., 2000).When tumour cells are treated with Paclitaxel, it induces cell arrest in G2-M phase of the cell cycle and initiates apoptosis that leads to cell death (Georgiadis et al., 1998).

It has been suggested that mitotic spindle assembly check point controls this paclitaxel induced apoptosis. Chromatids are attached to spindle fibres by their kinetochores (a protein

present at centromere). If kinetochores are not properly attached with spindle microtubules, it will result in activation of signal transduction cascade which will stop the cell from progressing into the next stage of anaphase by inhibiting anaphase promoting complex (Kops et al., 2005; Lara-Gonzalez et al., 2012; Foley and Kapoor, 2013), and this involves check point genes such as *hs*-MAD2 (Sorger et al., 1997; Li et al., 1997), BUB1, (Taylor and McKeon, 1997) BUB3, (Sorger et al., 1997; Taylor et al., 1998) PP2A, (Minshull et al., 1996) and MAPK (Minshull et al., 1994) . After paclitaxel treatment BUB3 expression was up-regulated suggesting this could lead to paclitaxel induced apoptosis (Taylor and McKeon, 1997; Martinez et al., 1999).

1.4.1.3 Role of p53 in paclitaxel induced apoptosis

P53 has also been linked to paclitaxel induced apoptosis. Approximately 96% of human cancers show mutations of p53 (Wang and Wang, 1996). Although some studies have shown no correlation between p53 expression and paclitaxel response (Safran et al., 1996), other studies suggest that loss of its normal function in cancer patients could sensitize them to paclitaxel therapy (Wahl et al., 1996; Perego et al., 1998; Zaffaroni et al., 1998). This increased sensitivity is associated with increased G_2 /M arrest (Wahl et al., 1996), and altered regulation of downstream targets of p53 such as p21 and MAP4. Upregulation of p21 by p53 helps the cells to come out of mitotic arrest (Barboule et al., 1997) and if there is no p21, the paclitaxel treated cells are killed by apoptosis. Microtubule associated protein 4 (MAP4) helps in stabilizing microtubules and in the presence of activated p53 it is down regulated and in the absence of normal p53 microtubules are stabilized by MAP4, making cells more sensitive to paclitaxel treatment (Wang et al., 2000).

1.4.1.4 Role of Bcl-2 family in paclitaxel induced apoptosis

Bcl-2 family members are categorized into two types i.e. Antiapoptotic and pro-apoptotic. Bcl-2 and Bcl-X_L are antiapoptotic proteins and Bcl-X_S, Bak, Bad and Bax are pro-apoptotic proteins. This family of proteins are also involved in Paclitaxel induced apoptosis (Strobel et al., 1996; Strobel et al., 1998). Paclitaxel treatment changes the expression of Bcl-2 family

and can trigger post-translational changes in Bcl-2 molecules. Paclitaxel treatment results in upregulation of expression levels of Bax and Bak and down regulates expression of Bcl-X_L (Liu and Stein, 1997; Srivastava et al., 1998), as well as phosphorylation for Bcl-2 in cells arrested at G_2 /M phase of the cell cycle which enhances paclitaxel induced apoptosis (Blagosklonny et al., 1997; Roth et al., 1998). How mitotic arrest by paclitaxel links with Bcl-2 is an area which remains to be explained.

1.4.1.5 Paclitaxel induced apoptosis by activation of JNK/SAPK

Jun amino terminal kinases (JNK) / stress activated protein kinases (SAPK) belong to the MAPK (mitogen activated protein kinases) family. These proteins are activated by stress, inflammation regulatory cytokines or growth factors and have been linked with apoptosis. It has been shown (Wang et al., 1998, Lee et al., 1998 and Amato et al., 1998) that JNK/SAPK is activated by Ras- and ASK1 (apoptotic signalling kinase 1) dependent signalling pathways after paclitaxel treatment (Figure 1.13). JNK/SAPK signalling transduction pathways were analysed using dominant negative signalling protein vectors and the results showed that for up to 16 hours of paclitaxel treatment, activation of JNK/SAPK is needed for early phase of paclitaxel induced apoptosis. The later stages of apoptosis were possibly due to mitotic arrest but not due to activation of JNK/SAPK (Wang et al., 1999). Other studies by (Maundrell et al., 1997 and Srivastava et al., 1999) reported that Bcl-2 phosphorylation was due to activation of JNK/SAPK after cancer cells were treated with paclitaxel, but this study was contrary to the studies of (Wang et al., 1999 and Attalla et al., 1998) which showed that JNK/SAPK induced apoptosis was independent of phosphorylation of Bcl-2. These contradictions in results could be possibly due to unknown cofactors in particular cell lines involved in regulation of Bcl-2 phosphorylation (Wang et al., 2000).

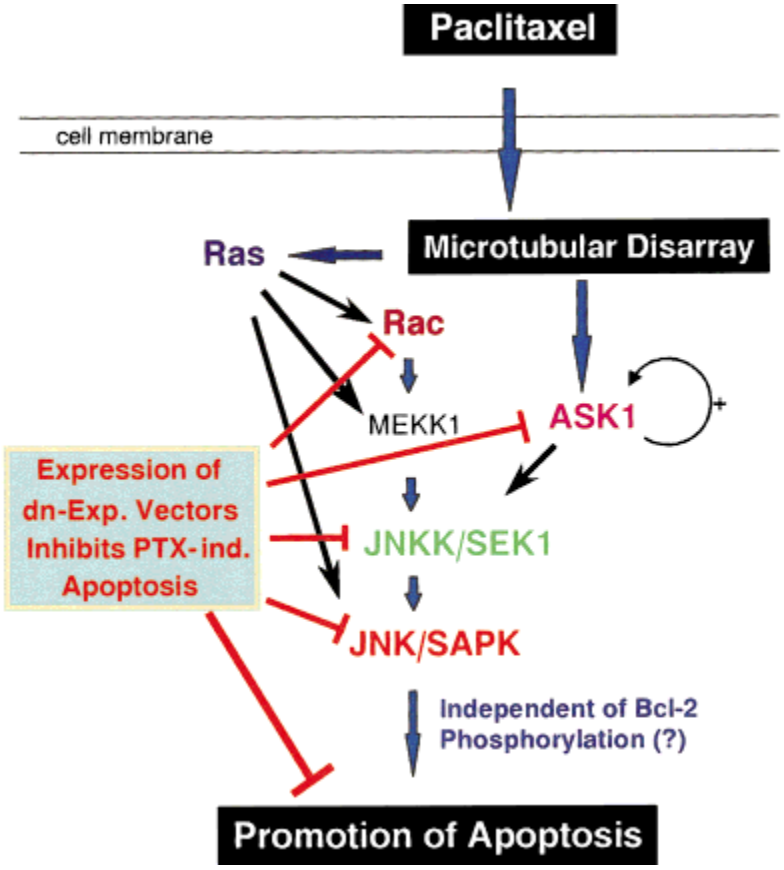


Figure 1.13: Induction of paclitaxel induced apoptosis (early phase up to 16 hours of paclitaxel treatment) due to activation of JNK/SAPK which can be inhibited by the expression of dominant negative signalling proteins of the JNK/SAPK signalling. PTX: paclitaxel; dn: dominant negative (Wang et al., 2000).

1.4.2 Salubrinal

Salubrinal (Figure 1.14) is a cell permeable compound which protects cells from ER stress induced apoptosis. It is soluble in DMSO, non-toxic, and its micro molar concentrations are very cyto-protective against ER stress inducers such as; tunicamycin (Boyce et al., 2005).

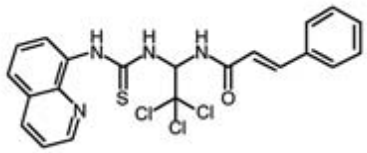


Figure 1.14: Salubrinal (3-Phenyl-*N*-[2, 2, 2-trichloro-1-[[(8-quinolinylamino) thioxomethyl] amino] ethyl]-2-propenamide) (Boyce et al., 2005).

1.4.2.1 Salubrinal: PERK inhibitor

Once ER-stress is induced, PERK is activated by dimerization and phosphorylation, it phosphorylates eIF2α, which enhances translation of the ATF-4 transcription factor, and UPR genes are expressed (figure 3.3). Salubrinal particularly inhibits the translation initiation factor eIF2α de-phosphorylation and protects the cells from ER stress induced apoptosis (figure 3.3). It does so by inhibiting the phosphatase activity of protein complex GADD34-PP1 comprised of a serine/threonine phosphatase PP1 and a non-enzymatic co factor GADD34 which dephosphorylate eIF2α (Boyce et al., 2005). The increased phospho-eIF2α produced causes global translational repression but enables translation of key ER-stress proteins (such as various chaperones), supporting a cyto-protective state.

1.4.3 Tunicamycin

Tunicamycin is an antibiotic naturally produced by *Streptomyces lyso-superificus* (Takatsuki et al., 1971). It is a white crystalline powder soluble in DMSO, water and some organic solvents. Tunicamycin is a mixture of homologous antibiotics which contain uracil: N-acetyl glycos-amine (GlcNAc), an 11-carbon aminodialdose called tunicamine, and an amide linked fatty acid (Tsvetanova et al., 2002). Tunicamycin is a reversible inhibitor of UDP-N-acetyl-D-hexosamine: polyprenol-phosphate N-acetylhexosamine-1-phosphate transferases (D-HexNAc-1-P-transferases) including the eukaryotic UDP-N-acetyl-glucosamine—dolichyl-phosphateN-acetyl-glucosamine-phospho-transferase (DPAGT1; also, called GPT). It has been suggested that tunicamycin either works as a non-competitive substrate–product–transition state analogue of DPAGT1/GPT (Heifetz et al., 1979) or as a substrate analogue

inhibitor of D-HexNAc-1-P-transferases, which shows it competes with the UDP-Hex NAc (UDP- N-acetyl glycos-amine in eukaryotes) for active site binding (Keller et al., 1979; Brandish et al., 1996).

1.4.3.1 Tunicamycin target N-linked glycosylation in ER

It has been suggested that tunicamycin works by inhibiting the first step of N-linked glycosylation of proteins via DPAGT1 (Keller et al., 1979; Brandish et al., 1996). N-linked glycosylation is a process in which glycan (oligosaccharide) is attached to the nitrogen of asparagine in the sequence (Asn-X-Ser or Asn-X-Thr). Within this sequence X could be any amino acid but not proline and glycan could be any monosaccharide such as N-acetyl-galactose-amine or N-acetyl-glucose-amine etc. (Imperiali and O'Connor, 1999). Failure in the N-linked glycosylation of proteins results in accumulation of unfolded proteins in the ER, and therefore activation of the unfolded protein response.

1.5 The WWOX tumour suppressor and cisplatin chemo-response

1.5.1 Cisplatin

In 1965, Dr Rosenberg discovered the biological functioning of cisplatin while studying the growth of bacteria *Escherichia coli*. The experiment was not specifically designed to study the activity of cisplatin, but it was about the effects of electric fields on the growth of bacteria. The apparatus used for the experiment consisted of platinum electrodes and when electric field was applied to bacteria in the presence of these electrodes it produced an inhibitory effect on the cell division in bacteria but did not completely stop the growth of bacteria. Upon further analysis, it was discovered that when electricity was applied to the platinum electrodes, a small amount of the platinum product was formed in the media containing ammonium chloride used for growing bacteria (Rosenberg et al., 1965), which produced an oxidative form of platinum - cis-diamminedichloroplatinum (II) also named cisplatin (Rosenberg et al., 1967). These exciting results encouraged the scientist to see if these inhibitory effects of platinum compounds can inhibit growth of cancer cells in animal tumour

models? Rosenberg studied the effects of platinum compounds on mouse tumour models and their findings showed that they showed inhibitory effects on sarcoma 180 and leukaemia L1210 (Rosenberg and Vancamp, 1969)

FDA approved cisplatin in 1978 and since then it has been continuously used as an anticancer drug for treatment of solid tumours. Cisplatin has been used as first line therapy either alone or in combination with other anticancer drugs (paclitaxel) for the treatment of many cancers including testicular, ovarian, cervical, head and neck and small cell lung cancers. It has been observed that during the start of cisplatin therapy patients' response is good but with time drug resistance issue is very common. Drug resistance along with dose limiting toxicity such as nephrotoxicity, compromise the effectiveness of cisplatin. The mechanism behind drug resistance of cells could be either acquired or intrinsic (Basu and Krishnamurthy, 2010).

Cisplatin interacts with chromosomal DNA for its antitumor activity (Sherman et al.,1985) but studies revealed that the actual amount of cisplatin that interacts with DNA is not much and therefore it cannot be postulated that the only biological activity of cisplatin is to inhibit the replication of DNA (Eastman, 1990). The efficiency of the chemotherapeutic drug is not only its DNA damaging ability, but it also depends on the capability of the cells by which they detect and respond to DNA damage (Kerr et al., 1994). Cells respond to DNA damage by repairing it and progressing through the cell cycle and if they cannot repair the damage, they will proceed to die (Kerr et al., 1994). Like other chemotherapeutic drugs, cisplatin induces apoptosis and therefore the signalling pathways involved in the regulation of apoptosis play significant role in deciding the cellular response to cisplatin (Basu and Krishnamurthy, 2010).

1.5.1.1 Structure of Cisplatin

Cisplatin or cis-diamminedichloroplatinum (II) is a neutral, square planer, coordination complex of divalent Pt (Figures 1.15 and 1.16) (Todd and Lippard, 2009). There are different forms of cisplatin based on its structural configuration, but cis configuration is required for its

antitumor activity (Zamble and Lippard, 1995). Cisplatin is a deep yellow or yellow orange crystalline powder at room temperature which is slightly soluble in water but soluble in dimethylprimanide and N, N-dimethylformamdie. Cisplatin is stable under normal temperature and pressure but slowly overtime it transforms into the *trans*-isomer (IRAC 1981). The structure of cisplatin consists of labile chloride groups and two relatively inert amine ligands. Cisplatin hydrolyses in water and the concentration of chloride is an important factor in regulating its hydrolysis. Blood plasma has high chloride concentration (103mM), so it prevents hydrolysis of cisplatin. When it enters into the cell, the concentration of chloride ion goes down to 4mM which eases the aquation process (Sedletska et al., 2005). In aqueous conditions cisplatin is a powerful electrophile and interacts with nucleophiles, nucleic acids and sulfhydryl groups of proteins (Basu and Krishnamurthy, 2010).

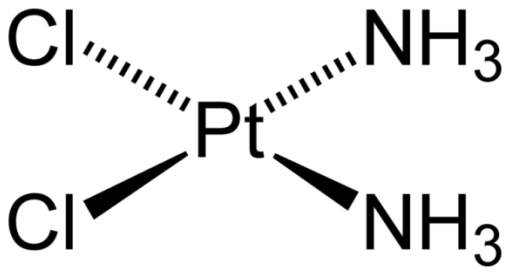


Figure: 1.15 Structure of cisplatin

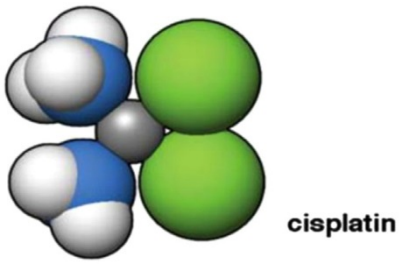


Figure: 1.16 Computational molecular structure of cisplatin (Goodsell, 2006)

1.5.2 Molecular Mechanism of cisplatin

1.5.2.1 Accumulation of cisplatin inside cells

How cisplatin accumulates inside cells? The first thought was cisplatin and its analogues enter cell via passive diffusion because its uptake is linear, non-saturable and could not battle with platinum analogues (Sherman et al., 1985; Eastman, 1990; Kerr et al., 1994; Sedletska et al., 2005). Sometimes decreased intake of cisplatin is associated with cisplatin resistance, although few or no changes were observed in the function of plasma membrane of cisplatin resistant cell lines, when compared with parent cells (Holzer et al., 2006; Holzer et al., 2004; Safaei et al., 2004). It was first proposed by Byfield et al., (1981) that active transport of cisplatin into cell takes place via carrier mediated transport. The transporters that facilitate the accumulation of cisplatin in cell include Na^+, K^+ -ATPase and members of solute carried (SLC) transporters (Hall et al., 2008; Andrews et al., 1991). Particularly one member of the SLC family, the plasma membrane copper transporter-1 (CTR1) was shown by some studies to be actively involved in transport of cisplatin into cells as a defect in *Ctr1* gene in yeast decreased the accumulation of cisplatin in yeast (Ishida et al., 2002; Safaei et al., 2005). A study by Hozler et al., (2004) also showed that in ovarian cancer cells, both cisplatin and copper caused the rapid down regulation of CTR1 via proteasome mediated pathway. The proteins involved in cisplatin export from the cell are ATP-dependent glutathione-conjugated efflux pump and copper transporters ATP7A and ATP7B (Safaei et al., 2004). When cisplatin resistant cells show reduced intracellular cisplatin, it is widely considered because of less uptake of cisplatin into the cells rather than increase in drug efflux (Kelland, 2007; Gately and Howell, 1993).

1.5.2.2 Binding of cisplatin with the DNA and signalling pathways

1.5.2.2.1 Formation of adducts

Several studies suggest that the main biological target of cisplatin is DNA (Sedletska et al., 2005; Jamieson and Lippard, 1999; Eastman, 1987). The platinum atom of cisplatin forms

covalent bonds with the N^7 position of purine bases and results in formation of 1, 2- or 1, 3-intra-strand crosslinks and a lower percentage of inter-strand crosslinks (Figure 1.17). The intra-strand crosslink between two adjacent G residues is considered as an important lesion responsible for cisplatin toxicity. The formation of cisplatin-DNA adducts disturbs DNA replication and transcription. The structure of DNA is distorted due to binding with cisplatin and formation of intra-strand cross links exposes a groove which serves as site for protein binding (Wand and Lippard, 2005). Approximately 96% of binding of cisplatin with DNA forms intra strand cross links and there is a linear relationship between platinum binding with the DNA and the cytotoxicity (Fraval and Roberts, 1979). The adducts formed in the DNA initiate signalling of damage recognition proteins. These proteins include hMSH2 or hMutSα complex (mismatch repair proteins MMR), HMG1 and HMG2 (non-histone chromosomal high mobility group) proteins, the human RNA polymerase I transcription upstream binding factor (hUBF) and transcription factor TATA binding protein (TBP) (Donahue et al., 1990; Fink et al., 1998; Chaney and Vaisman, 1999). Although the main function of these proteins is to sense DNA damage and induce downstream signalling pathways but sometimes they are involved in some other functions as well, for example HMG1 interaction with DNA adducts promotes cytotoxic effects but also inhibits the repair mechanism (Huang et al., 1994). This dual function of HMG1 was later verified by a study of He et al., (2000), which showed that overexpression of HMG1 in breast cancer cells increased sensitivity towards cisplatin. Other proteins such as hUBF and TBP cause suppressed transcription by RNA polymerase I because of sequestering at the damaged DNA sites (Jordan and Carmo-Fonesca, 2000). Cisplatin can therefore initiate both pro-apoptotic and pro-survival signalling pathways (Siddik, 2003).

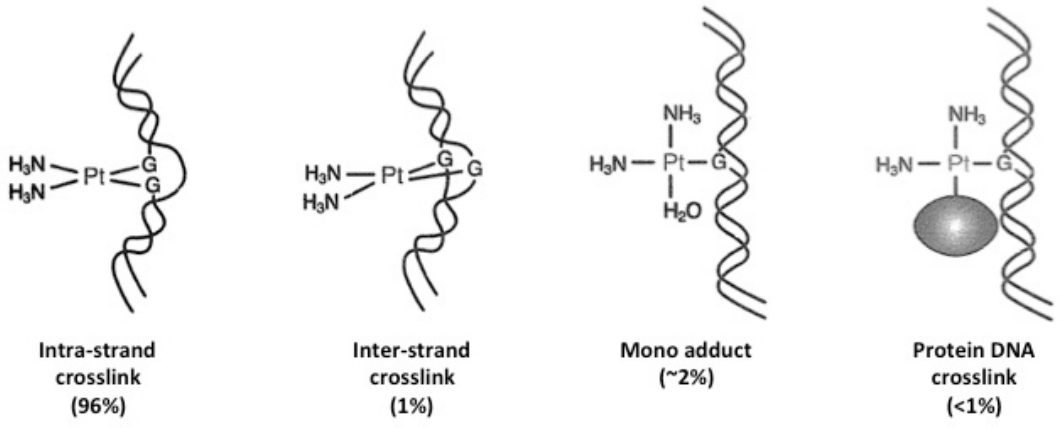


Figure: 1.17 Formation of different DNA lesions due to interaction of Chlorine anions with hydroxy groups (mostly at N^7 of purines) [www.conconilab.ca/projects/]

1.5.2.2.2 Cisplatin and DNA damage signalling

Formation of DNA adducts by cisplatin activates many pathways and one of them is activation of cell cycle check points. DNA damage by cisplatin causes a short-lived S-phase arrest and then longer G2/M arrest of the cell cycle (Shapiro and Harper, 1999; Shi et al., 1994). Although the relationship between cytotoxic effect of cisplatin and cell cycle arrest is not very clear but still cell cycle arrest is widely considered as a cytotoxic effect of cisplatin because G2/M check point arrest enhances the sensitivity of the cells towards cisplatin (Demarcq et al., 1994; O'Connor and Fan et al., 1996). During G2/M arrest, the nucleotide excision repair (NER) mechanism tries to protect the cell by repairing the DNA adducts but if it fails, cell will proceed to apoptosis. This mechanism of activation of cell cycle check points due to DNA adducts leading to repair, are linked with p53 tumour suppressor protein (Morgan and Kastan, 1997; Bullock and Fresht, 2001).

1.5.2.2.3 Activation of p53 and cisplatin induced DNA damage

P53 is a tumour suppressor protein, also known as 'guardian of genome' is mutated in approximately 96% of human cancers (Hollstein et al., 1991). The involvement of p53 in

activation of cell cycle check points, is due to activation of two upstream kinase, ATM (ataxia telangiectasia mutated protein) and ATR (ATM- and Rad3-related protein). Among these two kinases, cisplatin activates ATR (Damia et al., 2001; Zhao H, Piwnica-Worms, 2001) phosphorylating p53 at serine-15 resulting in activation of p53 protein (Appella E, Anderson, 2001). Further modification of p53 take place by ATR mediated CHK1 kinase which phosphorylates serine 20 (Shieh et al., 2000). Several studies reveal the importance of p53 in cancer cells. Absence of functional p53 in tumour cells makes them resistant to cisplatin which could be reversed by reconstitution of WT p53 (Kigawa et al., 2002; Kanamori et al., 1998). Another study by Pestell et al., (2000) showed that ovarian cancer cell lines (A2780) lacking p53 expression sensitized to cisplatin and showed reduction in DNA adduct repair and loss of control of G1-S check point.

Some other proteins which are part of p53 signalling pathways are also involved in p53 activity and cisplatin cytotoxicity. Aurora kinase A is protein which is overexpressed in multiple human cancers and it is an important protein of the p53 pathway, activating it by phosphorylation at serine 315, causing ubiquitylation by murine double minute 2 (MDM2) and proteolysis. Knockdown of Aurora A kinase reduces the phosphorylation of p53, increases the stability of p53 and causes cell cycle arrest at G2/M. Mammalian cells in the absence of aurora A became more sensitive to cisplatin induced apoptosis and this can be reversed by increased expression of aurora A (Katayama et al., 2004).

Cyclin G1 is another gene which can be transcriptionally activated by p53 and it plays an important role in p53-Mdm2 (multi double minute 2) auto regulated module. Knock down of cyclin G1 in MCF7 cells by siRNA caused the accumulation of p53 protein due to DNA damage activating ATM protein (Ohtsuka et al., 2003; Ohtsuka et al., 2004).

There are several other mechanisms by which p53 can determine cisplatin induced cell death. P53 induced cell death due to cisplatin requires degradation of FLIP (FLICE -like inhibitory protein) (Abedini et al., 2008). P53 counteract and bind to antiapoptotic functioning

of Bcl-xL resulting in enhanced cisplatin induced cell death (Kutuk et al., 2009). Overexpression of phosphate and tension homolog (PTEN) in cisplatin resistant ovarian cancer cells also requires p53 mediated cell death (Yan et al., 2006). Activation of nutrient sensor AMP-kinase (AMPK) has been observed in AGS cells and HCT116 colorectal cancer cells due to cisplatin and inhibition of AMPK caused induction of p53 resulting in cisplatin induced cell death (Kim et al., 2008).

1.5.2.2.4 Cisplatin and JNK

c-Jun N-terminal kinase (JNK) has a role in regulation of anticancer activity of cisplatin. JNK can be activated by stress response or DNA damage. JNK pathway is activated by cisplatin via p73, a pro-apoptotic member of p53 family. Cells having defects in JNK pathway showed resistance towards cisplatin (Zanke et al., 1996). It has also been reported that JNK along with c-Abl are also involved in MMR mechanism which induces cell death due to cisplatin-DNA adducts (Tsai and Yuan, 2003).

1.5.2.2.5 Apoptosis induced by cisplatin

Apoptotic cell death induced by cisplatin can involve both the extrinsic pathway and intrinsic pathway. Initiation of extrinsic pathway takes place when ligands bind with TNFα (tumour necrosis factor-α) receptor family. In next step, adaptor molecules help oligomerization and recruitment of pro-caspase-8 and form a complex called death inducing signalling complex (DISC). The intrinsic pathway can be activated because of stress response or DNA damage. In this pathway cytochrome c is released from mitochondria which activates pro-caspase9 which form an active apoptosome complex by interacting with APAF-1 (apoptosis promoting activating factor -1) (Nunez et al., 1998; Kischkel et al., 1995).

1.5.2.2.6 Cisplatin and calcium signalling

Intracellular chloride ions play an important role in determining the response to cisplatin. When the concentration of intracellular chloride ions is low, cisplatin hydrolyses into charged reactive species such as monoaquated [cis-(NH) PtCI(HO)]$^{+}$ and diaquated [cis-(NH)Pt(HO)]2+ forms (Jennerwein and Andrews, 1995; Aggarwal et al., 1980) which are 1000 times more reactive than normal cisplatin and functions by inhibiting the respiratory activity of the mitochondria. Thus, calcium ions move out from mitochondria causing a temporary increase in the cellular calcium levels which disturbs calcium homeostasis and disrupts normal cellular functions (Aggarwal, 1993).

1.5.2.2.7 Cisplatin and MAPK

Mitogen activated protein kinases (MAPK) are serine/threonine family of proteins kinases which are involved in the regulation cell survival and growth by coordination with extracellular signals (Chang and Karin, 2001; Marshall, 1995). Some studies show that cisplatin activates ERK (extracellular signal regulated kinase) in many types of cells but whether this activation prevents or promotes cell death is controversial (Wang et al., 2000; Yeh et al., 2002). Activation of ERK by cisplatin causes p53 mediated DNA damage by phosphorylating p53 which further up regulates p21, GADD45 (45kd growth arrest and DNA damage) and Mdm2 (mouse double minute 2 homolog) (DeHaan et al., 2001).

1.5.2.2.8 Cisplatin and AKT

Akt is family member of serine/threonine kinases which is activated downstream of PI3K (phosphoinositide 3 kinase) which is very important for its role in cell survival. BAD (Bcl-2 associated death promotor) is a pro-apoptotic member of Bcl-2 family of proteins. Akt phosphorylates BAD at ser 136 due to DNA damage by cisplatin. Cells with cisplatin induced DNA damage need phosphorylation of BAD for their viability. A study by Hayakawa et al., (2000) showed that cisplatin Induced DNA damage phosphorylates BAD Ser112 by ERK cascade and BAD Ser 136 by PI3K-PKB/AKt pathways and inhibition of either of these

cascades in ovarian cancer cells sensitized them to cisplatin. These results show that inhibition of BAD by either of these cascades can suppress the apoptotic function of BAD.

1.5.2.2.9 c-Abl and cisplatin induced DNA damage

C-Abl is a tyrosine kinase receptor which responds to DNA damaging agents. C-Abl can move between nucleus and cytoplasm controlled via nuclear localization motifs and nuclear export signals which are important for apoptosis induced by DNA damage (Peyer et al., 2007). C-Abl responds to DNA damage by moving from cytoplasm to the nucleus and then form a complex by phosphorylation of MEK kinase 1, activating JNK/SAPK (stress activated protein kinase) (Kharbanda et al., 2000). It has also been shown that MMR (mismatch repair) recognizes DNA damage induced by cisplatin and activates c-Abl and JNK and cells lacking MMR function do not respond to c-Abl (Nehme et al., 1999). P73 which is a member of p53 initiate apoptosis by binding with MMR/c-Abl (Gong et al., 1999). The pro-apoptotic ability of p73 can be further enhanced by JNK which preventing the proteasomal degradation of p73 and increases its stability by binding with a transcription co activator Yap1 and triggering recruitment of p300 (Levy et al., 2008). C-Abl is substrate for caspase and it is cleaved for cisplatin induced apoptosis (Machuy et al., 2004).

1.5.2.2.10 DNA damage and repair mechanisms

Cisplatin induced DNA damage can undergo DNA repair mechanism to counter the cytotoxicity of cisplatin. Both inter-strand and intra-strand disrupt the structure of DNA which is recognised by the cellular proteins for repairing cisplatin induced DNA damage. Cisplatin induced DNA damage caused by intra-strand link is mainly repaired by nucleotide excision repair (NER). In NER DNA repair mechanism the damaged DNA is repaired by removal of cisplatin-DNA adduct. The first step is to find out the location of the lesion in the DNA. Once the lesion is recognised it is excised and up to 30 base pair single strand DNA portion containing lesion is removed. In the next step the synthesis and ligation of the repair patch at the pre-existing strand takes place (Costa et al., 2003). Importance of NER mechanism has

been shown in some studies such as, deficiency of the gene involved in NER can cause a disorder called Xeroderma Pigmentosum (XP). The cells derived from XP patients are very sensitive to cisplatin (McKay et al., 2001).

Although cisplatin induced DNA damaged is preferably repaired by NER mechanism but there are some other DNA repair mechanisms as well such as; double strand base repair (DSB), base excision repair (BER), inter-strand cross link repair and direct reversal of damage. Depending upon the type of lesion, the repair mechanism responds and fixes the DNA damage (Hoeijmakers, 2001). Among all these DNA repair mechanisms, NER is the most extraordinary pathway because it can remove most of the DNA lesions which occurs due to intra-strand cross links in the DNA due to cisplatin. There are two distinct sub-pathways by which NER can detect the DNA damage, which are Global genome NER (GG-NER) and transcription coupled NER (TC-NER) (Bohr et al., 1985; Mellon et al., 1987).

1.5.2.2.11 The NER mechanism

The detailed mechanism of Global genome NER (GG-NER) and transcription coupled NER (TC-NER) pathways is shown in figure 1.18. The first step shows probing and detection of lesion in the strand of DNA due to distortion in double helix which is recognised by damage sensor protein XPC/HR23B in GG-NER pathway, whereas in TC-NER pathway at the site of lesion transcription is stalled and RNA polymerase displaces itself from the site of lesion and increases its affinity towards NER proteins CSA and CSB (Cockayne syndrome protein) forming a complex and allow them to access the site of DNA damage. These two pathways have different mechanisms for detection of the lesion in the DNA strand but once a lesion is recognized, the repairing mechanism is same. In next step, further recognition of DNA damage and verification takes place by recruitment of XPA and RPA at the site of DNA damage and when damage is fully detected then transcription initiation factor IIH (TFIIH) is also recruited at the site of DNA damage. This TFIIH complex mediates both transcription and DNA repair. Next, the helicases components XPB and XPD of TFIIH are recruited at the

site of lesion and they unwind the DNA duplex surrounding the DNA adduct. In next incision step, the structure specific endonucleases XPG and ERCC1-XPF incise the DNA adduct at positions 3’ and 5’ respectively and remove 24-32 base nucleotides containing the DNA lesion. The trimeric proliferating cell nuclear antigen (PCNA) ring, recruited by XPF-ERCC1 after the 5’ incision, binds DNA Pol δ, DNA Pol κ or DNA Pol ε to fill the gap by synthesis of DNA. The filling of the gap is a quick process once the 5’ incision has been done. DNA ligase 1 or 3 is used to do sealing and this final step completes the NER pathway (Marteijn et al., 2014; Hanawalt et al., 2002).

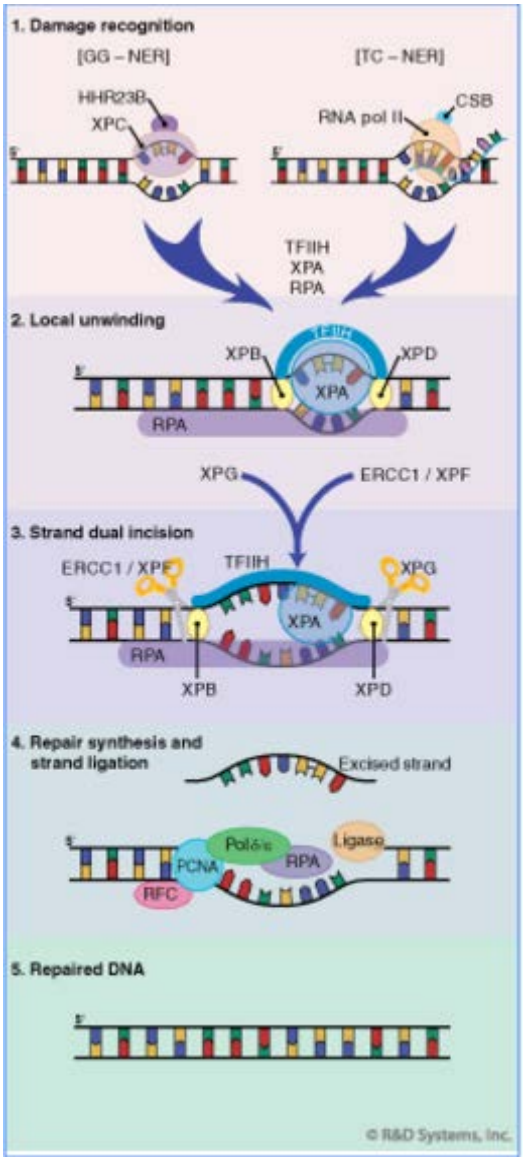


Figure: 1.18 Steps of Nucleotide Excision Repair (NER) mechanism. © R&D systems, Inc

The two pathways by which NER can detect the DNA damage are Global genome NER (GG-NER) and transcription coupled NER (TC-NER). Simplified steps of NER mechanism: Step (1) DNA recognition (2) Unwinding of DNA (3) Incision of DNA strand (4) synthesis of DNA and ligation (5) DNA repair completed. Human Rad23 homolog (HHR23B); Xeroderma pigmentosum complementation group C (XPC); CSA and CSB (Cockayne syndrome protein); Xeroderma pigmentosum complementation group A (XPA); Replication protein A (RPA); transcription initiation factor IIH (TFIIH); Xeroderma pigmentosum type B (XPB); Xeroderma pigmentosum type D (XPD); Xeroderma Pigmentosum Complementation Group G (XPG) protein; Excision repair 1 endonuclease non-catalytic subunit (ERCC1); trimeric proliferating cell nuclear antigen (PCNA) ring.

1.5.3 Fragile sites in genome

Chromosomal aberrations in tumour cells usually take place due to genomic instability. Various exogenous factors (UV radiation, ionizing radiation, chemotherapy) and endogenous factors (reactive oxygen species) can cause DNA damage (Jackson, 2002). Fragile sites are very susceptible to DNA breakage and several studies show they are involved in chromosomal aberrations responsible for cancer (O'Keefe and Richard, 2006). Fragile sites are regions of the human genome with breaks or gaps on metaphase chromosomes during partial inhibition of DNA synthesis (Richards, 2001). Fragile sites are termed as common or rare fragile sites based on their frequency in the population and the way they are induced. According to Genome Database (GDB), the frequency of rare fragile sites (RFS) is humans is less than 5% and they are segregated in Mendelian manner in specific families, whereas common fragile sites (CFS) are part of the human chromosomal structure are found in all individuals. Rare fragile sites are breakable due to expansion of a CGG repeat or an AT-rich island and are also capable of forming stable secondary structures, whereas common fragile sites contain no common sequences but show extensive regions of high flexibility and stable secondary structure formation. Previous studies suggest that all the fragile sites are late replicating portions of the genome and after exposure to inducing agents, replication could be even further delayed, and some alleles of fragile sites may remain unreplicated in late G2 (Hansen et al., 1997; Le Beau et al., 1998; Hellman et al., 2000; Handt et al., 2000; Pelliccia et al., 2008). Rare fragile sites are induced by folate deficient conditions or by chemical which bind to AT-rich DNA e.g. beneril (Sutherland et al., 1984). On the other hand, common fragile sites are induced by low doses of an inhibitor of DNA polymerase known as aphidicolin (APH) (Glover et al., 1984). Contrary to these findings, a study by Mrasek et al., (2010) on peripheral blood lymphocytes of three unrelated individuals showed that aphidicolin induced both RFS and CFS showing that they depend less on inducing chemical originally described before. Rare fragile sites are caused by repeat sequences which are unstable in nature (Sutherland and Baker, 2000) but there are no expanded repeat

sequences in common fragile sites (Smith et al., 1998). Some dietary and environmental factors such as caffeine, ethanol, pesticides and cigarettes smoke can also significantly increase breakage of fragile sites (Richards, 2001). When cells are cultured under normal conditions, fragile sites are stable but when they are treated with inhibitors of replication they become highly prone to breakages, exchanges and chromosomal rearrangements or deletions (Glover and Stein, 1987, 1988).

1.5.3.1 Role of fragile sites in cancer

About 90 CFS has been reported in the human genome having variable frequency of their expression. The most frequently expressed common fragile sites are FRA3B (3p14.2), FRA16D (16q23.2), FRAXB (Xp21.1) and FRA6E (6q26) (Smith et al., 2007). Many cancer genes have been reported to be present at or within fragile sites (Popescu, 2003). Studies also suggest that many oncogenic viruses target these chromosomal regions and integrate within them (Popescu, 2003). All this information suggests the possibility of fragile sites contribution towards development of cancer. A recent study by (Gandhi et al., 2010) in human papillary thyroid carcinoma suggests that the formation of cancer specific translocations was due to contributions of DNA breakages at fragile sites. All the common fragile DNAs which has been examined to date show AT-rich flexibility islands and show the capability of making secondary structures that are much more stable than other genomic regions (Mishmar et al., 1998; Zlotorynski et al., 2003). These fragile regions can span mega-bases of DNA having gaps or breaks throughout (McAvoy et al., 2007).

There are some other proteins which are important for maintenance of fragile sites such as ATR (Ataxia-telangiectasia and Rad3 Related)-dependent DNA damage checkpoint pathway and breakage of fragile site could enhance if there is deficiency of ATR (Casper et al., 2002; Casper et al., 2004) and its downstream targets i.e. BRCA1 (Arlt et al., 2004) and CHK1 (Durkin et al., 2006). These studies show that at fragile sites under replication stress the polymerases involved in replication could detach from the helicase resulting in single-stranded long DNA (ssDNA) regions which encourage the formation of stable secondary

structures. The replication stress activates ATR pathway and ATR bind with its partner protein ATRIP (ATR interacting protein) and recognize ssDNA coated with replication protein A (RPA) resulting in its activation. Once activated, it phosphorylates CHK1 resulting in activation of signalling cascades which end up in cell cycle arrest (Durkin and Glover, 2007).

1.4 Hypotheses

Hypothesis 1: Paclitaxel cytotoxicity is in part mediated through induction of ER stress (Chapter 3)

In this chapter I will attempt to answer the following:

- Can paclitaxel induce ER stress (*in vitro)* in ovarian (A2780, SKOV3, PEO1) and colorectal (HCT116) cancer cells lines?

- Can paclitaxel induced cell death be blocked by inhibiting the PERK pathway of ER-stress using Salubrinal?

- Can paclitaxel induced cell death be blocked by inhibiting ER-stress associated apoptotic pathways, such as Caspase-4, Caspase-12 or JNK?

Hypothesis 2: Expression of WWOX correlates with good progression free and overall survival in patients with high levels of ER stress markers (Chapter 4)

In this chapter I will attempt to answer the following:

- Do ovarian cancer patients with higher WWOX protein expression show improved overall and progression free survival?

- Do ovarian and colorectal cancer patients with higher WWOX mRNA expression show improved overall and progression free survival?

- Does the influence of WWOX expression on patient survival depend upon elevated expression of the ER-stress marker GRP-78?

Hypothesis 3: WWOX increases cisplatin induced cell death in an ER-stress dependent manner (Chapter 5)

In this chapter I will attempt to answer the following:

- Does cisplatin induce ER-stress response in cancer cell lines?

- Does WWOX expression increase cisplatin induced cell death in an ER-stress dependent manner?

Chapter 2 Materials and methods

2.1. Cell culture

Various cancer cell lines such as, ovarian cancer (A2780, SKOV3 and PEO1), skin cancer (SK-MEL-28), prostate cancer (PC3, LNCap) and colorectal cancer (HCT116, HCT116-p53null, HCT116-p21null and HCT116-baxnull) were used for experiments. The cells were cultured in RPMI-1640 medium (without HEPES) (Gibco, 31870-074) or DMEM(1X) (Gibco, 11880-28) mixed along with 10% FBS (foetal bovine serum) (PAA Gold A15-152, Sigma # F2442), 0.4 mM L-glutamine (PAA, M11-004 or Gibco #25030-024), 100 U/ml penicillin, 100 µg/ml streptomycin (PAA, P11-010 or Sigma # P4333), 1% amphotericin B (Sigma, A2942) in an incubator maintained at 37°C and 5% CO_2. A class II laminar flow safety cabinet was used to provide sterile environment for the cell culture work.

2.1.1 Passaging of cell lines

The adherent cell lines were grown in 75 cm^2 flasks until they reached 90-100% confluence (dependent upon cell line). The cells were washed with PBS (phosphate buffered saline) (Oxoid, BR0014) and then detached from the flask using enzyme trypsin (Sigma, T4549) before being split into new flasks containing RPMI medium and placed in an atmosphere of 5% CO_2 at 37°C.

2.2 Cell culture experiments:

2.2.1 Cell culture assays

Cell culture experiments performed were independent of each other and these experiments were repeated multiple times. At first, the suitable number of cells per well and suitable drug concentration was selected by treating various cell numbers with various drug concentrations. A drug dose which killed at least 50% of the cells after 24 hours was selected for the experiments. In the same way, the cell number selected was after looking at the survival percentage following drug treatments (see appendices S2.1). The concentrations used for the inhibitors were according to the manufacturer's instructions.

2.2.1.1 Cell culture experiments for paclitaxel and inhibitors studies

In these experiment log-phase various cancer cells per well (table 2.1) (in triplicate for each treatment) were plated in 96-well plate and placed in the incubator at 37^{o}C and 5% $CO_{2.}$ After 48 hours of incubation, media was removed from the wells and replaced with fresh media, DMSO (0.18 %), and media containing different concentration of drug solutions and plates were placed in the incubator for 72 hours at 37^{o}C and 5% CO_2. Salubrinal (Merck, 324895) was used as PERK inhibitor. Caspase-4 (Z-YVAD-FMK) (R&D, FMK005), Caspase-12 (Z-ATAD-FMK) (R&D # FMK013) and JNK inhibitor (SP600125) (Tocris Bioscience #1496) were used to inhibit Caspase-4. -12 and JNK respectively. Tunicamycin (Sigma, T7765) an inducer of ER-stress, was used as positive control. Paclitaxel (Sigma, T7402) was used to see if it generates any ER-stress.

Table 2.1: Cell type and cell numbers used in the experiments (see appendices S2.1)

Cell type	Cell numbers/well
A2780	15,000
SKOV3	6000
HCT116, HCT116-p53null, HCT116-p21null, HCT116-baxnull	6000
PEO1-H8 PEO1-FP2	12,000 12,000

2.2.2 Cell culture experiments for gene expression (GRP-78 or XBP-1) analysis

HCT116, PC3, LNCap and SK-MEL-28 cells (table 2.2) were plated in 6-wells plates and placed in incubator for 24 hours at 37^{o}C and 5% $CO_{2.}$ After 24 hours, media was removed from the wells and replaced with either fresh media or paclitaxel drug solutions of various concentrations (8nM, 16nM, 32nM, 64nM, 128nM and 40µM) and plates were placed in the

incubator. After 72 hours, plates were removed from the incubator and confluence in the wells was recorded.

Table 2.2: Cell types and cell numbers used in PCR experiments

Cell Type	Cells/well (6-well plate)
HCT116	350,000
PC3	250,000
LNCap	300,000
SK-MEL-28	300,000

2.2.3 Cell culture experiments for WWOX analysis

A2780, HCT116 and LNCap cancer cells (table 2.3) were plated in 6-wells plates and placed in an incubator for 24 hours at 37^{o}C and 5% CO_2. Next day, plates were removed from the incubator and media was removed from the wells and replaced with either fresh media, DMF or cisplatin and plates were placed in the incubator. Plates were removed from the incubator after 24 or 48 hours and confluence in the wells was observed under the microscope. Dead cells were clearly visible in cisplatin treated wells and confluence was low as compared to untreated and DMSO only treated cells.

Table 2.3: Cell types and cell numbers used for WWOX and FHIT experiments

Cell Type	Cell Numbers	Cisplatin concentration
HCT116	350,000	5µg/ml
A2780	500,000	4µg/ml
LNCap	600,000	2.5µg/ml

2.3 Trichloroacetic acid (TCA) fixing and sulforhodamine B (SRB) staining

After 72 hours of incubation, the cells in 96-well plates were fixed with 10%TCA (trichloroacetic acid) (Sigma T6399) and incubated for 1 hour at 4^{o}C. In the next step, plates were washed cold water, dried (by air or 37^{o}C) and stained with 0.4% SRB (sulforhodamine B) (Sigma, 230162) solution. Plates were then placed on a shaking platform for about 30 minutes and then washed with 1% acetic acid (Sigma, 320099) and dried once again (by air or 37^{o}C). Cell bound dye was re-solubilized with 10mM Tris (Sigma, 93362) and the absorbance was measured at 570nm wavelength using a spectrophotometer.

2.4 Extraction of RNA from cell lysates and DNASE I treatment of purified RNA in solution

Extraction of RNA was done by using kit (Isolate II RNA mini kit # Bioline-52072). In the first step cells were lysed using lysis buffer (from kit) mixed with β-mercaptoethanaol (β-ME). Cell were homogenised with the help of a cell scraper. In next step, 70% ethanol was added to the filtered lysates. The on-column method for digestion of DNA using enzyme DNASE I was proved not successful for me at early stages of my work, so I performed digestion of DNA in solution for enhanced removal of DNA contamination traces. For this purpose, DNase I enzyme was mixed with reaction buffer (from kit) and incubated for 10 minutes at 37^{o}C. In the next step (ethanol precipitation step), sodium acetate (3M, pH 5.2) was added followed by absolute ethanol. After centrifugation and drying, RNA pellet was re-suspended in water.

2.4.1. RNA concentration measurement

RNA concentration (ng/µl) was quantified and the purity was checked with Nanodrop 2000 spectrophotometer (Thermo scientific). The purity parameters were between the range of 1.8 – 2.1 for (260/280) and 2.0 – 2.3 for (260/230).

2.5 Synthesis of cDNA

Once the purity of RNA was checked by Nanodrop, the next step was to calculate the volume of RNA in 1µg by using the values obtained from Nanodrop spectrophotometer. 1µg equivalent of RNA was used to prepare all the samples of cDNA. For this purpose, Bioline tetro cDNA synthesis kit (BIO-65043) was used. This kit contains all the reagents (table 2.4) required for the preparation of complementary DNA (cDNA) from messenger RNA. The prepared cDNAs will be suitable for PCR using gene specific primers. The following reagents (table 2.4) were used in this method.

Table 2.4: Reagents used for the preparation of cDNA

Reagents	Volume
mRNA	(1µg equivalent volume) µl
Primer: Oligo (dT)18	1µl
10mM dNTP mix	1µl
5x RT Buffer	4µl
RiboSafe RNase Inhibitor	1µl
Tetro Reverse Transcriptase (200u/µl)	1µl
DEPC-treated water	to 20µl

Samples were mixed gently by pipetting and incubated at 45^{o}C for 30 minutes. The reaction was terminated by incubating at 85^{o}C for 5 minutes. The samples were chilled in ice and stored at -20^{o}C

Two types of cDNA samples were prepared, one with all the reagents and one with everything but excluding Ribosafe Rnase inhibitor (R) and tetro reverse transcriptase (T). Both types of samples will be run for PCR but samples without RT will help to confirm if there is any contamination of DNA in mRNA samples. All the cDNAs used either for reverse transcriptase PCR or Real time PCR were checked for genomic DNA contamination.

2.6 Polymerase chain reaction (PCR) for gene expression of GRP-78, XBP-1 and WWOX

After the preparation of cDNAs from cancer cell lines, in the next step polymerase chain reaction (PCR) was performed to see the gene expression of Glucose regulated protein (GRP-78), X-box binding protein 1 (XBP-1) and WWOX (primers sequence shown in table 2.7). The kit used for these experiments was Biotaq Red DNA Polymerase kit (Bio-21108). The total volume of the PCR master mix prepared was 25µl. cDNA template added to master mix was 2µl. Samples were run in Bio-Rad T100 thermal cycler for 35 cycles. The table 2.5 shows the reagents, volumes and concentrations used for these experiments. The table 2.6 show the cycling conditions used for PCR.

Table 2.5: Preparation of master mix for PCR

Reagents	**Final concentration**	**Volume/PCR tube**
5X My Taq red reaction buffer	1X	5µl
Forward Primer	0.8µM	1µl
Reverse Primer	0.8µM	1µl
My Taq red DNA polymerase	1unit	0.2µl
Water dH_2O		15.8µl

Table 2.6: PCR cycle conditions (30-35 cycles)

Cycle Steps	**Temperature ^oC**	**Time**
Initial heat	94	2 minutes
denaturation	94	20seconds
Annealing	55 53 (For WWOX)	20seconds
Extension	72	40seconds
	72	2 minutes

Table 2.7: Primers sequence used for PCR reactions

Primers	Sequence	Product size (bp)
GRP-78	F: CGTGGAATGACCCGTCTGTG R: CTTTGGTTGCTTGGCGTTGG	252
XBP-1	F: GCTCGAATGAGTGAGCTGGA R: AACTGGGCCTGCACCTG	263
β-actin	F: CTACGTCGCCCTGGACTTCGAGC R: GATGGAGCCGCCGATCCACACGC	385
GAPDH	F: TGTTGCCATCAATGACCCCTT R: CTCCA CGACGTACTC AGCG	292
WWOX (PCR)	F: CCGTGTCATTGTGGTCTCCT R: TATAAGCCAAGCATCGCCCAA	122
WWOX (QRT-PCR)	F: CAAGGACGGCTGGGTTTACT R: TGGCAAATCTCCTGCCACTC	100

At the end of the PCR cycles, samples were transferred from thermocycler onto ice or stored at 8^{o}C. 1% agarose gel (Sigma, A9539) was prepared and samples were run on the gel at 100V for 1 hour and then images of gel were observed under UV (Geldoc- it TS2 imager). Only for XBP-1 PCR samples, 2% agarose gel was prepared. 100bp DNA ladder (TrackIt^{TM}, Invitrogen # 10488-058) was used to match the product length of the desired gene of interest.

2.7 Quantification of gene expression by Real Time Polymerase chain reaction (QRT-PCR)

For QRT-PCR, the first step was to optimize conditions for the PCR and achieve a good standard curve. For this purpose, different primer sets (WWOX & FHIT) and conditions were used and once a desired standard curve was achieved, the next step was to run the cDNA sample mixed with the master mix and carry out PCR. The thermocycler machine used for these experiments was Rotor Gene 6000 (Rotor-Gene Q). Reference cDNA (prepared from 1µg RNA in 20µl) was used to prepare a standard curve from 5 serial dilutions (1:5, 1:25, 1:125, 1:625 and 1:3125). For analysis of the WWOX gene, cDNA from PEO1-H8 cells was used; for FHIT gene analysis, cDNA from HCT116 cells was used; for β-actin analysis, cDNA from SKOV3 cells was used. Sample cDNA from each treatment was diluted 1:20. The QRT-PCR kits used were (Qiagen # 204054, Bioline # 94005 and Biosystems # PB20.11-05). Table 2.8 shows the PCR cycling conditions.

Table 2.8: QRT-PCR cycling conditions

Conditions	Time	Temperature
PCR initial heat	2 or 5 minutes	95^{o}C
3 step cycling @ 45 cycles		
Denaturation	10 seconds	95^{o}C
Annealing	20 seconds	55^{o}C
Extension	20 or 30 seconds	60^{o}C
Acquiring @ Green	1 second	72^{o}C

2.7.1 Relative quantification analysis of qRT-PCR experiments

Relative quantification method was used for analysis of qRT-PCR experiments. All experiments were repeated in triplicate. The expression of each gene was determined by comparing Ct values against the relevant standard curve for that gene. Normalised expression for each gene was calculated from the ratios of each gene of interest to β-actin expression. Mean normalised expression and standard error were calculated by combining the triplicate experiments. Statistical significance was analysed by using Student T-test. $P < 0.05$ was considered statistically significant.

2.8 Kaplan-Meier survival data

Kaplan-Meier graphs were used to analyse the survival data from both sets of patients i.e. IHC and RNAseq (from TCGA). For Kaplan-Meier analysis tables were prepared using excel spread sheets containing the raw data and the variables such as survival time and status of the event (dead=1/ censored= 0). For analysis of WWOX, FHIT or GRP-78 expression alone, patients were split into higher than median expression or lower than median expression. For combined analysis of WWOX and GRP-78, patients were divided into four equal groups using the median WWOX and median GRP-78 expression levels (giving high WWOX/high GRP-78; high WWOX/low GRP-78; low WWOX/high GRP-78 and low

WWOX/low GRP-78 groups). Data was analysed by Statistical package for the Social Science (SPSS) software, where the Kaplan-Meier analysis and log rank test was performed.

2.9 Ethical approval for IHC data

Ovarian tumour specimens were available as formalin fixed, paraffin embedded tissue blocks from patients who underwent surgery and received treatment at the Hammersmith Hospital, London, United Kingdom. All slides were reviewed by Dr Mona El-Bahrawy and representative blocks of the tumours were selected for the study. Ethics committee approval was obtained from Hammersmith and Queen Charlotte's and Chelsea Research Ethics Committee (REC reference: 05/Q0406/178).

2.10 Protein analysis of NF-kB2 and PAR2

2.10.1 Western Blotting

After 24 or 72 hours of drug treatment, cells were washed with cold PBS. Lysis buffer (IX Laemmili buffer) (0.5M Tris hydrochloride (pH 6.8), SDS 20% stock, glycerol (100%) stock and distilled water) mixed with protease inhibitor cocktail (P8340-1ML) was added to the cells and lysates were prepared with the help of cell scrapper. Lysates were homogenised with a 27G needle and syringe. Lysates were then centrifuged at 4^{o}C and 12,000 rpm in a centrifuge machine. 80µl of lysate was transferred into another tube which was used for BCA assay. 12µl of DDT (dithiothreitol) (1M) was added into remaining 120µl of lysate (0.1M final conc. of DTT) and tubes containing lysates were heated for 5 minutes at 95^{o}C. All the tubes containing lysates were stored at -20^{o}C or used for the next step. To quantify the total protein concentration of the lysates, Bicinchoninic acid (BCA) assay kit (Pierce^{R} # 23227) was per manufacturer's protocol. Protein samples were prepared by using the right volumes of lysates, lysis buffer (IX Laemmili buffer) and loading buffer (IX Laemmili buffer containing 0.1M DTT and bromophenol blue) and heated for 5 minutes at 95^{o}C. The proteins were separated using Mini-Protean^{R} TGX^{TM} precast gels (12%) (BIO-RAD # 456-1043) using 1X

Running buffer (trizma base 25mM, glycine 192mM, SDS 20% and water). Prepared lysate samples were loaded in to the wells of the gel along with a protein ladder (EZ RUN^{TM} # BP3603) and run @ 200V. In the next step, proteins were transferred onto a nitrocellulose membrane (0.45µm) (BIO-RAD # 1620-0115) in the presence of 1X Transfer buffer (trizma base 25mM, glycine 192mM, water, methanol and SDS 20%). Once the transfer was complete, membranes were then blocked in 5% fat free milk in PBS-T (phosphate buffer saline-tween) for 2 hours. Membranes were blocked with primary antibodies (diluted appropriately in 5% blocking solution) overnight at 4^{o}C on a shaking platform. Next day, membranes were washed six times with PBS-T and blocked with secondary antibodies (diluted appropriately in 5% blocking solution) for 1 hour at room temperature on a shaking platform. Membranes were washed 6 times with PBS-T. Membranes were incubated with Pierce^{R} ECL 2 western blotting substrate per manufacturer's protocol. X-ray films (CL-XPosure^{TM} film 5 x7 inches) (Thermo scientific # 34088) were used to visualise the protein bands. The details of the antibodies used are in appendix 2.

2.11 RNA interference experiments

2.11.1 esiRNA knockdown of NF-kB expression in A2780 and HCT116 cells

A2780 and HCT116 log phase cells were counted by haemocytometer and approximately 700,000 cells/well were plated in 6-wells plates in the presence of RPMI media mixed with 10% FBS, 1% Penicillin/streptomycin, 1% Amphotericin B and 1% L-glutamine and placed in the incubator for 24 hours. Next day esiRNA treatments were prepared and for this purpose RPMI serum free media was used. 200µl of serum free media was transferred into 3 tubes A, B and C. In tube A 4µl (53.3pmol) of esiRNA (Mission esiRNA for human NF-kB2 # EHU067311-50UG), in tube B 4µl (53.3pmol) of GFP (Mission esiRNA targeting EGFP # EHUEGFP) and in tube C only serum free media was added. 12µl of transfection reagent (S1452-100UL) was added into all three tubes and incubated at room temperature for 15 minutes. Media was removed from the wells of 6-wells plates and replaced with contents of tubes A, B, C and a well with serum free media only. Plates were incubated for 24 hours at

37^{o}C in the presence of 5% CO_2. Next day cells were trypsinized and re-suspended in RPMI media and counted in haemocytometer. Approximately 500,000 cells per well were plated in new 6-wells plates and placed in incubator. Next day, plates were removed from the incubator and media was replaced with either fresh media or DMSO or 4µg/ml cisplatin drug solution. After 72 hours' plates were removed from the incubator and cell lysates were prepared for western blotting according to the protocol described in section 2.10.1.

2.11.2 esiRNA knockdown of NF-kB expression in A2780 using different cell numbers

In this experiment, different cell numbers were used for siRNA knockdown of NF-kB. 500,000, 700,000 and 900,000 log phase cells of A2780 were plated in 6 -wells plates in the presence of RPMI media mixed with 10% FBS, 1% Penicillin/streptomycin, 1% Amphotericin B and 1% L-glutamine and placed in the incubator for 24 hours. The siRNA knockdown was done according to the protocol described in section 2.11.

2.11.3 Knockdown of NF-kB expression in A2780 using different esiRNA concentrations

700,000 log phase A2780 cells were plated in 6 -wells plates in the presence of RPMI media mixed with 10% FBS, 1% Penicillin/streptomycin, 1% Amphotericin B and 1% L-glutamine and placed in the incubator for 24 hours. The siRNA knockdown was done according to the protocol described in section 2.11 but the only difference was 3µl of esiRNA (Mission esiRNA for human NF-kB2 # EHU067311-50UG) was used.

2.12 Fluorescence activated cell sorting (FACS) analysis

The first step was calibration of the instrument followed by preparation of samples. All samples were prepared as single cell suspension and filtered using a 70µm cell strainer prior to acquisition. EDTA was used to minimize cell clumping. Samples were kept dilute avoiding any clouding. FACS buffer (PBS+2%FCS+0.05% sodium azide) was used for the cells. Single cell suspension cultures were loaded on the SIP and acquired. For control unstained and single stain fluorescence were used.

Chapter 3: The role of ER stress in paclitaxel response

3.1 Introduction

3.1.1 ER stress or unfolded protein response (UPR)

In addition to its role in mitotic arrest, several studies suggest that apoptosis induced by Paclitaxel is due to endoplasmic reticulum (ER) stress also known as unfolded protein response (UPR) (Liao et al., 2008). UPR is a stress response to unfolded proteins in the ER resulting from disturbance in cellular redox regulation, viral infections or glucose deprivation (Ma & Hendershot 2004). The purpose of UPR is to re-establish the normal ER function by maintaining the homeostasis, through inhibition of translation and up-regulation of molecular chaperones (Chunyan et al., 2005).

All organisms have a mechanism by which proteins are properly folded for their proper functioning. In all eukaryotes, much of the protein assembly takes place in endoplasmic reticulum where they are properly folded before being passed on to the Golgi apparatus (Kaufman, 2002). Only properly folded proteins can leave ER to other intracellular organelles or to be displayed on the cell surface. Misfolded proteins are not allowed to leave ER and form a complex with molecular chaperones inside lumen of ER or they are degraded through a 26S proteasome governed by a process called ER-associated degradation (ERAD). Misfolded proteins can also be destroyed by autophagy (Malhotra and Kaufman, 2007). The process of protein folding can only work efficiently if conditions (environmental, genetic and metabolic) are suitable or it can affect the survival of the cell. The lumen of ER is an oxidizing environment where sulphide bonds are prepared and this process of formation of sulphide bonds is governed by multiple protein disulphide isomerase (PDIs) which make sure proper disulphide bonds are formed. ER serves as primary site for storage of Ca^{2+} which is very important for folding of proteins and functioning of calcium dependent molecular chaperones (GRP-78, GRP-94 and calreticulin). These chaperones, especially GRP-94, slow down the protein folding and stabilize them (Ma and Hendershot, 2004).

Failure in this process results in unfolded protein response (UPR) (Schröder and Kaufman, 2005).

3.1.1.1 Signal transduction events associated with ER stress

In a mammalian cell the unfolded protein response is initiated by one ER chaperone, Glucose regulated protein (GRP-78) and three ER transmembrane proteins; Activating transcription factor 6 (ATF6), Inositol requiring enzyme 1 (IRE1) and double stranded RNA-activated protein kinase like ER kinase (PERK) (Figures 3.4 and 3.5) (Harding et al., 2002) and (Zhang and Kufman, 2004). During non-stress conditions GRP-78 binds to the luminal domains of PERK, IRE1 and ATF6, and maintains them in an inactive state but during stress conditions in ER, when misfolded proteins accumulate, GRP-78 prefers to bind to these misfolded proteins and release (PERK, IRE1 and ATF6) allowing their activation and results in unfolded protein response (Rao & Bredesen, 2004). After dissociation from GRP-78, PERK dimerizes, auto phosphorylates, and triggers phosphorylation of eukaryotic translation initiation factor 2 on the alpha subunit (eIF2α). This attenuates mRNA translation, thereby preventing influx of newly synthesized polypeptides into the ER lumen of the stressed cell (Kaufman, 2004). Some selected mRNAs such as ATF4 evade this inhibition of translation. ATF4 on activation can increase levels of chaperones, restore cellular redox homeostasis, and help the ER to degrade or fold proteins (Harding et al., 2000).

During stress conditions, another transmembrane protein IRE1 is released by GRP78. IRE1 is a bifunctional protein with kinase and endoribonuclease (RNase) activity. It dimerizes, and auto phosphorylates to activate its RNase activity. This activation results in removal of a 26-nucleotide intron from X-box binding protein (XBP1) mRNA. Spliced XBP-1 is a transcriptional factor which activates many UPR target genes (Malhotra and Kaufman, 2007).

ATF6 is a bZIP family transcription factor. Following the activation of ER stress, GRP-78 release ATF6, which then translocate to the Golgi apparatus by vesicular transport. ATF6 is

cleaved in Golgi apparatus by proteases and the cytoplasmic portion translocates to the nucleus where it binds to ER stress element (ERSE) to activate transcription of ER chaperone genes such as BIP/GRP-78, GRP-94 and calreticulin (Kaufman, 2004).

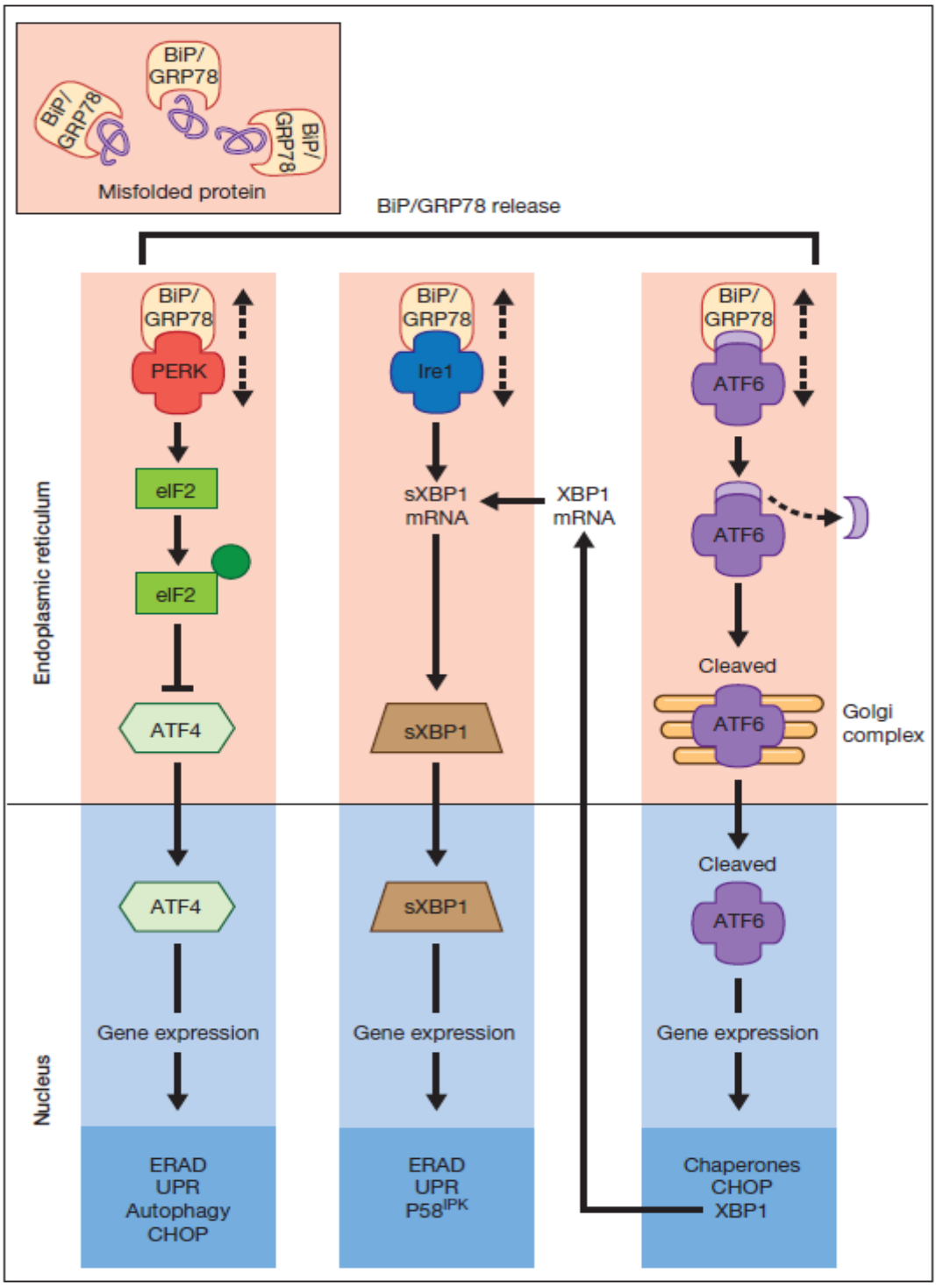


Figure 3.1: **Signal transduction events associated with ER stress** (Smith, 2013)

Misfolded proteins aggregate causing Grp78 to release three ER resident transmembrane proteins (PERK, IRE1 and ATF6) and activate them. These signalling pathways lead to unfolded protein response (UPR) including inhibition of translation, increased chaperone production, ER associated protein degradation (ERAD), autophagy and apoptosis.

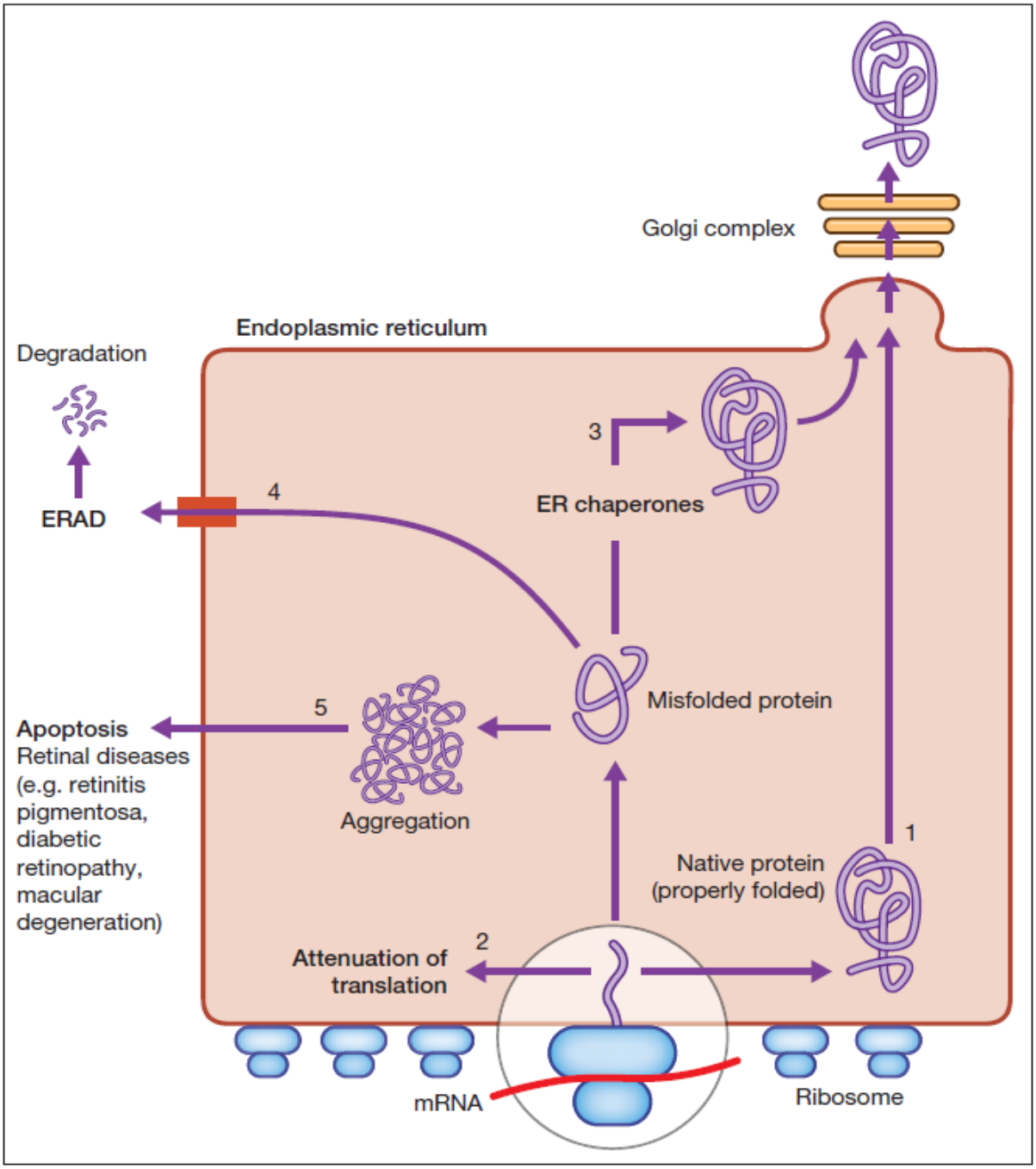


Figure 3.2: **Overview of the unfolded protein response (UPR).** Ribosome attaches itself to ER membrane for translation of nascent protein. Failure to process these proteins leads to UPR including (2) temporary suspension of protein synthesis; (3) increased protein folding capacity by transcriptional induction of ER chaperone genes; (4) disposal of the misfolded protein by ER-associated degradation (ERAD), or upregulation of apoptosis (5) causing cell death (Smith, 2013).

3.1.2 Apoptosis induced by ER stress

When UPR fails in maintaining the proper protein folding capacity of the ER then ER stress signals the induction of apoptosis (Figures 3.5 and 3.6). Various mechanisms which could generate ER stress induced apoptosis include:

1- Apoptosis mediated by CHOP
2- Apoptosis mediated by IRE1
3- Apoptosis mediated by caspases
4- Apoptosis mediated by mitochondria

3.1.2.1 Apoptosis mediated by CHOP

CHOP a member of CCAAT/enhancer binding protein (C/EBP) family is a bZIP transcription factor (Ron and Hebener, 1992). CHOP/GADD153 (growth arrest and DNA damage 153) is activated by prolonged ER stress and induces apoptosis (Harding et al., 2000; Okada et al., 2002). Although it can be transcribed by IRE1, ATF6 and PERK pathways, PERK is considered the most significant pathway (Ma et al., 2002; Harding et al., 2000; Okada et al., 2002). CHOP activates transcription of genes such as GADD34, ERO1α, DR5, TRB3 and carbonic anhydrase VI which can initiate apoptosis. eIF2α is de-phosphorylated by GADD34 on serine 51 resulting in translational recovery which will increase protein synthesis. Continuous synthesis of proteins during ER stress can cause cell death by UPR (Marciniak et al., 2004; Novoa et al., 2001). Transcription of ERO1α (an oxidoreductase) increases the oxidizing capacity of the ER (Marciniak et al., 2004), and carbonic anhydrase VI lowers intracellular pH (Sok et al., 1999) which will enhance the amount of misfolded proteins. DR5 (death receptor 5) can activate caspase cascades (Yamaguchi et al., 2004). Anti-apoptotic protein Bcl-2 is transcriptionally repressed by CHOP resulting in downregulation of it expression. CHOP increases reactive oxygen species (ROS) which are also involved in ER stress induced apoptosis (Harding et al., 2003; Haze et al., 1999).

3.1.2.2 Apoptosis mediated by IRE1

IRE1 responds to ER stress by recruiting adaptor protein tumour necrosis factor (TNF) receptor associated factor 2 (TRAF2) to the ER membrane (Urano et al., 2000). This is controlled by c-jun NH_2 –terminal inhibitory kinase (JIK) which communicates with both IRE1 and TRAF2 (Yoneda et al., 2001). IRE1 and TRAF2 recruit and activate ASK1 and the downstream JNK pathway causing apoptosis (Nishitoah et al., 2002; Nishitoah et al., 1998). TRAF2 is also involved in activation and regulation of Caspase-12 (Yoneda et al., 2001). Cyclin AMP-dependent transcription factor ATF-3 is also activated by IRE1 and TRAF2 complex, generating apoptosis (Zhang et al., 2001). The mechanisms by which JNK initiate apoptosis are not fully clear but perhaps regulate Bcl-2 family of proteins as well (Lai et al., 2007).

3.1.2.3 Apoptosis mediated by Caspases

It has been reported that Caspases 2, 3, 4, 7, 9 and 12 participate in apoptosis induced by ER stress. Caspase-12 (in mice) is positioned on the cytoplasmic side of the ER and is cleaved and activated during ER stress by Calpains (a family of Ca^{2+} dependent cysteine proteases) (Tan et al., 2006; Nakagawa et al., 2000a; Nakagawa et al., 2000b). Mitochondrial pro-apoptotic Bcl-2 family members BAX and BAK positioned at the ER membrane interacts with Caspase-12 and initiate apoptosis (Cheung et al., 2006; Di Sanoet al., 2006; Hitomi et al., 2004). Caspase-12 activates caspase-9, which sequentially activates caspase-3 causing apoptosis (Morishima et al., 2002).

Caspase-7 also responds to ER stress and activates caspase-12 by cleavage (Rao et al., 2001), as can TRAF2 (Yoneda et al., 2001). The ER stress induced apoptotic role of caspse-12 in humans is questionable, however, because in human caspase-12 is inactive due to several mutations (Fischer et al., 2002). It has been reported that in human caspase-4 is homologue of mice caspase-12 and it serves the functions of caspase-12. It has been shown

that ER stress is responsible for cleaving caspase-4 and when caspase-4 was knocked down it reduced apoptosis induced by ER stress (Hitomi et al., 2004).

3.1.2.4 Apoptosis mediated by mitochondria

Several studies suggest the role of mitochondrial Bcl-2 family of proteins in ER stress induced apoptosis. Both Bax and Bak are present on mitochondria and ER membrane (Scorrano et al., 2003; Zong et al., 2003). During ER stress conditions. Bax and Bak undergo oligomerization and conformational change in the ER membrane and initiate apoptosis by two separate pathways (Zong et al., 2003). In mitochondrial independent pathway, the conformation change in the ER allows Ca^{2+} to move from ER into the cytoplasm which lowers the concentration of Ca^{2+} inside ER (Zong et al., 2003; Scorrano et al., 2003). The high concentration of Ca^{2+} in the cytosol results in activation of m-calpain which in turn activates caspase-12, inducing apoptosis as described above (Nakagawa et al., 2000a; Morishima et al., 2002; Tan et al., 2006). In mitochondrial dependent pathway, Ca^{2+} from the cytosol enters into mitochondria and causes depolarization of the inner mitochondrial membrane resulting in the release of cytochrome c (Crompton, 1999). Released cytochrome c activates formation of an apoptosome comprised of apoptosis protease activating factor1 (Apaf-1), pro-caspase9, cytochrome c and ATP. This apoptosome complex activates caspase-9 which then activates caspase-3 leading to apoptosis (Crompton, 1999; Shibue and Tanigichi, 2006). Apart from Bax and Bak, during ER stress conditions other BH3 domain family members such as PUMA (p53 up-regulated modulator of apoptosis) and NOXA (neutrophil NADPH oxidase factor) are also up regulated by p53 (Li et al., 2006).

BIK (Bcl2 interacting killer) is also present in ER and its pro-apoptotic nature helps ER for the deployment of Bax and Bak at its surface (Mathai et al., 2005). Expression of Bcl2 is supressed due to high expression of CHOP which cause activation of pro-apoptotic Bcl2 proteins (McCullough et al., 2001). JNK can phosphorylate Bcl2 resulting in inhibition of Bcl2

binding to pro-apoptotic Bax and Bid proteins (Bassik et al., 2004). JNK can also phosphorylate Bim (Bcl2 interacting mediator of cell death), releasing Bim to activate caspase-12 during ER stress conditions (Lei and Davis, 2003). These findings show the role of CHOP and JNK in mediating ER stress induced apoptosis by regulating mitochondrial Bcl2 proteins.

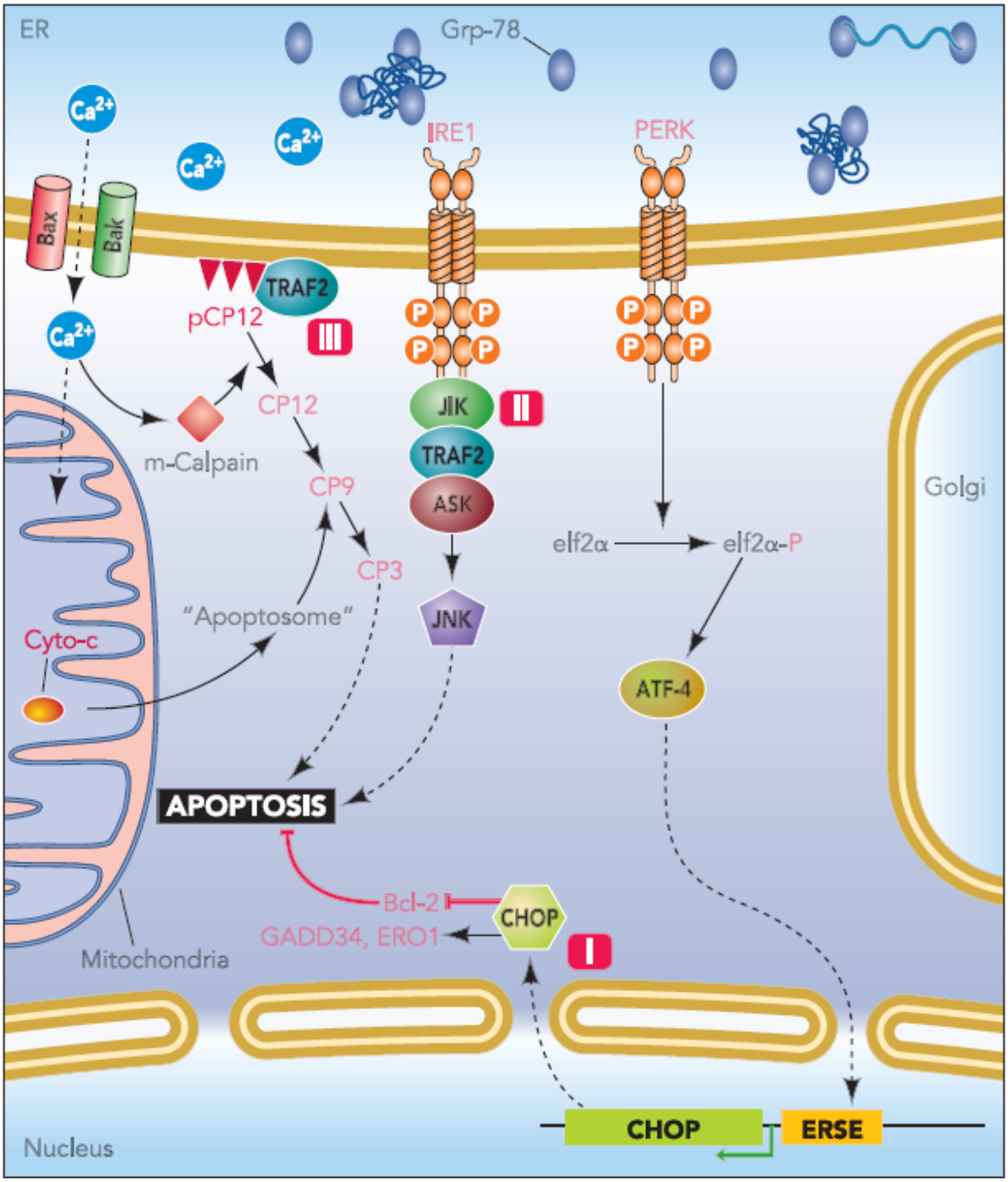


Figure 3.3: ER stress pathways involved in apoptosis. *I*: PERK activates and phosphorylates eIF2α which enhances translation of ATF4 transcription factor which upregulate target genes via ERSE promotor resulting in induction of CHOP. Activated CHOP downregulates the expression of the anti-apoptotic protein Bcl-2 and induces GADD34 and ERO1. *II*: After activation IRE1 binds JIK and recruits TRAF2, which leads to the activation ofASK1 and JNK. JNK phosphorylates Bcl-2 and BH3-only protein (Bim), initiating mitochondria-mediated apoptosis (not shown). *III*: Attached on the inner

side of ER membrane, TRAF2 dissociate from procaspase-12 (pCP12) hence allowing activation of procaspase-12. Under ER stress conditions Bax and Bak residing in the ER membrane oligomerize and permits the release of Ca2+ from the ER to the cytosol, resulting in activation of m-Calpain, which subsequently cleaves and activates pCP12. Activation of caspase-12 (CP12) cleaves and activates procaspase-9, which then activates downstream caspases such as caspase-3. In addition, Ca2+ released from the ER is taken up by the mitochondria, altering mitochondrial inner membrane depolarization and allow cytochrome c to move into the cytoplasm. The cytochrome c forms an apoptosome (consisting of Apaf-1, cytochrome c, ATP, and procaspase-9), activation of procaspase-9, and subsequent downstream caspases leading to cell apoptosis (Lai et al., 2007).

3.2 Experimental Approach

In this chapter the aim was to see induction of ER stress in ovarian cancer cells (A2780, SKOV3, PEO1-H8 and PEO1-FP2) and colorectal cancer cells (HCT116, HCT116-p53null, HCT116-p21null, HCT116-baxnull) following paclitaxel treatment, and to determine the key ER-stress pathway(s) used by paclitaxel to kill cancer cells. The key ER stress pathways under investigation were PERK and apoptotic pathways involving Caspase-4, Caspase-12 and JNK. Before the start of experiments the first step was the optimization of suitable drug dose concentrations to be used for experiments (see details in appendices section S2). Ovarian and colorectal cancer cells were treated with paclitaxel to induce ER stress and salubrinal was used to inhibit induction of ER stress via PERK pathway. Inhibitors such as Z-YVAD-FMK and Z-ATAD-FMK were used to inhibit ER stress related apoptotic components Caspase-4 and Caspase-12 respectively, while SP600125 was used to inhibit c-jun (JNK) arm of apoptosis. Tunicamycin was used as positive control of ER stress activation. Cell survival experiments were performed by using SRB staining technique. SRB is a dye widely used to study *in vitro* cytotoxicity. SRB works by binding to protein constituents of the cells in a stoichiometric manner. The principle behind this technique is, the extracted dye from stained cells is directly proportional to the cell mass. In my experiments the survived cells after treatment with drugs or inhibitors will retain this dye which on measurement with spectrophotometer will show the cell survival. The aim was to characterise the ER-stress related cell death mechanisms induced due to paclitaxel treatment. After treatment of cancer cells with paclitaxel, ER stress may be induced via PERK pathway which will be inhibited by salubrinal. Similarly, ER stress induced by apoptosis will be inhibited by using inhibitors of Caspase-4, Caspase-12 and c-jun (JNK). Induction of paclitaxel induced ER stress in cancer cells will be confirmed by using these inhibitors.

3.3 Results

3.3.1 Paclitaxel treatment and PERK pathway inhibition in A2780 and SKOV3

In this experiment, ovarian cancer cells A2780 and SKOV3 were treated with paclitaxel in combination with salubrinal (Table 3.1). The aim of these experiments was to determine if salubrinal could protect the cells from paclitaxel and tunicamycin. Results of A2780 (Figure 3.4) show the relative cell survival percentage of drug and inhibitor treated cells as compared to untreated and DMSO treated cells. In A2780 relative cell survival of paclitaxel treated cells was 58% as compared to untreated or No Drug (ND) 100% which shows paclitaxel has reduced the number of cells possibly due to ER stress induced apoptosis. Cells treated with tunicamycin (an ER stress inducer) showed cell survival of 88% as compared to ND but it is not significantly less. Cells treated with two different concentrations of salubrinal (an ER stress inhibitor) (30µM and 60µM) did not show any significant effect on cell number as compared to ND. Cells with DMSO treatment showed almost no change in their relative cell survival which was 98% as compared to ND. Combination of paclitaxel and two different salubrinal concentrations (60µM and 30µM) showed relative cell survival of 52% and 50% respectively as compared to 58% of pacliatxel only treated cells which show that salubrinal did not reverse the effects of pacliatxel. Cells treated with combination of tunicamycin and two different concentrations of salubrinal (30µM and 60µM) showed 90% and 88% relative cell survival respectively as compared to 88% of tunicamycin only treated cells. It is unsurprising that salubrinal did not reverse the effect of tunicamycin because tunicamycin itself did not cause any significant change in cell survival as compared to ND.

Results of SKOV3 (Figure 3.4) show the relative cell survival percentage of drug and inhibitor treated cells as compared to untreated and DMSO treated cells. Paclitaxel treated cells showed 10% relative cell survival as compared to ND possibly due to ER stress induced apoptosis. Combination of paclitaxel and two different salubrinal concentrations (60µM and 30µM) showed relative cell survival of 16% and 18% respectively showing that salubrinal did not reverse the effects of paclitaxel massively. Tunicamycin treated cells

showed 90% relative cell survival as compared to ND. The combination of tunicamycin and two concentrations of salubrinal (60µM and 30µM) showed 96% and 92% realtive cell survival showing the reversal effect of salubrinal is negligible. Salubrinal only (60µM and 30µM) treatments showed no significant effect on cell number as compared to ND. DMSO also did not show any effect on cell number as compared to ND.

Surprisingly in these results my positive control of ER stress i.e. tunicamycin did not show any significant effect on cell numbers as compared to ND. This suggests maybe it is not inducing ER stress in these ovarian cancer cells. However, paclitaxel showed less cell numbers as compared to ND, but surprisingly salubrinal did not reverse this effect which raises this question that may be the reduced cell number in paclitaxel treated cells was not due to ER stress but some other cytotoxic effect of paclitaxel which salubrinal could not reverse.

Table 3.1: Explanation of codes for drugs and inhibitors combinations used in graphs

ND	No drug
Sal 60	Salubrinal 60μM
Sal 30	Salubrinal 30μM
Tun	Tunicamycin 200ng/ml
T+S60	Tunicamycin 200ng/ml + Salubrinal 60μM
T+S30	Tunicamycin 200ng/ml + Salubrinal 30μM
Pac	Paclitaxel
P+S60	Paclitaxel+ Sal 60μM
P+S30	Paclitaxel+ Sal 30μM

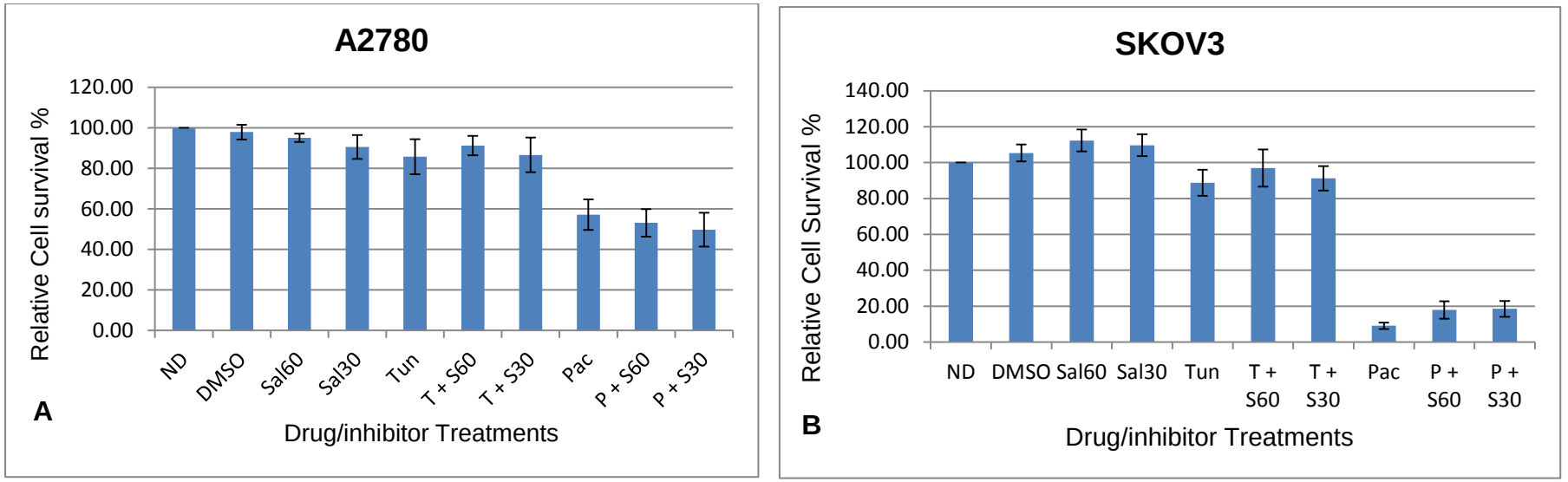


Figure 3.4: Relative cell survival in ovarian cancer cell lines after ER stress inhibition by salubrinal.

(A) A2780 cells were treated with paclitaxel (P or Pac) (16 nM), salubrinal (Sal or S) (30 μM and 60 μM), tunicamycin (Tun or T) (200 ng/ml), or in combination with each other. **(B)** SKOV3 cells were treated with paclitaxel (P or Pac) (20 nM), salubrinal (Sal or S) (30 μM and 60 μM), tunicamycin (Tun or T) (200 ng/ml), or in combination with each other. After 72 hours, spectrophotometric analysis was used (post SRB staining) to observe cell number. Experiments were repeated seven times for A2780 and six times for SKOV3. Means ± SEM are shown.

3.3.2 Paclitaxel treatment and PERK inhibition in HCT116 isogenic cells

Drug responses differ between cell lines due to different genetic backgrounds. Since salubrinal failed to reverse paclitaxel response in A2780 or SKOV3 cells, we tried repeating these experiments using colorectal cancer HCT116 isogenic cell lines. These were HCT116 cells but lacking p53, p21 and bax genes, so they are named as HCT116, HCT116-p53null, HCT116-p21null and HCT116-baxnull.

3.3.2.1 Is the PERK pathway required for the paclitaxel-induced reduction in cell number in HCT116 isogenic cell lines?

The aim of these experiments was to see if PERK pathway is required for paclitaxel induced reduction in cell numbers. HCT116 isogenic cell lines were treated with paclitaxel, salubrinal, tunicamycin alone or in combination with each other and surviving cell numbers determined by SRB staining. The results showed that (Figure 3.5) paclitaxel reduced the number of cells as compared to ND and DMSO treated cells.

After paclitaxel treatment HCT116 showed (62%), HCT116-p53null (78%), HCT116-p21null (54%) and HCT116-baxnull (59%) cell numbers as compared to untreated and DMSO treated cells. The cells treated with combination of paclitaxel and two different concentrations of salubrinal (60μM and 30μM) did not show reversal of the paclitaxel response but rather an even greater reduction in cell survival, HCT116 (34% & 36%), HCT116-p53null (34% & 40%), HCT116-p21null (28% & 34%) and HCT116-baxnull (30% & 36%) respectively as compared to paclitaxel-only treated cells.

Tunicamycin treated HCT116 cells showed (45%), HCT116-p53null (45%), HCT116-p21null (68%) and HCT116-baxnull (48%) relative cell numbers as compared to ND cells. The cells treated with combination of tunicamycin and salubrinal (60μM and 30μM) showed that HCT116 showed (30% and 32%), HCT116-p53null (28% and 30%), HCT116-p21null (28%

and 36%) and HCT116-baxnull (28% and 36%) cell numbers as compared to tunicamycin only. These results show salubrinal did not reverse the effects of tunicamycin. Interestingly salubrinal itself caused a reduction in cell survival and both concentration of salubrinal showed approximately 40%-44% cell numbers as compared to ND. Salubrinal is an ER stress inhibitor but it looks like it is having some type of cytotoxic affect as well which is contrary to what we observed in the results of ovarian cancer A2780 and SKOV3 cells (Figure 3.4).

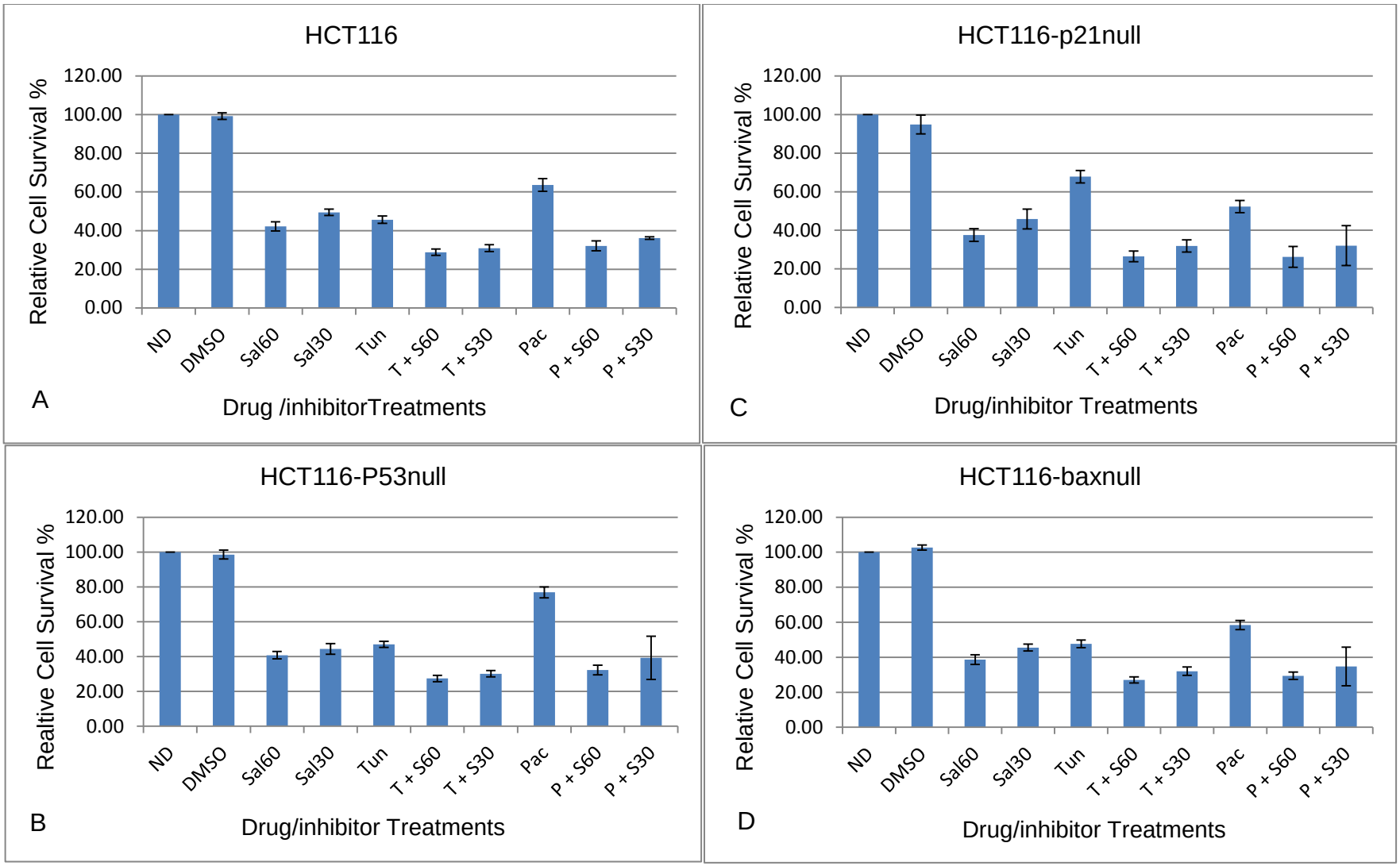


Figure 3.5: Relative cell survival in colorectal cancer cell lines after ER stress inhibition by Salubrinal HCT116 isogenic cell lines were treated with either paclitaxel (P or Pac) (1nM), salubrinal (S) (30 μM and 60 μM), tunicamycin (T or Tun) (75 ng/ml), or in combination with each other. After 72 hours, spectrophotometric analysis was used (post SRB staining) to observe cell survival. Experiments were repeated three times independently of each other. Means ± SEM are shown

3.3.2.2 Is Caspase-4 required for the paclitaxel-induced reduction in cell number in HCT116 isogenic cell lines?

The data from the previous section suggest that PERK pathway is not relevant to paclitaxel response, so in these experiments importance of UPR associated caspases was observed.

The aim of these experiments was to see if I can reverse the effects of paclitaxel induced cell death in HCT116 isogenic cell lines by inhibiting caspase-4 using a specific caspase-4 inhibitor (Z-YVAD-FMK) which was a cell permeable, fluoromethyl ketone (FMK) peptide with no cytotoxic effects. It inhibits apoptosis by irreversibly binding to catalytic site of caspase-4 protease.

HCT116 isogenic cells were treated with caspase-4 inhibitor, tunicamycin, paclitaxel or in combination with each other. Results show that paclitaxel treatment alone did not show any big difference in cell numbers as compared to ND (Figure 3.6). HCT116, HCT116-p53null, and HCT116-baxnull cells showed approximately 98% and HCT116-p21null cells showed 88% survival as compared to ND. The combined treatment of paclitaxel and caspase-4 inhibitor did not show much difference in cell numbers as compared to paclitaxel alone for all four cell lines. Previously I have shown paclitaxel causes reduced cell numbers as compared to ND, but in these experiments, paclitaxel did not show a visible reduction in cell numbers which is different to previous results. This suggests a possible problem with the paclitaxel treatment in these experiments, although the result was consistent in all three replicate experiments.

However, tunicamycin treated cells showed a reduction in cell numbers as compared to ND. All four cell lines showed cell numbers between 58%-62% as compared to ND, but combined treatment of caspase-4 inhibitor and tunicamycin showed no reversal of this response in HCT116, HCT116-p53null and HCT116-baxnull cells. Whereas in HCT116-p21null combined caspase-4 inhibitor and tunicamycin treatment showed 79% cell survival compared to tunicamycin alone (68%). This reversal effect of caspase-4 inhibitor looks

minimal. DMSO (95%-100%) only treatment showed almost no change as compared to ND in all four cell lines. Overall these results could not establish any involvement of caspase-4 in paclitaxel or tunicamycin response, although there may have been a problem with the paclitaxel response in these experiments.

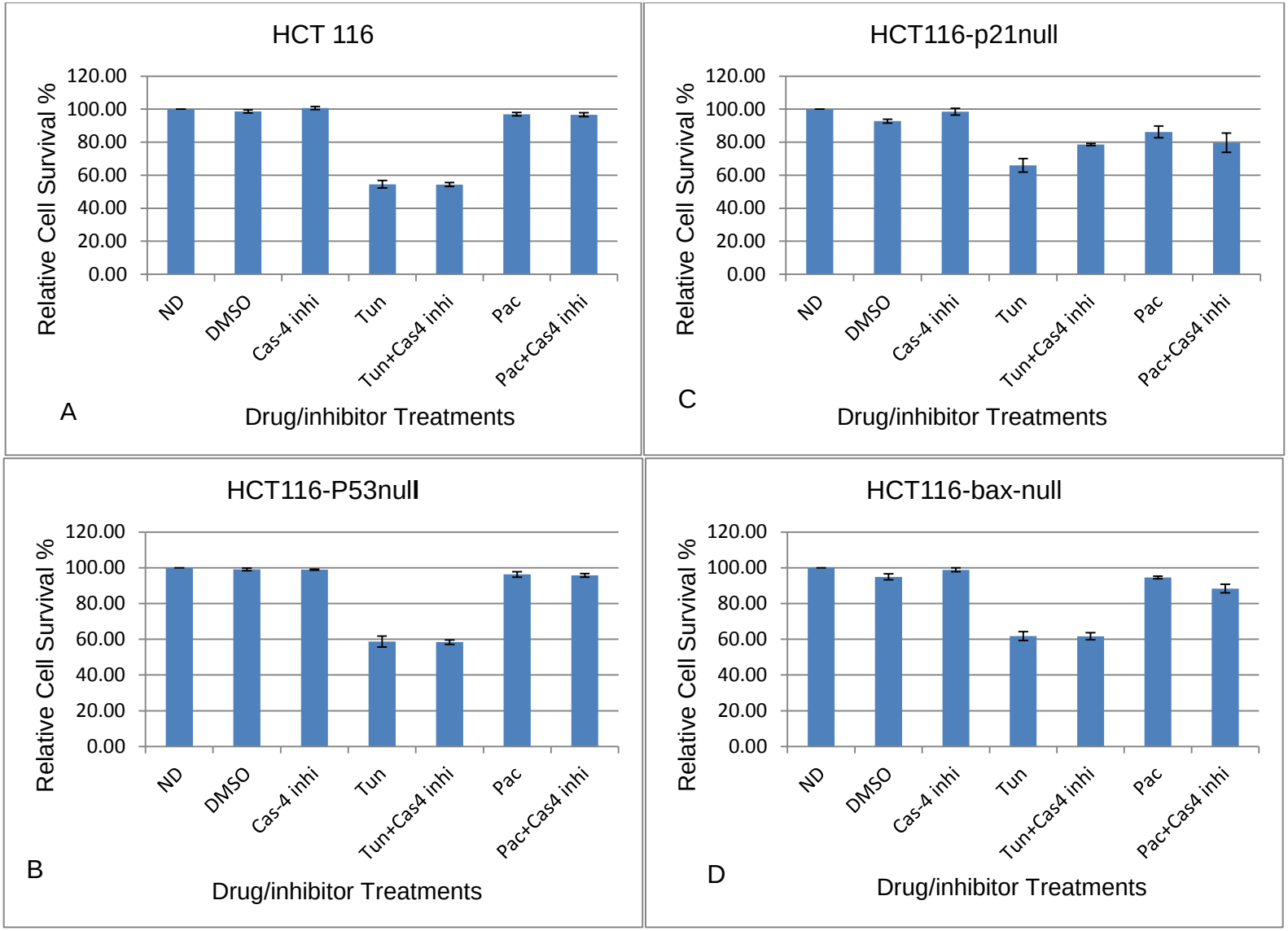


Figure3.6: Relative cell survival in colorectal cancer cell lines after ER stress inhibition by inhibitor for Caspase-4 (Z-YVAD-FMK)

HCT116 isogenic cell lines were treated with either paclitaxel (Pac) (1nM), Caspase-4 (Z-YVAD-FMK) inhibitor (10 μM), tunicamycin (Tun) (75 ng/ml), or in combination with each other. After 72 hours, spectrophotometric analysis was used (post SRB staining) to observe cell survival. Experiments were repeated three times independently of each other. Means ± SEM are shown.

3.3.2.3 Is Caspase-12 required for the paclitaxel-induced reduction in cell number in HCT116 isogenic cell lines?

I used specific Caspase-12 inhibitor (Z-ATAD-FMK), a synthetic peptide which irreversibly inhibits caspase-12 and its activity. This inhibitor is a cell permeable fluoromethyl ketone (FMK) peptide with no cytotoxic effects. The aim was to determine whether caspase-12 inhibition could reverse paclitaxel or tunicamycin response.

HCT116 isogenic cells were treated with caspase-12 inhibitor, tunicamycin, paclitaxel or in combination with each other. Paclitaxel treated HCT116, HCT116-p53null, HCT116-p21null showed approximately 90% and HCT116-baxnull showed 79% cell survival as compared to ND (Figure 3.7). The combined treatment of paclitaxel and caspase-12 inhibitor did not show much difference in cell numbers as compared to paclitaxel itself. But my main drug paclitaxel itself did not showed reduced cell numbers as it showed in my previous experiments; suggesting this data may not be reliable.

Tunicamycin treatments showed reduced cell numbers in all four HCT116 isogenic cell lines but the combined treatment of tunicamycin and caspase-12 inhibitor did not reverse the effects of tunicamycin. Caspase-12 inhibitor treatment alone and DMSO treatment did not show any significant difference as compared to ND.

My data shows no indication of Caspase-4 or Caspase-12 involvement in paclitaxel response. However, the lack of clear cytotoxic effect of paclitaxel in these experiments makes this result uncertain. I had planned to repeat these experiments again with fresh reagents, however subsequent data made this unnecessary (see below).

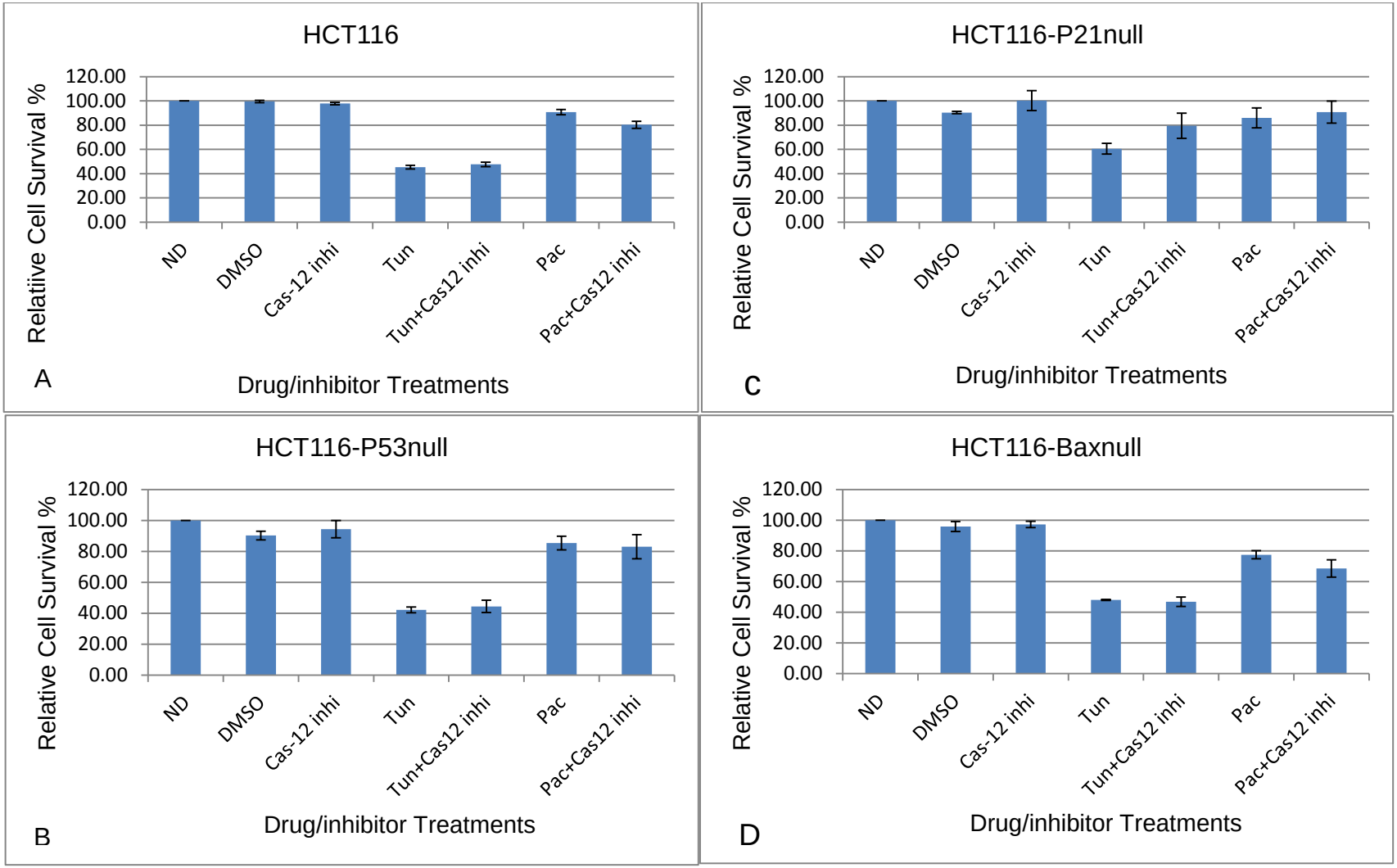


Figure3.7: Relative cell survival in colorectal cancer cell lines after ER stress inhibition by inhibitor for Caspase-12 (Z-ATAD-FMK)

HCT116 isogenic cell lines were treated with either paclitaxel (Pac) (1nM), Caspase-12 (Z-ATAD-FMK) inhibitor (10 μM), tunicamycin (Tun) (75 ng/ml), or in combination with each other. After 72 hours, spectrophotometric analysis was used (post SRB staining) to observe cell survival. Experiments were repeated three times independently of each other. Means ± SEM are shown.

3.3.2.4 Is JNK pathway required for the paclitaxel induced reduction in cell numbers?

Another important regulator of ER-stress mediated apoptosis is the JNK pathway. Here in my experiments I have used JNK (SP600125) inhibitor which is a powerful, cell permeable anthrapyrazolone inhibitor of JNK that competes with ATP to inhibit the phosphorylation of c-Jun. I hypothesize that treatment of cancer cell with paclitaxel and tunicamycin will induce ER stress induced apoptosis and JNK inhibitor will inhibit this ER stress induced apoptosis.

HCT116 isogenic cells were treated with JNK inhibitor, tunicamycin, paclitaxel or in combination with each other (Figure 3.8). Paclitaxel treated HCT116 showed (68%), HCT116-p53null (50%), HCT116-p21null (34%) and HCT116-baxnull (80%) cell survival as compared to ND (Figure 3.18). When combined treatment of paclitaxel and JNK inhibitor was used HCT116 showed (40%), HCT116-p53null (59%), HCT116-p21null (35%) and HCT116-baxnull (55%) cell numbers as compared paclitaxel only. Although paclitaxel has showed reduced cell numbers in these experiments, JNK inhibition failed to reverse the effects of paclitaxel and in some cell, lines appears to have further reduced cell survival.

Tunicamycin only treated HCT116 and HCT116-p21null showed (60%) cell numbers, while HCT116-p53null showed (45%) and HCT116-baxnull showed (80%) cell numbers as compared to ND. When combined treatment with tunicamycin and JNK inhibitor, HCT116 showed (44%), HCT116-p53null (45%), HCT116-p21null (40%) and HCT116-baxnull (70%) as compared to tunicamycin only treatment. In all these cell lines JNK inhibition did not reverse the effect of tunicamycin and in some cell lines appears to have further reduced cell survival.

Consistent with its effects in combination with paclitaxel or tunicamycin, JNK inhibitor treatment alone caused a reduction in cell survival, HCT116 showed (70%), HCT116p21-null (58%), HCT116-p53null and HCT116-baxnull showed (80%) cell numbers as compared to ND. DMSO only treatment did not show any difference as compared to ND.

In these experiments paclitaxel and tunicamycin showed visible reduction in cell numbers in HCT116 isogenic cells as compared to ND but JNK inhibitor did not reverse these effects.

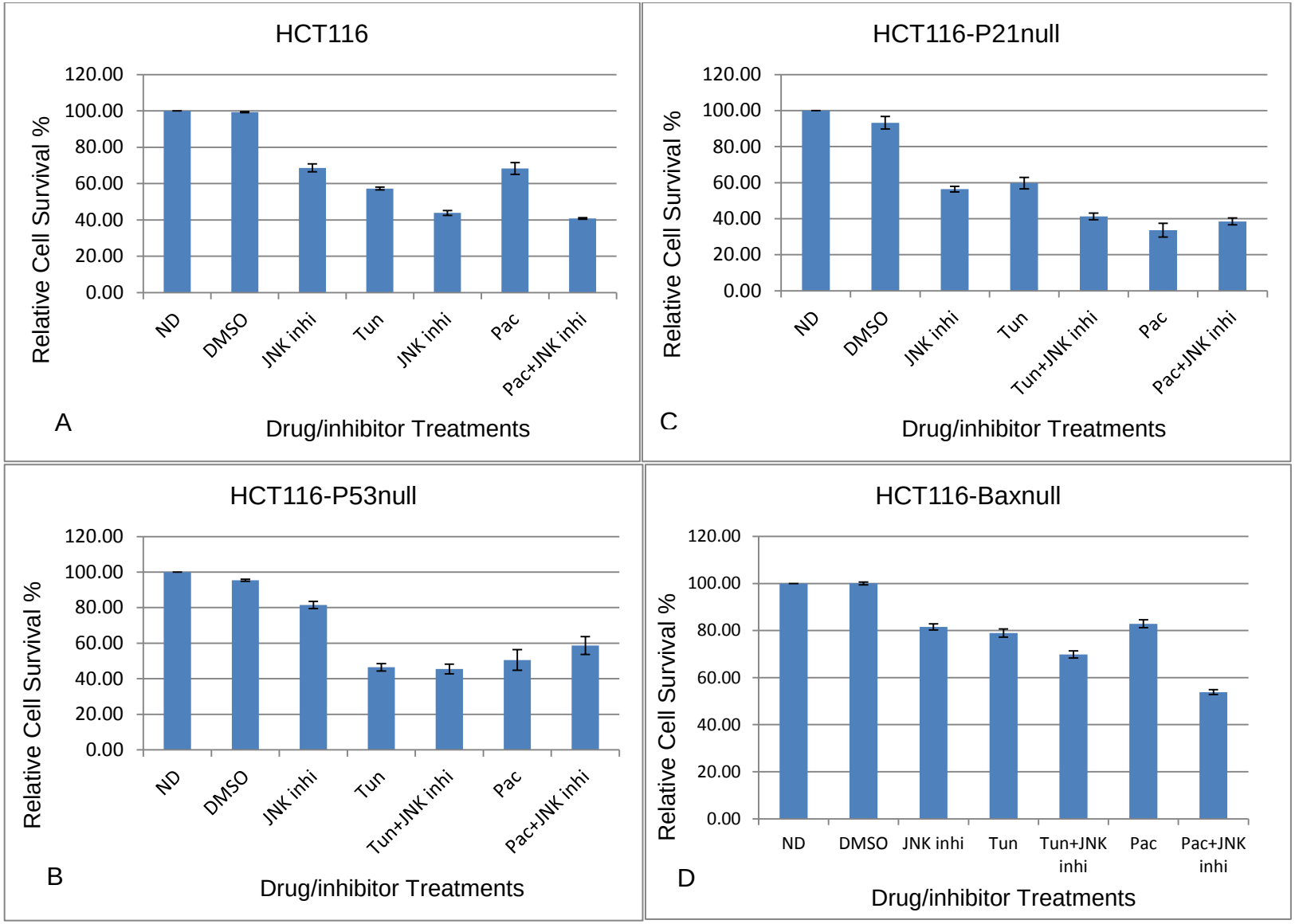


Figure3.8: Relative cell survival in colorectal cancer cell lines after ER stress inhibition by inhibitor for JNK (SP600125)

HCT116 isogenic cell lines were treated with either paclitaxel (Pac) (1nM), JNK inhibitor (SP600125) (10 μM), tunicamycin (Tun) (75 ng/ml), or in combination with each other. After 72 hours, spectrophotometric analysis was used (post SRB staining) to observe cell survival. Experiments were repeated three times independently of each other. Means ± SEM are shown.

3.3.3 Paclitaxel treatment and ER stress inhibition in PEO1 cells

Unpublished preliminary work in Dr Paige's group had identified a link between paclitaxel response and ER-stress in the PEO1 cell line. Given that none of the inhibitors used in the above experiments were shown to reverse paclitaxel response we decided to repeat these experiments using PEO1 cells. In these experiments, ovarian cancer cells lines PEO1-H8 and PEO1-FP2 were treated with paclitaxel in combination with salubrinal. PEO1 is an ovarian cancer cell line but PEO1-H8 is a transfected version of PEO1 cells expressing the WWOX protein (which may influence paclitaxel response, unpublished data) and PEO1-FP2 is vector only transfected cell line (Gourley et al., 2005; Gourley et al., 2009).

3.3.3.1 Paclitaxel treatment and PERK pathway inhibition in PEO1 cells

In these experiments, I used *WWOX* and vector only transfected cell lines. My previous results of ovarian and colorectal cell lines did not show any positive effects of the inhibitors I used in those experiments. As described in introduction the literature shows *WWOX* sensitises cancer cells towards apoptosis, and preliminary data in our group has suggested a link between paclitaxel response and ER-stress in PEO1 cells. Therefore, my aim was to see if using *WWOX* transfected cells could enhance the ER-stress induced apoptosis (more reduction in cell numbers) and enable us to see reversal of these effects by inhibiting PERK pathway.

Results (Figure 3.9) show that paclitaxel treated PEO1-H8 cells showed 36% cell numbers as compared to ND. Combined treatment of paclitaxel with two salubrinal concentrations (60μM and 30μM) showed 25% and 20% cell numbers respectively, indicating that salubrinal has not reversed the effects of paclitaxel but has enhanced them.

Tunicamycin treated PEO1-H8 cells showed 32% cell numbers as compared to ND. Combined treatment of tunicamycin with two salubrinal concentrations (60μM and 30μM) showed 40% and 35% cell survival respectively. Salubrinal did not reverse the effects of tunicamycin.

Both salubrinal only treatments showed 85% cell numbers as compared to ND. DMSO only treatment did not show any reduction in cell numbers as compared to ND

Results (Figure 3.9) show that paclitaxel treated PEO1-FP2 cells showed 24% cell numbers as compared to ND. Combined treatment of paclitaxel with two salubrinal concentrations (60μM and 30μM) showed 15% and 12% cell numbers respectively. This shows salubrinal has not reversed the effects of paclitaxel but had enhanced them.

Tunicamycin treated PEO1-FP2 cells showed 80% cell numbers as compared to ND. Combined treatment of tunicamycin with two salubrinal concentrations (60μM and 30μM) showed 55% and 54% cell numbers respectively. Salubrinal did not reverse the effects of tunicamycin which is positive control for ER stress, but again has enhanced them.

Both salubrinal only treatments showed 59% and 60% cell numbers as compared to ND. DMSO only treatment did not show any reduction in cell numbers as compared to ND.

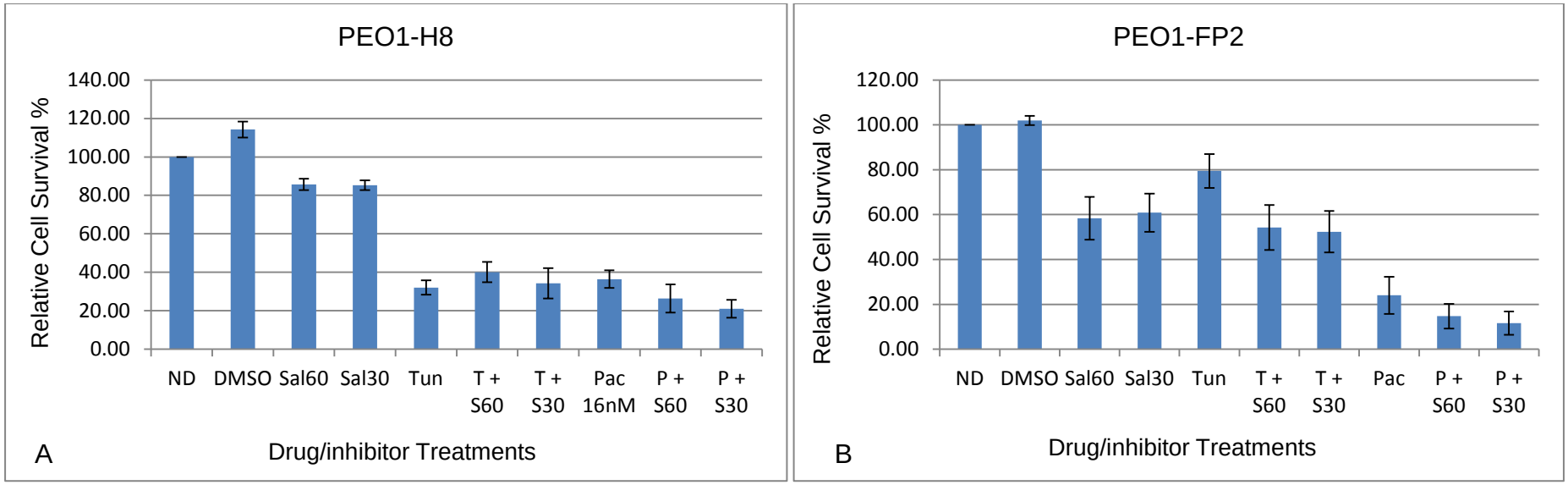


Figure 3.9: Relative cell survival in ovarian cancer cells after ER stress inhibition by salubrinal

PEO1-H8 and PEO1-FP2 cells were treated with paclitaxel (Pac or P) (16 nM), salubrinal (Sal or S) (30 μM and 60 μM), tunicamycin (Tun or T) (200 ng/ml), or in combination with each other. After 72 hours, spectrophotometric analysis was used (post SRB staining) to observe cell survival. Experiments were repeated four times. Means ± SEM are shown.

3.3.4 Examining the induction of ER stress markers caused by paclitaxel and tunicamycin by reverse transcriptase- polymerase chain reaction (PCR)

My results show no indication that inhibiting the PERK pathway, JNK pathway or caspases 4 or 12 can reverse the effect of paclitaxel in any of the cell lines used above. This was true even in the PEO1-H8 and PEO1-FP2 cell lines for which previous unpublished data had suggested a link between paclitaxel response and ER-stress. This could indicate that these specific components of the ER-stress pathway are not required for paclitaxel response, that paclitaxel does not induce the ER-stress at all in these cells, or that there are technical issues (such as efficacy of the inhibitors) affecting the results. To explore these possibilities, I next decided to screen a panel of cell lines to identify a suitable model system in which paclitaxel clearly induces ER-stress, as previously seen in Dr Paige's group (unpublished data).

The aim therefore is to see if paclitaxel induces markers of ER stress or not. Under stress conditions in the ER, proteins are misfolded. Cells try to adapt to the misfolded proteins and try to stabilize them to maintain cellular homeostasis. This adaptation towards misfolded proteins in the ER protects the cells by increasing the folding capacity inside ER and enhancing the production of chaperons such as GRP-78. Therefore, if paclitaxel treatment induces ER-stress we should see an increase in GRP-78 expression. ER stress also activates IRE1. IRE1 is a bifunctional protein with kinase and endoribonuclease (RNase) activity. It dimerizes, and auto phosphorylates to activate its RNase activity. This activation results in removal of a 26-nucleotide intron from X-box binding protein (XBP1) mRNA. Spliced XBP-1 is a transcriptional factor which activates many UPR target genes (Malhotra and Kaufman, 2007). Generally, XBP-1 mRNA is expressed as un-spliced form and so it encodes un-spliced XBP-1 also termed as pXBP-1 (U). Under ER stress environment this un-spliced mRNA transforms into spliced form and encodes spliced version of XBP-1, also termed as pXBP-1 (S). If paclitaxel does induce ER-stress, we should see an increase in XBP-1 (S). I am using various doses of paclitaxel at two times points in my experiments. The

aim is to see if ER stress is generated in cancer cells after treatment with paclitaxel after 24 or 72 hours. A range of different cell lines was used to increase the chances of identifying a suitable model system in which paclitaxel induces ER-stress.

3.3.4.1 Can paclitaxel treatment generate ER stress?

For these experiments the first step was optimizing the conditions for PCR. The intensity of the PCR product on the agarose gel depends upon the amount of starting template added. For this purpose, preliminary experiments were performed using different numbers of PCR cycles and different amounts of template (data not shown). The conditions where PCR amplification was not saturated were selected for the experiments. For GRP-78 and XBP-1 (S) 35 cycles and for actin or GAPDH 30 cycles were selected. Prostate (PC3 and LNCap) and skin cancer (SK-MEL-28) cell lines were treated with a range of doses of paclitaxel or DMSO for 24 or 72 hours, and expression of GRP-78 and XBP-1(S) by RT-PCR was analysed.

3.3.4.1.1 Paclitaxel treatment and ER stress induction in PC3 cells

The results in (Figure 3.10) show that no amplification was observed in the 'no reverse transcriptase' controls showing that no genomic DNA contamination was present. Similar intensity of beta actin expression was seen in all samples showing equal loading of template. There was a slight increase in expression of GRP-78 at 32nM and 64nM concentrations as compared to 16nM and the DMSO treated controls, but it does not show increased and spliced XBP-1. Also, when I repeated this experiment it showed no change in either GRP-78 and XBP-1 expression levels, suggesting that ER-stress is not induced by paclitaxel in these cells. In the same cell at 24 hours' time point also showed similar results (Gel images not shown).

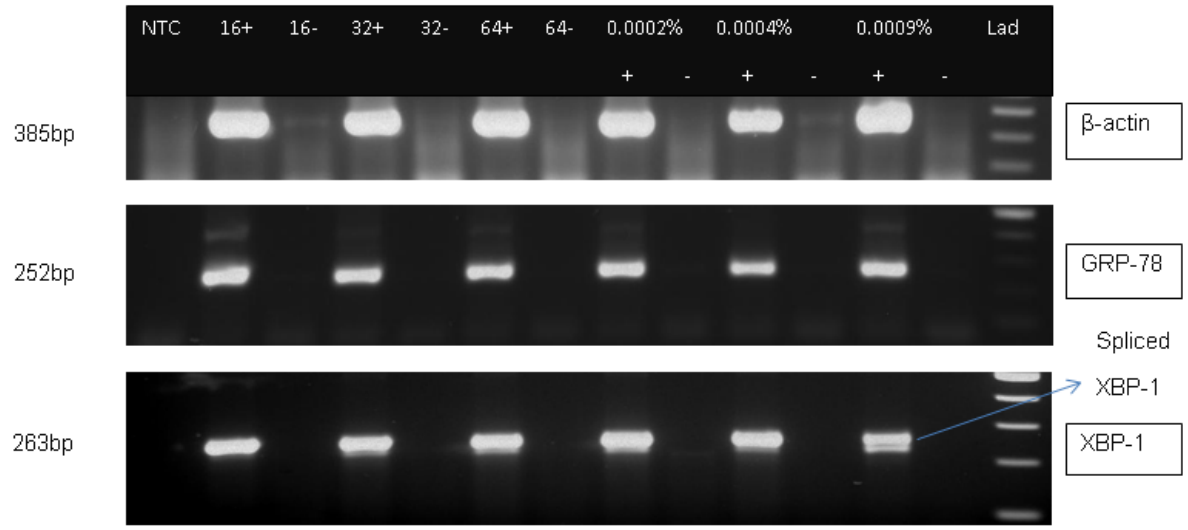


Figure 3.10: Gel images of PC3 after treatment with paclitaxel for 72 hours.

Samples include NTC (no template control), paclitaxel treated samples (16nM, 32nM, 64nM) and DMSO (0.0002%, 0.0004%, 0.0009%) treated samples. (+) represents cDNA samples from the different treatments and (-) indicates no reverse transcriptase controls to confirm that no genomic DNA was present.

3.3.4.1.2 Paclitaxel treatment and ER stress induction in LNCap cells

LNCap cells were treated with paclitaxel for 24 or 72 hours. The results (Figures 3.11; 3.12) show that no amplification was observed in the 'no reverse transcriptase' controls showing that no genomic DNA contamination was present. Similar intensity of beta actin expression was seen in all samples showing equal loading of template except for the 40µM dose- which is likely due to the large amount of cell death that was observed in the wells treated with this dose leading to low mRNA yield. The results show there was no increase in expression levels of GRP-78 and XBP-1 as compared to untreated at both time points (24 hours, Figure 3.24 or 72 hours, Figure 3.25). This shows there was no induction of ER-stress following paclitaxel treatment in these cells.

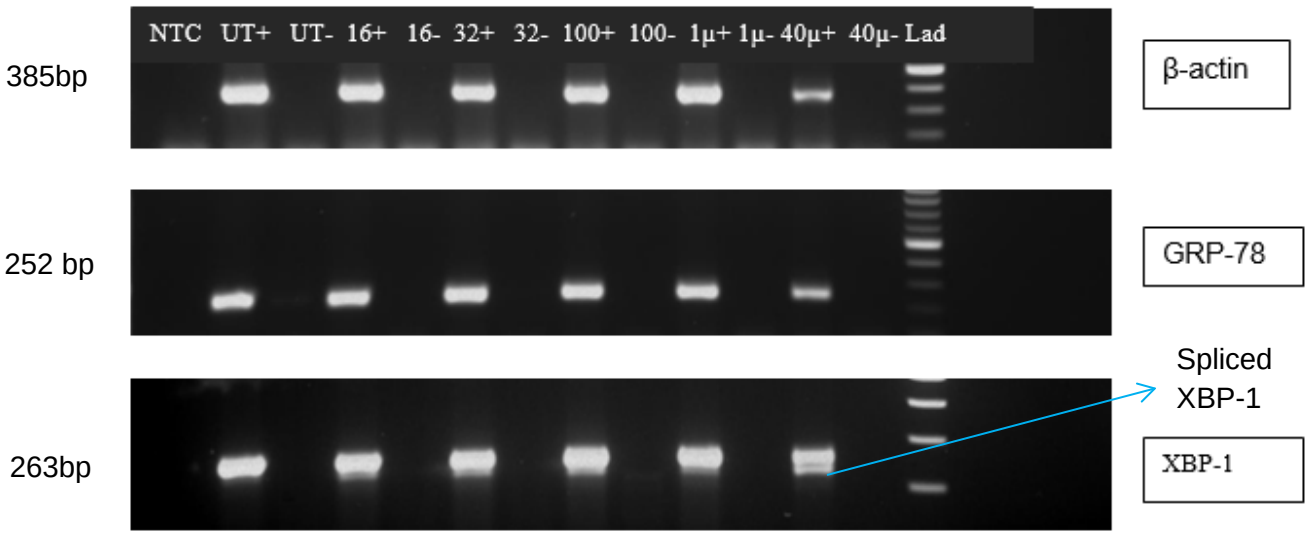


Figure 3.11: Gel images of LNCap after treatment with paclitaxel for 24 hours.

Samples include NTC (no template control), UT (untreated), and paclitaxel (16nM, 32nM, 100nM, 1µM and 40µM) treated samples. (+) represents cDNA samples from the different treatments and (-) indicates no reverse transcriptase controls to confirm that no genomic DNA was present.

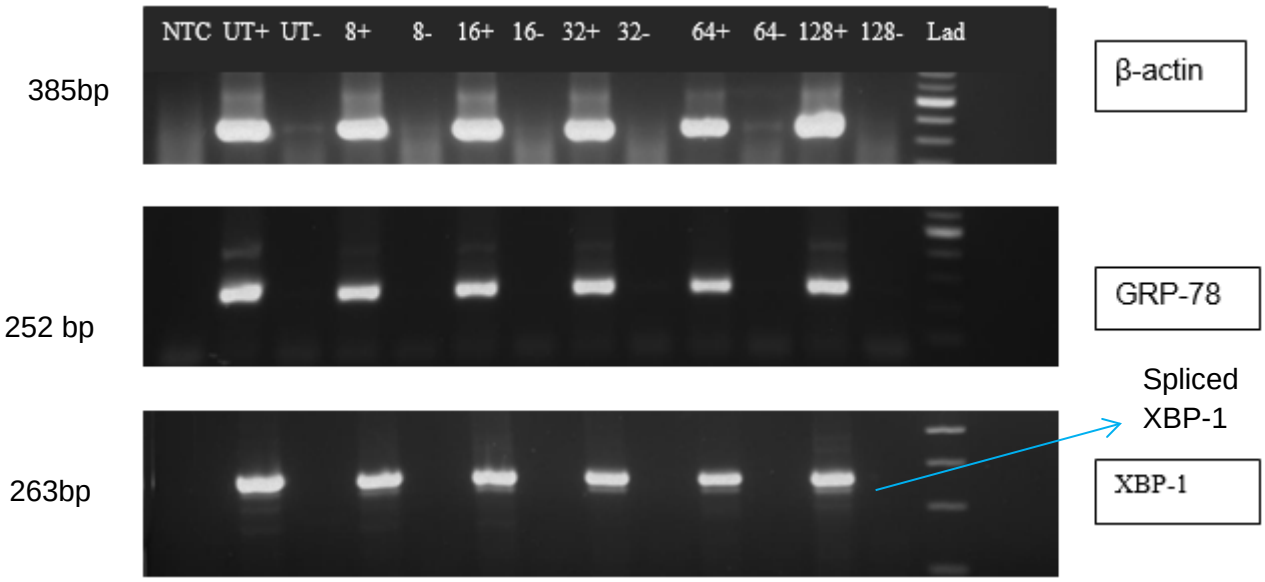


Figure 3.12: Gel images of LNCap after treatment with paclitaxel for 72 hours.

Samples include NTC (no template control), UT (untreated), and paclitaxel (8nM, 16nM, 32nM, 64nM and 128 nM) treated samples. (+) represents cDNA samples from the different treatments and (-) indicates no reverse transcriptase controls to confirm that no genomic DNA was present.

3.3.4.1.3 Paclitaxel treatment and ER stress induction in SK-MEL--28 cells

In this experiment melanoma cells SK-MEL-28 were treated with doses of paclitaxel. The results (Figure 3.13) show that no amplification was observed in the 'no reverse transcriptase' controls showing no genomic DNA contamination was present, except for the 128nM dose. Similar intensity of beta actin expression was seen in all samples showing equal loading of template. The results (Figure 3.13) showed there was no increase in expression levels of GRP-78 or XBP-1(S). Again, it shows there was no induction of ER-stress.

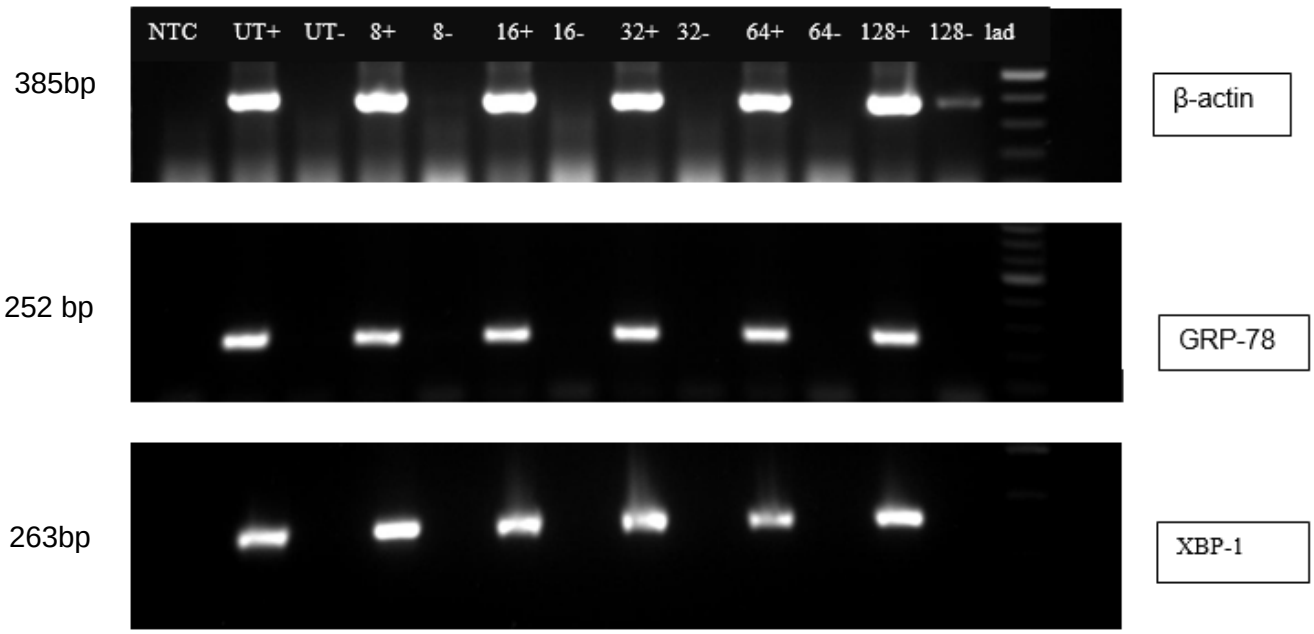


Figure 3.13: Gel images of SK-MEL-28 after treatment with paclitaxel for 72 hours.

Samples include NTC (no template control), UT (untreated), and paclitaxel (8nM, 16nM, 32nM, 64nM and 128 nM) treated samples. (+) represents cDNA samples from the different treatments and (-) indicates no reverse transcriptase controls to confirm that no genomic DNA was present.

These results do not indicate that paclitaxel induced ER stress in these cell lines. I also examined the expression of GRP78 and XBP-1(S) in HCT116 cells (appendices S9.1) but results again showed no change in expression following paclitaxel treatment and indicate that these lines are not a suitable working model where I can see induction of ER stress.

3.3.5 Discussion

3.3.5.1 ER stress inhibition in ovarian and colorectal cancer cells (*in vitro*)

Paclitaxel is an anti-mitotic drug that binds to microtubules and stabilises them which leads to mitotic cell cycle arrest (Bhalla et al., 1993). How binding of paclitaxel with microtubules and stabilizing them leads to initiation of downstream mechanisms causing apoptosis is not fully understood but research in the signalling molecules activated by paclitaxel show that there are multiple mechanisms which could be responsible for apoptosis (Wang et al., 2000). In my study, I hypothesize that cell death induced by paclitaxel is due to ER stress.

3.3.5.1.1 ER stress inhibition via PERK

PERK activates and phosphorylates eIF2α resulting in translation attenuation and enhancing the translation of ATF4 (a transcription factor) which induces expression of UPR target genes. The hypothesis behind these experiments was, paclitaxel kills the cells by activation of the PERK pathway of ER-stress.

The results showed paclitaxel reduced cell numbers as compared to untreated cells, but salubrinal (PERK inhibitor) did not protect the cell death caused by paclitaxel. This shows that the inhibitor for PERK is not working or that the way paclitaxel is killing the cells is not due to PERK activation. However, this could be investigated by repeating salubrinal treatment of cells, and using some of the cells in SRB cell survival assay to show that there is still no effect on survival, and using the rest to extract protein and measure phospho-eIF2a levels to see whether salubrinal had stopped PERK working or not? (If it is working it should affect the amount of phospho-eIF2a present). Previous studies have shown that paclitaxel induced ER stress mediated cell death in cancer cells. A study by Liao et al. (2008) showed that when leukemic U937 cells were treated with paclitaxel for 3-6 hours, it activated PERK pathway which was observed as increased expression of GADD34 and ATF4 mRNA. This study also showed that after 6-12 hours of treatment, there was increased expression of CHOP mRNA. ATF4, CHOP and GADD34 are all targets of PERK that have been linked to

ER-stress induced apoptosis. But in my experiments inhibiting PERK did not affect cell death. There is some resemblance between my results and a study by Drexler, (2009). This study showed that apart from the protective role of salubrinal, it can also work as pro-apoptotic in leukemic cells. In this study K562 (chronic myeloid leukaemia) cells were treated with proteasome inhibitor (PSI), histone deacetylase inhibitor VPA and salubrinal either alone or in combination with each other. PSI induces ER stress and VPA protects from transcriptional repression in leukemic cells. The results showed that salubrinal did not reduce the cytotoxic effects of PSI and PSI+VPA but instead stimulated apoptosis. These findings of Drexler et al., resemble to my results (Figure 3.5 and 3.9) where salubrinal combined with paclitaxel did not reduce the toxicity of paclitaxel but instead showed more reduction in cell numbers as compared to paclitaxel only treatment. Drexler et al. showed that Salubrinal and VPA alone were not toxic to the cells; however, in my results (Figure 3.5 and 3.9) salubrinal itself showed toxicity and showed reduced cell survival as compared to untreated or DMSO treated cells. Further analysis by Drexler et al. showed that the enhanced cell death due to combination of salubrinal with other two inhibitors was due to continuous synthesis of nascent proteins and this was confirmed by using translational inhibitor CHX. The results of (Drexler, 2009) are in agreement with another study by (Bastola et al., 2016) where VCP inhibitors were used alone or in combination with salubrinal in ovarian cancer cells. Valosin containing protein (VCP) also known as p97 AAA-ATPase was found to be a necessary gene in ovarian cancer cells as compare to non-ovarian cancer cells. VCP is associated with ERAD, cell cycle and autophagy. DBeQ, CB-5083 and ML240 were used as inhibitors of VCP. The findings of this study showed the inhibitors of VCP reduced viability of cells, caused G1 cell cycle arrest, induced cell death by apoptosis (Annexin V assay and DAPI staining) and triggered UPR (confirmed by using tunicamycin as positive control). Consistent with the findings of (Drexler, 2009) in leukemic cells, findings of (Bastola et al., 2016) showed that when ovarian cancer cells (OVCAR10, SKOV3, OVSAHO, OVCAR8, OVCAR5 and RMG1) were treated with a combination of salubrinal and VCP inhibitors it increased cytotoxicity at 8 hours and 72 hours. This was due to synergistic effect

of salubrinal and VCP inhibitors. The fact that salubrinal can work as pro-apoptotic was also confirmed by another study of (Koizumi et al., 2012) which showed that in SW1353 (chondrosarcoma cells) salubrinal enhanced radiation induced cell death. Another study by (Teng et al., 2014) showed that salubrinal in combination with TRAIL (Tumour necrosis factor related apoptosis inducing ligand) enhanced the expression of Bim (a CHOP regulated pro-apoptotic protein).

I also used tunicamycin (an established ER stress inducer) as a positive control for ER stress. Tunicamycin works by inhibiting the first step of N-linked glycosylation of proteins via DPAGT1 resulting in aggregation of misfolded proteins leading to UPR (Keller et al., 1979; Brandish et al., 1996). In the colorectal cancer cells, the results of tunicamycin are like paclitaxel results. The combination of tunicamycin and salubrinal showed more reduction in cell numbers as compared to tunicamycin only. This is consistent with the findings of Drexler, (2009) and Bastola et al. (2016) above. Surprisingly in my results in the ovarian cancer cell lines tunicamycin did not show any significant reduction in cell numbers, raising the possibility that it was not inducing ER stress. My results in these cells were opposite to a study by Long at al., (2005) which show that when PC12 cells were treated with a range of doses of tunicamycin for 37 hours, it showed ER stress induced apoptosis and salubrinal inhibited the ER stress mediated apoptosis induced by tunicamycin is a dose dependent manner. In this study when salubrinal was converted into a bio-tinylated derivative by addition of a trichloro-methyl group, it enhanced the activity of salubrinal. The results of this study were consistent to another study by Boyce et al., (2005) which showed that when PC12 cells were treated with tunicamycin it induced ER stress and salubrinal protected the cells from apoptosis up to 36 hours. This study also confirmed that salubrinal induces eIF2α phosphorylation in dose dependent manner in PC12 cells and when a derivate of salubrinal did not protect cells from apoptosis, it was found it did not induce phosphorylation of eIF2α. Another reason why ovarian cancer cells in my results did not respond to tunicamycin could be due to an issue with the uptake of the tunicamycin. In a study by Reiling et al., (2011) it

was shown that MFSD2A (a member of facilitator super family of proteins) is required for the uptake of tunicamycin into the cells. MFSD2A is located on the plasma membrane and knock down of MFSD2A by siRNA or gene depletion showed that it induced tunicamycin resistance and it's over expression enhanced the sensitivity towards accumulation of tunicamycin in the cells. Cancer cells are prone to mutations and may be in the ovarian cancer cells in my study MFSD2A did not function properly and it affected the uptake of tunicamycin into the cells.

My results of ovarian cancer cells also show similarity to a study by Gazit et al., (1999) which showed that MCF-7 breast cancer cell lines showed resistance towards tunicamycin but not to thapsigargin. Interestingly, another study by McCormack et al., (1997) showed that mouse lymphoid cells showed resistance towards thapsigargin but responded to tunicamycin by inducing transcription. This shows depending upon the genetic background some cancer cells may respond to ER stress and some may not. This shows that induction of ER stress can show contradictory consequences in different cancer cells.

3.3.5.1.2 ER stress inhibition via Caspase -12 and -4

Caspase12 and caspase 4 are involved in ER stress induced apoptosis (Nakagawa et al., 2000; Hitomi et al., 2004). So far, the presence of functional caspase-12 in humans is controversial and caspase-4 is considered as its alternative in humans. The results of Caspase-4 inhibitor (Z-YVAD-FMK) showed that paclitaxel alone and combined with caspase-4 inhibitor did not show cell death in HCT116 isogenic cell lines excluding HCT116-p21null which showed 20% cell death as compared to untreated, but caspase-4 inhibitor did not reverse it. Similarly, in the caspase-12 inhibitor experiments, paclitaxel showed little reduction in cell numbers (10-15%) as compared to untreated and caspase-12 inhibitor did not reverse it. It has been shown by many studies that paclitaxel causes cell death due to its cytotoxicity (Bhalla et al., 1993; Wang et al., 2000) but it is not evident in my results, and likely suggests a problem with the drug solution used.

However, tunicamycin showed reduction in cell numbers in both experiments of caspase-12 and caspase-4 inhibitor treatments. In both experiments tunicamycin showed about 40-50% reduction in cell numbers as compared to untreated cells, and combination of caspase-4 inhibitor or caspase-12 inhibitor with tunicamycin did not protect the cells from ER stress in all HCT116 isogenic cell lines apart from p21-null where it showed a minimal, but not statistically significant, reversal of tunicamycin effect.

In these experiments caspase-12 and caspase-4 inhibitors were used to block their functioning and protect the cells from ER stress induced apoptosis, but it did not reverse the effects of tunicamycin. Previous studies suggest that human caspase-4 and mouse caspase-12 respond to ER stress by inducing apoptosis and cell lacking the expression of caspase-12 and caspase-4 were not fully protected from ER stress (Nakagawa et al., 2000; Hitomi et al., 2004). Contrary to this study, (Obeng and Boise, 2005) showed that when murine cells FL5.12 and human cells U266, lacking caspase-4 and caspase-12, when treated with ER stress inducing agents such as tunicamycin, thapsigargin or brefeldin-A, showed induction of ER stress, and Bcl-x_L expression in both cell lines inhibited apoptosis induced by ER stress inducing agents. This study also showed that cell death induced by tunicamycin was due to caspase-dependent necrosis and in human myeloma cells; caspase-4 activity is not induced by tunicamycin. However, other studies, e.g. (Kalai et al., 2003), show that caspase-12 expression is more restricted in murine organs and cells, suggesting there are some other mechanisms of ER stress present in the cells. Tunicamycin has showed reduction of cell numbers in my experiments but caspase-12 and -4 inhibitors did not reverse it and this could be because cell death by tunicamycin may not involve caspase-12 and caspase-4 and so the inhibitors of these two caspases did not reverse tunicamycin effects, although it is also possible that the inhibitors did not effectively inhibit caspase function in these experiments.

3.3.5.1.3 ER stress inhibition via JNK in colorectal cancer cells

JNK is another arm of ER stress induced apoptosis. SP600125 is an anthrapyrazolone inhibitor of JNK that competes with ATP to inhibit the phosphorylation of c-Jun. In my results paclitaxel showed reduction in cell numbers in all HCT116 isogenic cells and only HCT116-baxnull showed less reduction in cells numbers as compared to other three cell lines. The involvement of JNK signalling pathway in paclitaxel induced cell death was verified by using JNK inhibitor SP600125. Results show JNK did not reverse the effect of paclitaxel. Previously it has been shown by many studies that paclitaxel activates JNK in ovarian cancer and some other types of cancers and paclitaxel induced cell death is due to activation of JNK (Kolomeichuk et al., 2008; Lee at al., 1998; Selimovic et al., 2008; Sunters et al., 2006; Wang et al., 1999). Again, these results could indicate that paclitaxel response does not require JNK activity, or that the inhibitor was not effective at blocking JNK function in these experiments.

However, not all studies have shown JNK to enhance paclitaxel induced cell death. In a study by Seino et al., (2016) the role of basal JNK activity towards sensitivity/resistance to paclitaxel was examined. Ovarian cancer cells were treated for 3 days with SP600125 to specifically inhibit the basal JNK activity ensuring there will be no interference of basal JNK with paclitaxel induced activation of JNK. The results showed that prior treatment with SP600125 followed by paclitaxel significantly enhanced the cell death as compared to combined SP600125 and paclitaxel treatment, suggesting inhibition of basal JNK activity may increase the sensitivity of ovarian cancer cells to paclitaxel. These results agree with my results of combined treatment with paclitaxel and SP600125 in HCT116 wild-type cells and HCT116-baxnull cells. In my experiments, I treated my cells with SP600125, one hour prior to paclitaxel treatment and the results in these lines showed an enhanced reduction in cell numbers.

In the other two cells lines HCT116-p53null and HCT116-p21null cells, paclitaxel killed cells but SP600125 showed very little effect on this response.

In the same experiment tunicamycin treatment showed reduction in cell numbers as compared to untreated, but SP600125 did not reverse its effects. As described earlier JNK is involved in ER stress induced apoptosis which has been explained by many studies such as a study by Urano et al., (2000), where it was reported that when AR42J cells (rat pancreatic acinar) were treated with ER stress inducing agents (tunicamycin, thapsigargin, dithiothreitol) it induced ER stress mediated apoptosis and C-Jun terminal kinase JNK was activated due to UPR. These findings are in agreement with another study by (Yoneda et al., 2001) where it was reported that JIK functions as regulator of JNK signalling during ER stress. When JIK was overexpressed, it enhanced the interaction between IRE1α and TRAF2 and JNK was activated by the influence of ER stress inducing agent tunicamycin. However, in my experiments combined treatment of tunicamycin and SP600125 showed no increasing of the cell numbers as compared to tunicamycin only treatment. It should be noted that although SP600125 is a JNK inhibitor and its function is to inhibit the pro-apoptotic activity of the JNK, but SP600125 itself can also become pro-apoptotic. It was shown in a study by Lu et al., (2010) that SP600125 synergistically enhanced DHA induced apoptosis in human lung adenocarcinoma cells (ASTC-a-1) by mitochondrial mediated cell death pathways. It has been shown previously that Dihydroartemisinin (DHA) can inhibit cancer cell growth by apoptosis. DHA mediates apoptosis through cell cycle arrest, activation of caspases and reduction of Bcl/bax expression etc. (Singh and Lai, 2004; Hou et al., 2008; Chen et al., 2009). The results showed SP600125 alone did not affect the growth of the cells but when ASTC-a-1 cells were pre-treated with SP600125, it enhanced DHA induced apoptosis. Role of JNK was also examined and it was reported that JNK was neither activated nor involved in the apoptosis induced by SP600125 and DHA. Further result showed that 1-hour pre-treatment with SP600125 enhanced the DHA induced apoptosis by translocation of Bax to mitochondria, release of cytochrome C and activation of caspase-9 and -3. Similarly, in my

results I pre-treated my cells with SP600125 for 1 hour and then mixed with the drug solutions, and this produced a small additional reduction in surviving cells compared to tunicamycin alone, though this was not significant. The ability of JNK to work as pro-apoptotic or anti-apoptotic depends upon the cell types, type of death stimuli and activation of other signalling pathways (Jing and Anning, 2005).

JNK inhibitor (SP600125) affects proliferation by inducing mitotic arrest at G_2/M phase and apoptosis in many human cancer cell lines such as breast cancer, prostate cancer, multiple myeloma and erythroleukemia (Hideshima et al., 2003; Jacobs-Helber and Sawyer, 2003; Du et al., 2004; Mingo-Sion et al., 2004). In my experiments, treatments of HCt116 isogenic cells with SP600125 alone showed 20% reduction is cell numbers in HCT116-p53null and HCT116-baxnull. HCT116 and HCT116-p21null cells treated with SP600125 showed 30% and 42% reduction in cell survival as compared to untreated. Although previous studies and manufacturing companies suggest SP600125 is not toxic to cell, but my results show it is somehow having some negative effect on cell numbers. A study by (Jemaa et al., 2012) show the cytotoxic effects of SP600125 on HCT116 cells and p53-deficient (*TP53*$^{-/-}$) HCT116 cells. The results showed that SP600125 induced polyploidization and cell death in p53 deficient cells by targeting MPS1 (mitotic kinase) as compared to wild type HCT116 cells. This is contrary to my results where HCT116 wild-type (30%) showed more reduction in cell numbers as compared to HCT116-p53null (20%). Another study by (Mili et al., 2016) showed the time dependent and dose dependent effects of SP600125 on HeLa cells for 24, 48 and 72 hours. The results of this study showed that various doses of SP600125 (10μM, 15μM and 20μM) induced a significant G2/M arrest of the cell cycle at 48 hours by enhanced phosphorylation of histone H3 and endoreduplication. SP600125 also induced delayed apoptosis due to Bcl-2 expression along with activation of caspase-3 and PARP cleavage. The findings of this study are similar to my results where SP600125 showed reduction in cell numbers depending upon the cell type possibly due to mitotic arrest or apoptosis.

3.3.5.1.4 Role of p53 pathway in paclitaxel response

I used four HCT116 isogenic cell lines. HCT116 cells were wild type and mutant HCT116-p53null, HCT116-p21null and HCT116-baxnull cells lack different components of the p53 pathway - p53, p21 and Bax respectively. The reason for using these mutant cell lines was to examine if they show different response to paclitaxel induced cell death as compared to wild type HCT116. HCT116 isogenic cell lines lacking p53, p21 and Bax did not show any prominent difference in paclitaxel and tunicamycin response compared with wild type HCT116. All four cell lines showed almost similar reduction in cell numbers when treated with paclitaxel or tunicamycin. Similarly, the response of inhibitors (salubrinal, JNK-inhibitor, Caspase-4 inhibitor and Caspase-12 inhibitor) did not show any difference in their inhibitory activity in p53-null, p21-null and Bax-null cells line compared to HCT116, providing no evidence for the p53 pathway in paclitaxel response. Previous studies suggest involvement of p53, p21 and Bax in response to paclitaxel/ER stress. Loss of normal p53 in patients sensitizes them to paclitaxel (Wahl et al., 1996; Vikhanskaya et al., 1998). Approximately 60% of human cancers exhibit p53 mutations (Wang and Wang, 1996). Low expression of p53 enhances cells progression to G2/M arrest leading to mitotic arrest (Wahl et al., 1996). Another study by Barboule et al., (1997) shows the relevance of p21 in MCF-7 cancer cells. This study shows that for paclitaxel induced mitotic arrest, p21 is not needed but it helps cells in exiting from mitotic arrest. Bax is a pro-apoptotic member of Bcl-2 family of proteins. This family of proteins are also involved in paclitaxel induced apoptosis (Strobel et al., 1996; Strobel et al., 1998). Although literature suggest involvement of the cell cycle regulating proteins p53, p21 and mitochondrial apoptotic protein Bax in response to paclitaxel, in my experiments all four HCT116 isogenic cell lines showed similar results.

The results in all HCT116 isogenic cells show that paclitaxel treatment showed reduction in cell numbers as compared to untreated. Combined treatment of paclitaxel and salubrinal did not reverse the effects of paclitaxel and instead showed more reduction in cell numbers as compared to paclitaxel only treatment.

3.3.5.1.5 ER stress inhibition in WWOX transfected ovarian cancer cells

My results of ER stress inhibition in A2780, SKOV3 and HCT116 isogenic cell lines did not show any evidence of reversal of ER stress, and we were concerned that these cell lines might not be suitable for examining the ER-stress related response to paclitaxel.

Previous work within Dr Paige's group (unpublished data) has shown induction of ER-stress due to paclitaxel treatment in PEO1 cell lines. So, the purpose of doing these experiments in PEO1 cells was to see if I can repeat those results again. Dr Paige's group previously showed that *WWOX* transfection into *WWOX*-null ovarian cancer cells, abolished in-vivo tumorigenicity due to alteration of integrin α3-mediated interactions between cancer cells and extracellular matrix (ECM), without any associated changes in in-vitro growth nor baseline or cisplatin induced apoptosis rates (Gourley et al., 2009). Recently, Dr Paige's group (unpublished data) showed that *WWOX* induces apoptosis following exposure or cells to paclitaxel and not cisplatin, and these effects were due to ER stress but not anti-mitotic action of paclitaxel. These results showed that paclitaxel triggered ER stress in ovarian cancer cells and when cells transfected with *WWOX* were treated with tunicamycin (an inducer of ER stress) they also showed decreased cell survival compared to vector transfected controls. The data also showed that this induction of ER stress was unaltered by *WWOX* but *WWOX* re-expression caused a significant shift towards an apoptotic response to ER stress, which can be reversed by WWOX siRNA. The key findings of this unpublished data were *WWOX* tumour suppressor sensitizes ovarian cancer cells to paclitaxel via ER stress induced apoptosis.

These findings were the reason I used *WWOX* and vector transfected PEO1 cells because *WWOX* overexpression can cause a significant shift towards an apoptotic response to ER stress. My results of PEO1-H8 showed reduction in cell numbers in paclitaxel and tunicamycin treated cells as compared to untreated but again salubrinal did not reverse the effect of paclitaxel and tunicamycin. In case of PEO1-FP2 paclitaxel showed reduction in

cell numbers as compared to untreated cells, which again were not reversed by salubrinal. Curiously, however, there was no obvious increase in paclitaxel response in the WWOX-transfected cells compared to the vector transfected controls, and it is not clear why this might be. Genetic analysis of these clones may be required to ensure that the frozen lines have been correctly labelled.

The results from the in vitro studies of ovarian and caner lines failed to show any evidence of induction of ER stress. Although, in vitro studies showed reduction in cell number after treatment with paclitaxel and tunicamycin but there was no evidence suggesting it was due to ER stress. Whilst it would be important to confirm that the inhibitor drugs have effectively blocked their target proteins, we had grown more and more concerned that the cell lines used were not a suitable model for investigating the cytoprotective role of ER-stress inhibition. To identify a suitable model, additional cell lines were screened by RT-PCR for evidence of ER-stress induction following paclitaxel treatment. For this purpose, the ER stress markers used in this study were GRP-78 and XBP-1(S).

3.3.5.2 Examining the effects of paclitaxel on ER stress markers (GRP-78 and XBP-1)

My results show the gene expression of ER stress markers GRP-78 and XBP-1(S) in prostate cancer cells PC3 after 72 hours of paclitaxel treatment. There was a slight increase in the expression of GRP-78 which was dose dependent but there was no increase in spliced expression of XBP-1(S). Increased expression of GRP-78 in these results was an indication of generation of ER stress but when this experiment was repeated three times, this data was not reproducible. Similarly, there was no sign of ER-stress induction following paclitaxel treatment in LNCap or SK-MEL-28 cells. I selected GRP-78 and XBP-1(S) because several studies suggest that paclitaxel enhance the expression of both of these ER stress markers. For example, in the study by Mhaidat et al., (2011) it was showed that when colorectal cancer cells SW480 were treated with 40µM dose of paclitaxel it showed an increased protein expression of GRP-78 which was time dependent. Tunicamycin treatment

was used as positive control for ER stress and it also showed a timely increase in protein expression of GRP-78 after paclitaxel treatment. Next in the same study, GRP-78 expression was knocked down with siRNA and it sensitized SW480 cells to paclitaxel induced apoptosis. My results are contrary to these results of (Mhaidat et al., 2011). I also used 40μM of paclitaxel for 24 hours (Figure 3.24) for LNCap cells, but it did not show any increase in the expression of GRP-78. Although it showed me enhanced expression of spliced XBP-1 but due to low mRNA yield, the concentration of cDNA of 40 μM samples was not equivalent to other samples and it can be seen by looking at the expression of B-actin which showed a thin band which was unequal as compared to the other samples. Also, this drug concentration was too strong for the cells because when observed under the microscope the confluence of the cells was very low and lots of dead cells were floating in the media. Another study using breast cancer cells (Wang et al., 2009) showed that 24 hours' treatment of paclitaxel and vinblastine (another chemotherapy drug) enhanced the protein expression of GRP-78 in three different breast cancer cell lines (ZR75-30, MCF-7 and T47D). This increase in the protein expression of GRP-78 was dose dependent (0.1μl, 0.5μl, 1μl, 2.5μl and 5μl) and time dependent (4-24 hours). In this study, further analysis of western blotting showed that, in MCF-7 cells paclitaxel and vinblastine induced the splicing of XBP-1, caspase-7 cleavage and phosphorylation of JNK and eIF2α. Again, the increase in all these ER stress proteins was dose and time dependent. Similar to the study of (Mhaidat et al., 2011), this study also showed that down regulation of GRP-78 by siRNA enhanced the sensitivity of breast cancer cells towards paclitaxel and vinblastine. So, these studies clearly show paclitaxel enhances GRP-78 and XBP-1 protein expression.

3.4 Conclusion

It is unclear why I could not find evidence of ER-stress activation following paclitaxel treatment in this broad range of cancer cell lines and different tumour types used. Further work is clearly needed to examine this and to explore the role of ER-stress in paclitaxel response. However, because of the lack of suitable model cell line for this work it was

decided to stop the in vitro investigation of paclitaxel response, and instead look for supportive evidence in clinical patient samples (see Chapter 4).

Chapter 4: Clinical relevance of ER stress and WWOX tumour suppressor

4.1 Introduction

In chapter 3, I have showed *in vitro* investigation of ER stress pathway in response to paclitaxel in various cancer cell lines. The studies (relative cell survival & gene expression) in chapter 3 did not demonstrate evidence linking ER stress pathway to paclitaxel chemo response, therefore I did not pursue that further. Although I could not see evidence of paclitaxel induced ER stress response in my experiments, but there are many studies that show paclitaxel induced ER stress response in cancer cells, such as a study by Wang et al., (2009) showed that taxol induced upregulation of GRP-78 and some other components of ER stress response in breast cancer cells and inhibition of GRP-78 sensitized breast cancer cells to paclitaxel induced apoptosis. Another study by Mhaidat et al., (2011) showed that paclitaxel induce activation of GRP-78 and when GRP-78 is inhibited, it sensitized colorectal cancer cells to paclitaxel induced apoptosis. Also, I have unpublished data from another study (Janczar et al.,) that suggests that WWOX enhances paclitaxel induced apoptosis by altering the ER-stress caused by paclitaxel into an apoptotic (rather than cytoprotective) outcome. Normal epithelial ovarian cells express WWOX but its expression is down regulated in ovarian tumours (Gourley et al., 2005). Loss of WWOX expression in linked with increased tumorigenesis and poor prognosis. 30% of ovarian carcinomas show loss of WWOX expression (Nunez et al., 2005).

4.1.1 Immunohistochemical staining (IHC) Analysis

IHC is a technique where monoclonal or polyclonal antibodies are used to determine the tissue distribution of an antigen of interest. It is widely used for diagnosis of cancers. The tumour tissues used for the study were from the patients who had chemotherapy and surgery at Hammersmith Hospital. Tumour samples are taken and preserved in paraffin. IHC study was done by Dr Mona El-Bahrawy and the anonymised patients' clinical data was provided by Dr Katherine Costello and Ms Nona Rama. The study was done according to approved ethics of Imperial College, London.

4.1.2 TCGA Analysis

There are nearly 200 different forms of cancer and many subtypes which make this disease very complex. Each type of cancer has different molecular profile and need specific treatment depending upon its type and sub-stage (Hanahan and Weinberg, 2000). Each cancer type has different epigenetic changes, somatic mutations, copy number variations and altered gene expressions. Understanding of all these genetic aberrations is very important for the treatment of cancer and to plan strategies about how to improve the therapeutics and better diagnostics (Stratton et al., 2009; Lengauer et al., 1998).

The Cancer Genome Atlas (TCGA) is a collaboration between the National Cancer Institute (NCI) and National Human Genome Research institute (NHGRI). In 2005 TCGA was launched as a public funded project to analyse the genomic data from high quality tumour sample through large scale genome sequencing and making it available on TCGA Data Portal. The data portal provides information about clinical information of patients that participated in the programme, metadata about the samples collected from patients, histopathology of samples and molecular information collected from samples such as; mRNA/miRNA expression, protein expression, copy number, etc). In addition to tumour samples, TCGA also included high quality non-tumour samples from the participants and analysed the germ line DNA to find the abnormalities in tumour samples compared with the normal samples to understand the oncogenic process. Comprehensive multi-dimensional maps of 33 types of cancer showing important genomic changes are available on TCGA website (The Cancer Genome Atlas, 2017). The information on TCGA website is available to public and researchers around the world. The findings can help for improved diagnosis, treatment and the measures to prevent cancer.

4.1.3 Experimental Approach

In this chapter I have looked at clinical samples to see whether I can find clinical evidence to support that paclitaxel response involves the ER stress pathway. The aims are (i) Does WWOX correlate with patient survival? (looking at PFS and OS. Looking at WWOX mRNA

and protein), (ii) Does GRP-78 correlate with patient survival? (looking at PFS and OS. Looking at WWOX mRNA and protein), and (iii) Does WWOX only affect patient survival in patients with low expression of the ER-stress marker GRP-78 (i.e. in patients without inherent activation of ER-stress). I have two approaches- analysing RNA expression in ovarian and colorectal cancers that are part of the TCGA study and analysing protein expression in ovarian cancers by IHC in collaboration with Dr Mona El-Bahrawy. IHC scoring data of ovarian cancer patients was provided to me by Dr Mona El-Bahrawy (details in section 4.2.1). For TCGA study I downloaded RNA-seq expression FPKM (fragments per kilo base of transcripts per million mapped reads) files for ovarian and colorectal cancer patients. RNA sequencing is a recently developed high throughout technology for transcriptome total RNA profiling which uses deep sequencing technologies. As compared to other methods, RNAseq can measure levels of transcripts and their isoforms very precisely. By using RNAseq, identification and quantification of rare and common transcripts, isoforms, novel transcripts, gene fusions and non-coding RNAs can be done swiftly in large samples (Wang et al., 2009). From the downloaded FPKM files, I extracted the required RNA expression information of WWOX and GRP-78 and used it for Kaplan Meier survival analysis.

4.2 Results

4.2.1 Immunohistochemical staining (IHC) of ovarian cancer tissue samples for analysis of WWOX and GRP-78 expression

From the anonymised tumour bank list provided by Ms Nona Rama, patients were selected. In order to keep the study based on homogeneous sample set of advanced ovarian cancer, I selected only tumours that were serous ovarian cancer, and were either stage 3 or 4 along with some stage 1c. Although 1c is officially considered as an early stage disease, the presence of cancer in lymph nodes indicate that this tumour actually has actually spread and often a 1c patient does have tumour cells that have spread into the peritoneum (i.e. stage 3) but are not yet detectable tumour masses. Also, I selected only those tumours that had been

treated using carboplatin and taxol. I am interested in the taxol response, but taxol is almost never given alone and so patients treated with carbo-taxol are the best I could get. I identified 50 samples for use in this study and was provided with anonymised overall and progression free survival data for these. From total of 50 patients, 46 had undergone carbo-taxol treatment while the other 4 also had treatment but it's not known. There were 36 stage 3 and 12 stage 4 patients. 1 patient was stage 1c and 1 patient with unknown stage. Table 4.1 show the summary of patients' clinical data.

Table 4.1: Summary of patient clinical data

Number of patients	50
Treatment Carbo-taxol Not Known	 46 (92%) 4 (8%)
Histology - Serous	50 (100%)
FIGO Stage 1c 3 4 Not known	 1 (2%) 36 (72%) 12 (24%) 1 (2%)
Median PFS time (±SD)	1.61 years
Number of relapsed patients	43 (86%)
Median OS time (±SD)	3.2 years

PFS = progression free survival, OS = overall survival, SD = standard deviation

4.2.1.1 IHC results in ovarian cancer

IHC of the ovarian cancer patients' tissue samples was done by Dr Mona El-Bahrawy and the scoring data of WWOX and GRP-78 staining was provided to me. From the total of 50 cases, 17 cases showed weak cytoplasmic WWOX staining, 29 cases showed moderate cytoplasmic WWOX staining and 4 cases showed strong cytoplasmic WWOX staining. For GRP-78, there were 26 weak cytoplasmic stained cases and 24 moderate cytoplasmic cases. However, some tumours showed some degree of on nuclear WWOX staining as well as shown in table 4.2.

Table 4.2: IHC results in ovarian cancer cells

Cytoplasmic WWOX staining	
Weak	17 (34%)
Moderate	29 (58%)
Strong	4 (8%)
Nuclear WWOX staining	
<50% of tumour cells	7 (14%)
>50% of tumour cells	3 (6%)
Cytoplasmic GRP78 staining	
Weak	26 (52%)
Moderate	24 (48%)

4.2.1.2 Analysis of GRP-78 expression by IHC and its effects on overall survival time

Table 4.3 below shows that there are 24 patients in high GRP-78 group and 26 patients in low GRP-78 group. Patients with moderate cytoplasmic GRP78 IHC staining were classified as high GRP-78 group, whereas patients with weak cytoplasmic IHC staining of GRP-78 were classified as low GRP-78 group. In high GRP-78 group 8 patients had died and 16 patients (66.7%) were still alive, whereas in low GRP-78 13 patients had died and 13 patients (50%) were still alive. Figure 4.1 show the graph showing Kaplan Meier survival curves for high and low GRP-78 groups. The vertical lines on blue and green survival curve represent cases which were censored. The blue curve represents better survival time in group of patients with high expression of GRP-78 compared to green curve which represents the group of patients with low GRP-78 expression. The p value (p=0.082) although not significant shows a trend towards significance (Table 4.4).

Table 4.3: Summary of patients in low and high GRP-78 groups

Case Processing Summary

GRP-78 Status	Total N	Dead Patients	Patients still alive	
			N	Percent
GRP-hi	24	8	16	66.7%
GRP-low	26	13	13	50.0%
Overall	50	21	29	58.0%

Table 4.4: Log Rank comparison of high GRP-78 and low GRP-78 patient groups

Overall Comparisons

	Chi-Square	df	P value
Log Rank (Mantel-Cox)	3.032	1	0.082

(df) represents degree of freedom

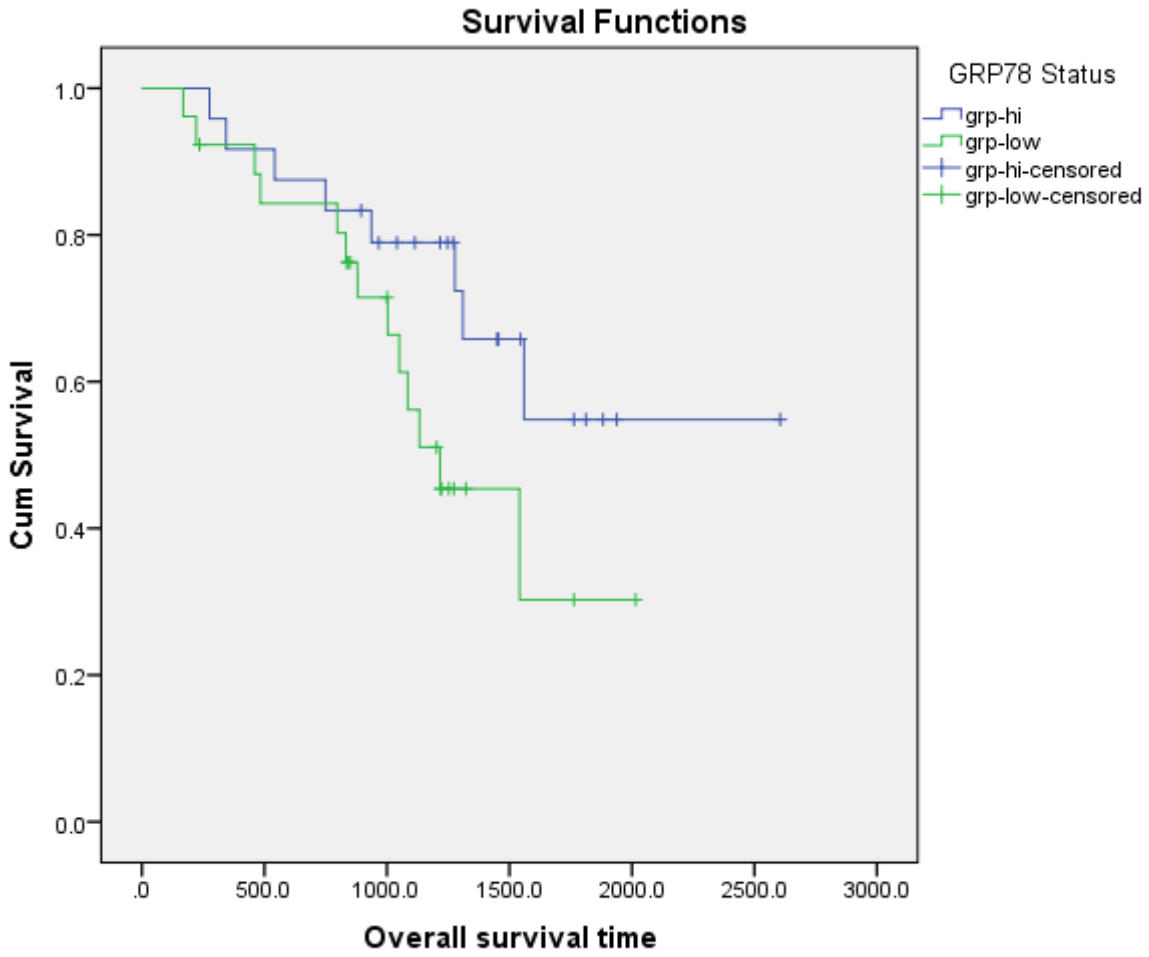


Figure 4.1: Kaplan Meier curves of ovarian cancer patients showing overall survival. The blue curve represents patients with high GRP-78 expression and green curve represents patients with low GRP-78 expression. The vertical line in blue curve represents the censored observations.

4.2.1.3 Analysis of WWOX expression by IHC and its effects on overall survival time

Table 4.5 below shows that there are 33 patients in high WWOX group and 17 patients in low WWOX group. Patients with moderate and strong cytoplasmic WWOX IHC staining were classified as high WWOX group, whereas patients with weak cytoplasmic IHC staining of WWOX were classified as low WWOX group. In high WWOX group 14 patients had died and 19 patients (57.6%) were still alive, whereas in low WWOX 7 patients had died and 10

patients (58.8%) were still alive. Figure 4.2 shows the Kaplan Meier survival curves for high and low WWOX groups. The results show both curves blue (high WWOX) and green (low WWOX) overlap each other and this show there was no effect of WWOX on overall survival time. Table 4.6 show (p=0.895) the survival difference between both curves was not significant.

Table 4.5: Summary of patients in low and high WWOX groups

Case Processing Summary

WWOX Status	Total N	Dead Patients	Patients still alive	
			N	Percent
WWOX-hi	33	14	19	57.6%
WWOX-low	17	7	10	58.8%
Overall	50	21	29	58.0%

Table 4.6: Log Rank comparison of high WWOX and low WWOX patient groups

Overall Comparisons

	Chi-Square	df	P value
Log Rank (Mantel-Cox)	0.017	1	0.895

(df) represents degree of freedom

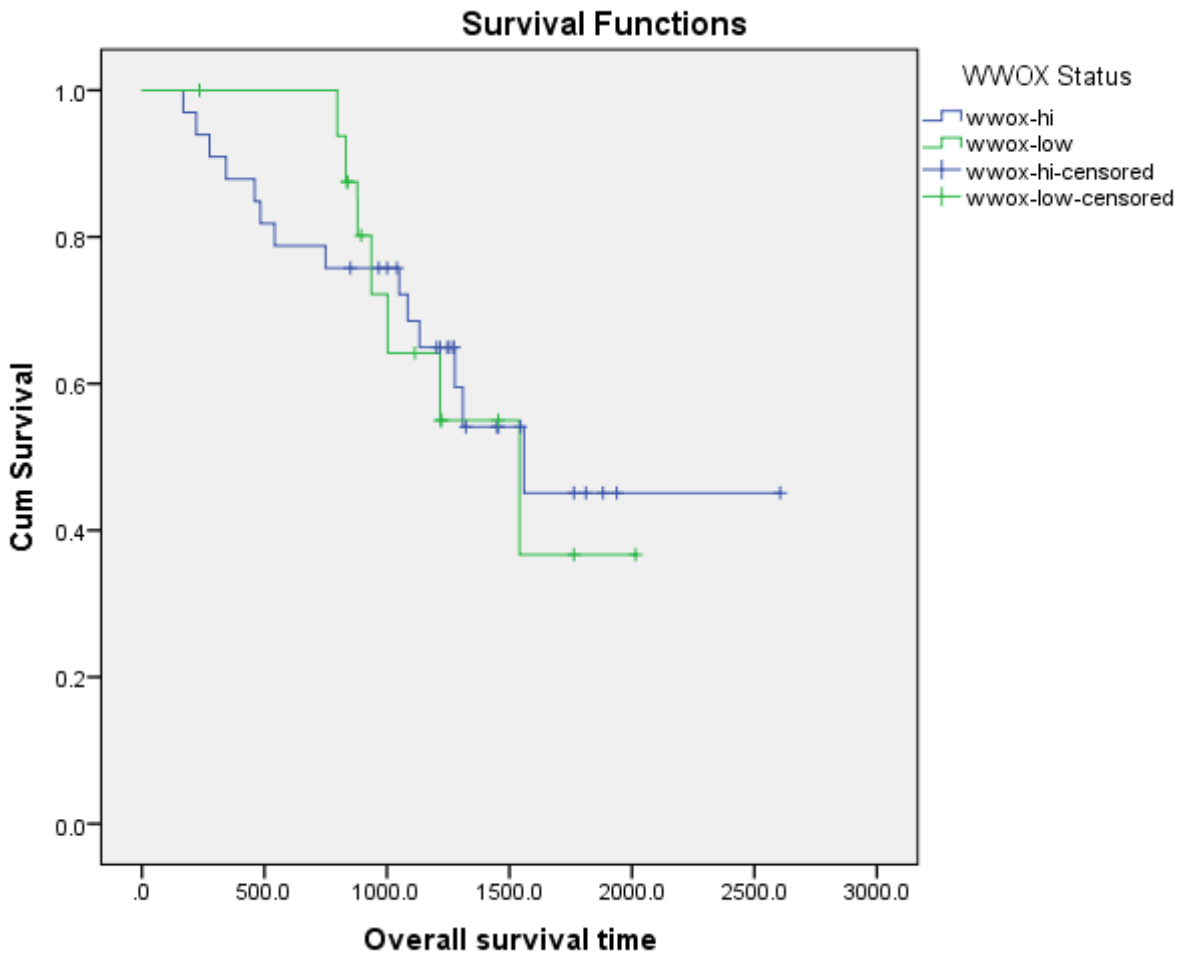


Figure 4.2: Kaplan Meier curves of ovarian cancer patients showing overall survival. The blue curve represents patients with high WWOX expression and green curve represents patients with low WWOX expression. The vertical line in blue curve represents the censored observations.

4.2.1.4 Analysis of combined WWOX/GRP-78 expression by IHC and its effects on overall survival (OS) time

Table 4.7 below shows that there were 19 patients for high-WWOX/high-GRP-78 and 12 patients for low-WWOX/low-GRP-78 groups. Similarly, there were 14 patients for high-WWOX/low-GRP-78 and 5 patients for low-WWOX/high-GRP-78 groups. For high-WWOX/high-GRP-78 group 7 patients had died and 12 (63.2%) were still alive, whereas for high-WWOX/low-GRP-78 group 7 patients had died and 7 (50%) were still alive. Similarly, for low-WWOX/high-GRP-78 group 1 patient had died and 4 (80%) were still alive, whereas for low-WWOX/low-GRP-78 group 6 patients had died and 6 (50%) were still alive. Figure 4.3 show the overall survival graph plotted by Kaplan Meier analysis showing curves for all four groups. All four groups are overlapping each other. The blue curve representing (high-WWOX/high-GRP-78) and yellow curve representing (low-WWOX/high-GRP-78) show better survival then purple (low-WWOX/low-GRP-78) and green curve (low-WWOX/high-GRP-78), reflecting the better survival seen in the high GRP-78 patients (shown in Figure 4.1). However, none of these differences reach significance (table 4.8, p=0.358) because of the small group size and the number of cases censored.

Table 4.7: Summary of patients in low or high WWOX or GRP-78 groups

Case Processing Summary

WWOX & GRP-78 Status	Total N	Dead Patients	Patients still alive	
			N	Percent
W-H/GR-H	19	7	12	63.2%
W-H/GR-L	14	7	7	50.0%
W-L/GR-H	5	1	4	80.0%
W-L/GR-L	12	6	6	50.0%
Overall	50	21	29	58.0%

Table 4.8: Log Rank comparison of high or low WWOX or GRP-78 patient groups

Overall Comparisons

	Chi-Square	df	P value
Log Rank (Mantel-Cox)	3.224	3	0.358

(df) represents degree of freedom

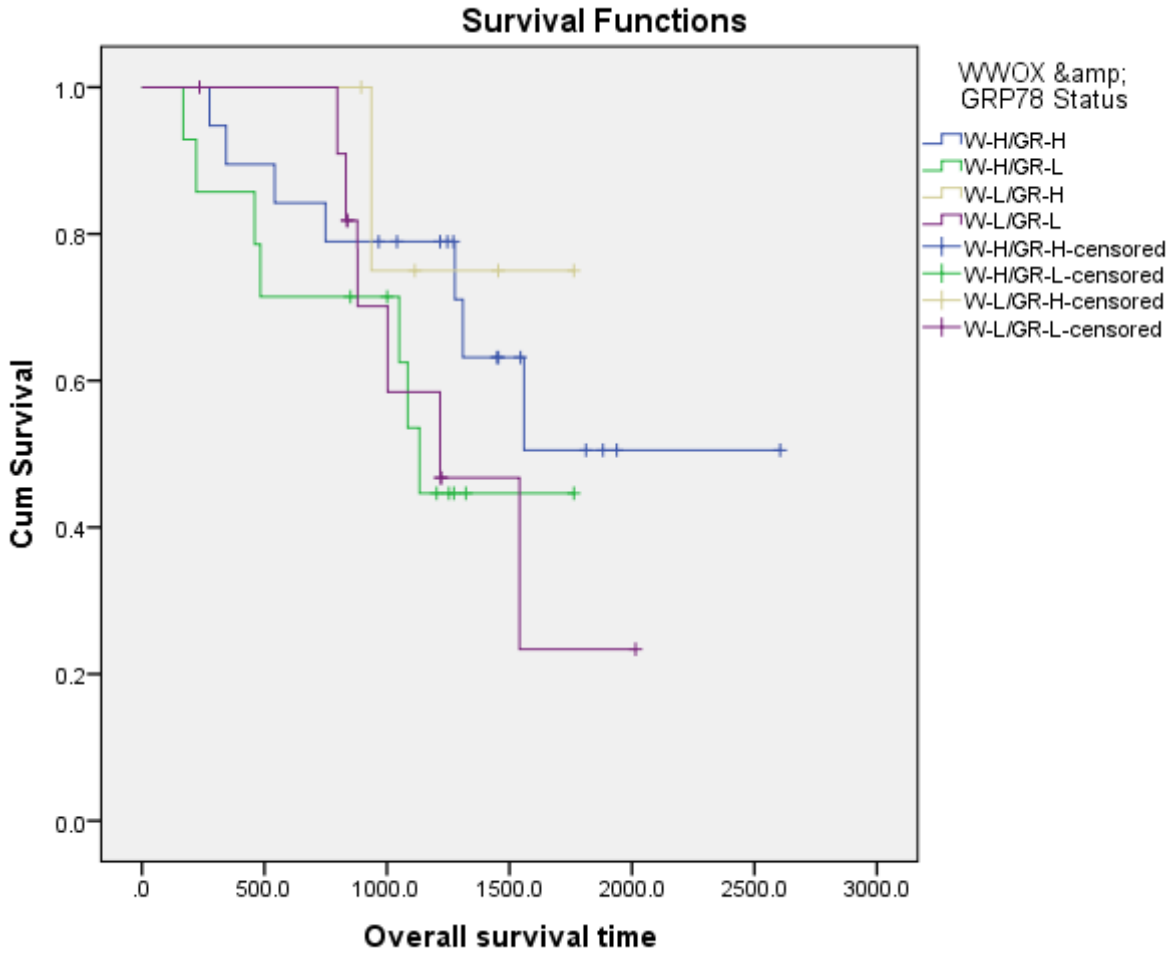


Figure 4.3: Kaplan Meier curves of ovarian cancer patients showing overall survival time. The curves blue, green, yellow and purple represents (high-WWOX/high-GRP-78), (high-WWOX/low-GRP-78), (low-WWOX/high-GRP-78) and (low-WWOX/low-GRP-78) respectively. The vertical lines in both curve represents the censored observations.

4.2.1.5 Analysis of GRP-78 expression by IHC and its effects on progression free survival (PFS) time

Table 4.9 below shows that there are 24 patients in high GRP-78 group and 26 patients in low GRP-78 group. In high GRP-78 group 21 patients had relapsed and 3 (12.5%) patients were still disease-free, whereas in low GRP-78 group 22 patients had relapsed and 4 patients (15.4%) were still disease-free. Figure 4.4 show the graph showing Kaplan Meier curves for high and low GRP-78 groups. Both blue (high GRP-78) and green (low GRP-78) curves are overlapping each other which show that there was no difference in terms of PFS within both groups. Table 4.10 show (p=0.829) there was no significant difference within both groups.

Table 4.9: Summary of patients in low and high GRP-78 groups

Case Processing Summary

GRP-78 Status	Total N	Patients who have relapsed	Disease free patients	
			N	Percent
GRP-hi	24	21	3	12.5%
GRP-low	26	22	4	15.4%
Overall	50	43	7	14.0%

Table 4.10: Log Rank comparison of low and high GRP-78

Overall Comparisons

	Chi-Square	df	P value
Log Rank (Mantel-Cox)	0.047	1	0.829

(df) represents degree of freedom

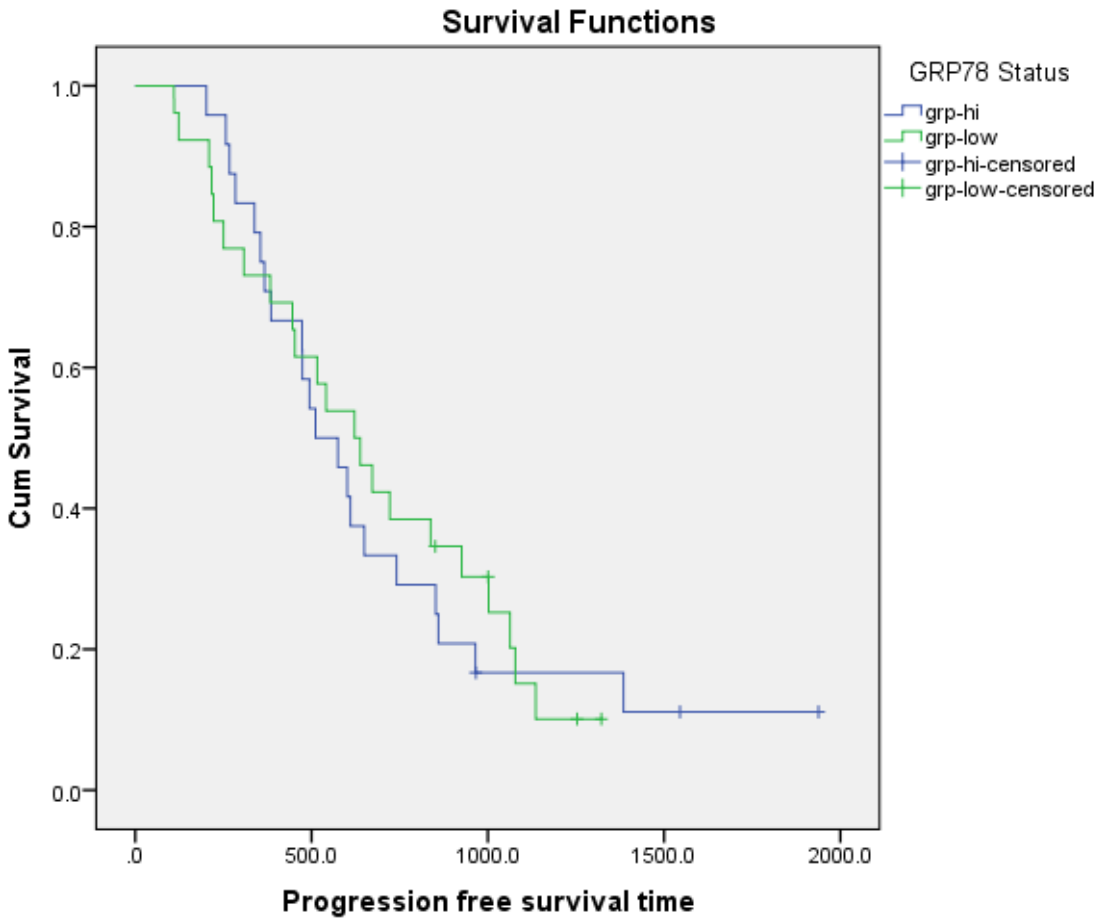


Figure 4.4: Kaplan Meier curves of ovarian cancer patients showing PFS. The blue curve represents patients with high GRP-78 expression and green curve represents patients with low GRP-78 expression. The vertical line in blue curve represents the censored observations.

4.2.1.6 Analysis of WWOX expression by IHC and its effects on progression free survival (PFS) time

Table 4.11 below shows that there are 33 patients in high WWOX group and 17 patients in low WWOX group. In high WWOX group 26 patients had relapsed and 7 patients (21.2%) were still disease-free, whereas in low WWOX group all patients had relapsed. Figure 4.5 show the graph showing Kaplan Meier curves for high and low WWOX groups. Blue curve representing high WWOX is overlapping green curve (low WWOX) from start to middle of the observation and then blue curve appears high showing better PFS. Although the difference is not significant (p=0.181, table 4.12) this may be due to small group sizes and the number of censored patients, and this data might indicate a trend towards significance.

Table 4.11: Summary of patients in low and high WWOX groups

Case Processing Summary

WWOX Status	Total N	Patients who have relapsed	Patients still alive	
			N	Percent
WWOX-hi	33	26	7	21.2%
WWOX-low	17	17	0	0.0%
Overall	50	43	7	14.0%

Table 4.12: Log Rank comparison of low and high WWOX

Overall Comparisons

	Chi-Square	df	P value
Log Rank (Mantel-Cox)	1.786	1	0.181

(df) represents degree of freedom.

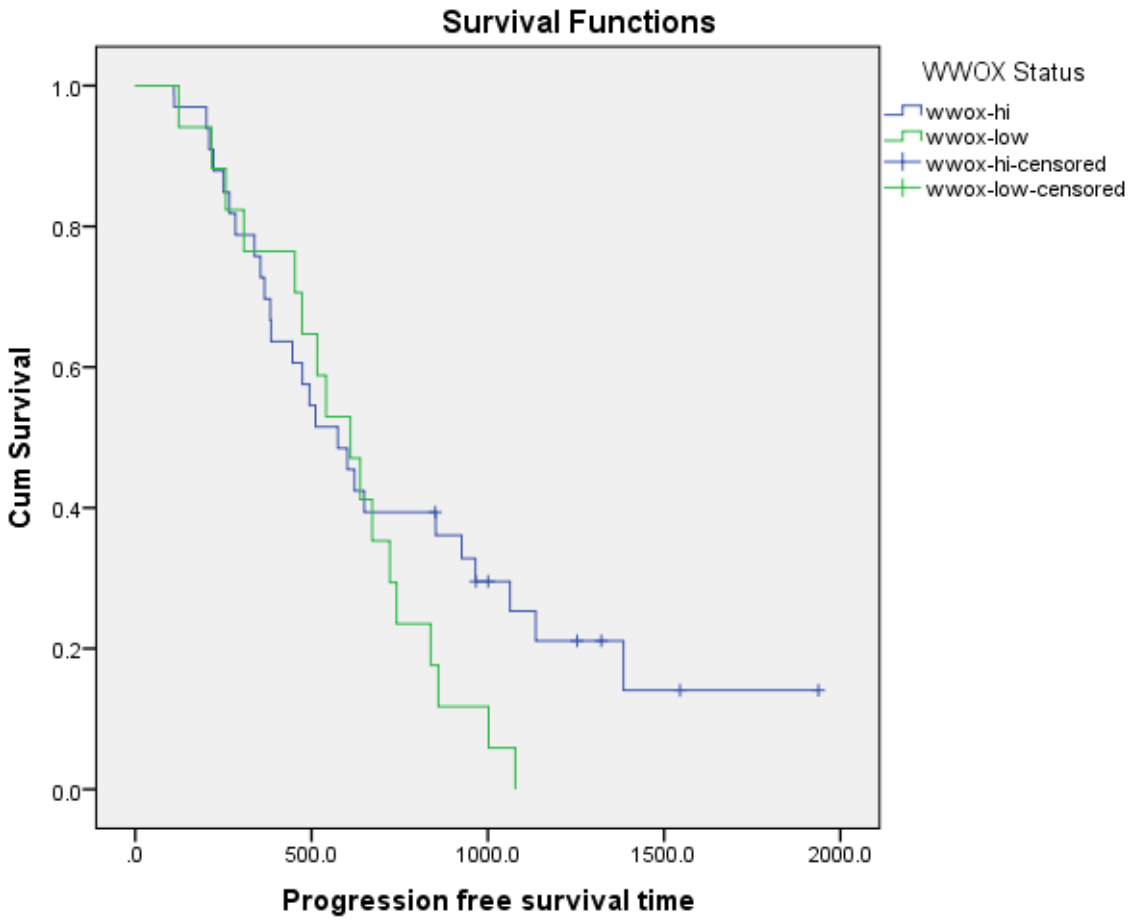


Figure 4.5: Kaplan Meier curves of ovarian cancer patients showing PFS. The blue curve represents patients with high WWOX expression and green curve represents patients with low WWOX expression. The vertical line in blue curve represents the censored observations.

4.2.1.7 Analysis of combined WWOX/GRP-78 expression by IHC and its effects on progression free survival (PFS) time

Table 4.13 below shows that there were 19 patients for high-WWOX/high-GRP-78 and 12 patients for low-WWOX/low-GRP-78 groups. Similarly, there were 14 patients for high-WWOX/low-GRP-78 and 5 patients for low-WWOX/high-GRP-78 groups. For high-WWOX/high-GRP-78 group 16 patients had relapsed and 3 (15.8%) were still disease-free, whereas for high-WWOX/low-GRP-78 group 10 patients had relapsed and 4 (28.6%) were still disease-free. For low-WWOX/high-GRP-78 group all 5 patients had relapsed, whereas for low-WWOX/low-GRP-78 group all 12 patients had relapsed. Figure 4.6 shows the progression free survival graph plotted by Kaplan Meier analysis. All four groups overlap each other and there is no significant difference (p=0.542) between all four groups as shown in table 4.14.

Table 4.13: Summary of patients in low or high WWOX or GRP-78 groups

Case Processing Summary

WWOX & GRP-78 Status	Total N	Patients who have relapsed	Patients still disease free	
			N	Percent
W-H/GR-H	19	16	3	15.8%
W-H/GR-L	14	10	4	28.6%
W-L/GR-H	5	5	0	0.0%
W-L/GR-L	12	12	0	0.0%
Overall	50	43	7	14.0%

Table 4.14: Log Rank comparison of high or low WWOX or GRP-78 patient groups

Overall Comparisons

	Chi-Square	df	P value
Log Rank (Mantel-Cox)	2.150	3	0.542

(df) represents degree of freedom

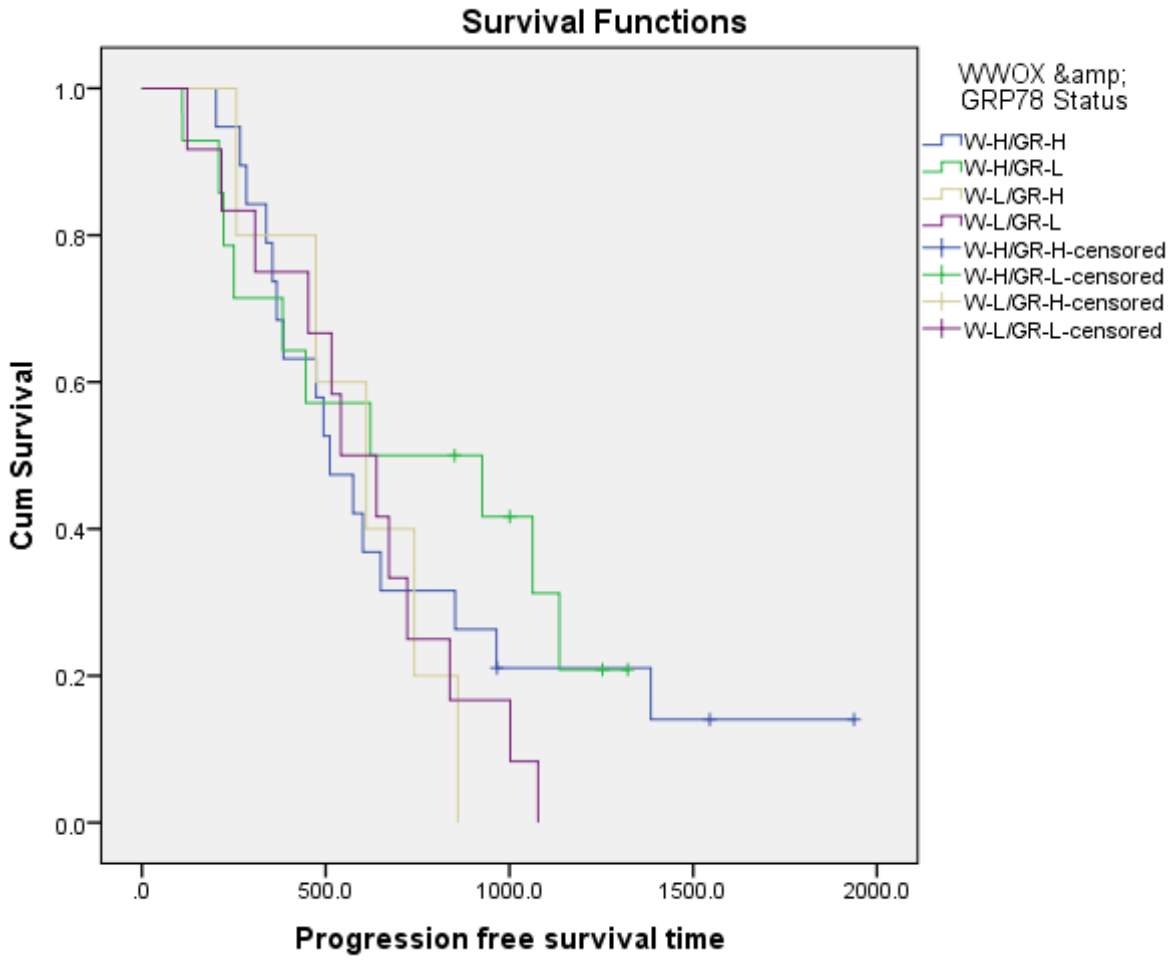


Figure 4.6: Kaplan Meier curves of ovarian cancer patients showing PFS. The curves blue, green, yellow and purple represents (high-WWOX/high-GRP-78), (high-WWOX/low-GRP-78), (low-WWOX/high-GRP-78) and (low-WWOX/low-GRP-78) respectively. The vertical lines in both curve represents the censored observations.

4.2.2 Analysis of RNA sequencing data of ovarian and colorectal cancer tumours

In order to look for evidence that might support a link between ER-stress and chemo response, I have looked for correlations between patient survival data and the RNA sequencing data of ovarian and colorectal tumours downloaded from TCGA Data Portal. As for the analysis of the IHC data above, I have analysed expression of the WWOX gene, and the ER-stress biomarker GRP78. From the TCGA database, RNA sequencing data of the required genes was selected and combined with the patient survival data in Microsoft Excel and, using SPSS software, Kaplan Meier analysis was performed, and survival data was analysed.

The downloaded RNA sequencing data from TCGA was from 376 ovarian cancer tumours (table 4.15, 4.16) and 388 colorectal cancer tumours (table 4.17). FPKM normalized RNAseq data was downloaded to allow direct comparison between the tumours. The cases were classified as high WWOX (higher than median WWOX expression) or low WWOX (median or lower WWOX expression), and high GRP78 (higher than median GRP78 expression) or low GRP78 (median or lower GRP78 expression). Kaplan Meier analysis and log rank test was used to examine the overall survival rate in the different groups to see if high or low expression shows any difference in terms of survival time.

Table 4.15: Summary of ovarian cancer patient's ethnicity (TCGA)

Ethnicity	Number of patients
Asian	11
Black	25
White	326
Native American	2
Pacific Islander	1
Unknown	10

Table 4.16: Summary of ovarian cancer patient's clinical data (TCGA)

Total number of patients	376
Number of Dead patients	230
Number of Alive patients	146
Median age of diagnosis	59 years
Minimum age of diagnosis	30.5 years
Maximum age of diagnosis	87.6 years
Median survival time	2.8 years
Minimum survival time	0.02 years
Maximum survival time	15 years

Table 4.17: Summary of colorectal cancer patients' clinical data (TCGA)

Total number of patients	388
Number of Dead patients	92
Number of Alive patients	296
Median age of diagnosis	69.3 years
Minimum age of diagnosis	34.1 years
Maximum age of diagnosis	90 years
Male patients	207
Female patients	181
Median survival time	1.93 years
Minimum survival time	0.01 year
Maximum survival time	12.3 years
Stage 1	69
Stage 2	163
Stage 3	114
Stage 4	49
Unknown stage	11

4.2.2.1 The effect of *WWOX* expression on overall survival (OS) time in ovarian cancer patients

The effect of WWOX expression on overall survival time in 376 ovarian cancer patients was analysed by Kaplan Meier analysis. The table 4.18 below show the case processing summary of ovarian cancer patients. From the total 376 patients, there were 188 patients for both high and low WWOX expression groups and among them for both high and low WWOX group, 115 patients had died and 73 patients (38.8%) were still alive. Figure 4.7 show the graph showing Kaplan Meier curves for high and low WWOX groups. The blue curve representing high WWOX expression is overlapping green curve representing low WWOX in the start of analysis but in the middle and end the high WWOX expression curve separates from green and shows greater survival. However, there is no statistical difference between both groups as shown in table 4.19 (p=0.218).

Table 4.18: Summary of patients in low and high WWOX groups

Case Processing Summary

WWOX high/low	Total N	Dead Patients	Patients still alive	
			N	Percent
WWOX High	188	115	73	38.8%
WWOX low	188	115	73	38.8%
Overall	376	230	146	38.8%

Table 4.19: Log Rank comparison of high WWOX and low WWOX patient groups

Overall Comparisons

	Chi-Square	df	P value
Log Rank (Mantel-Cox)	1.514	1	0.218

(df) represents degree of freedom

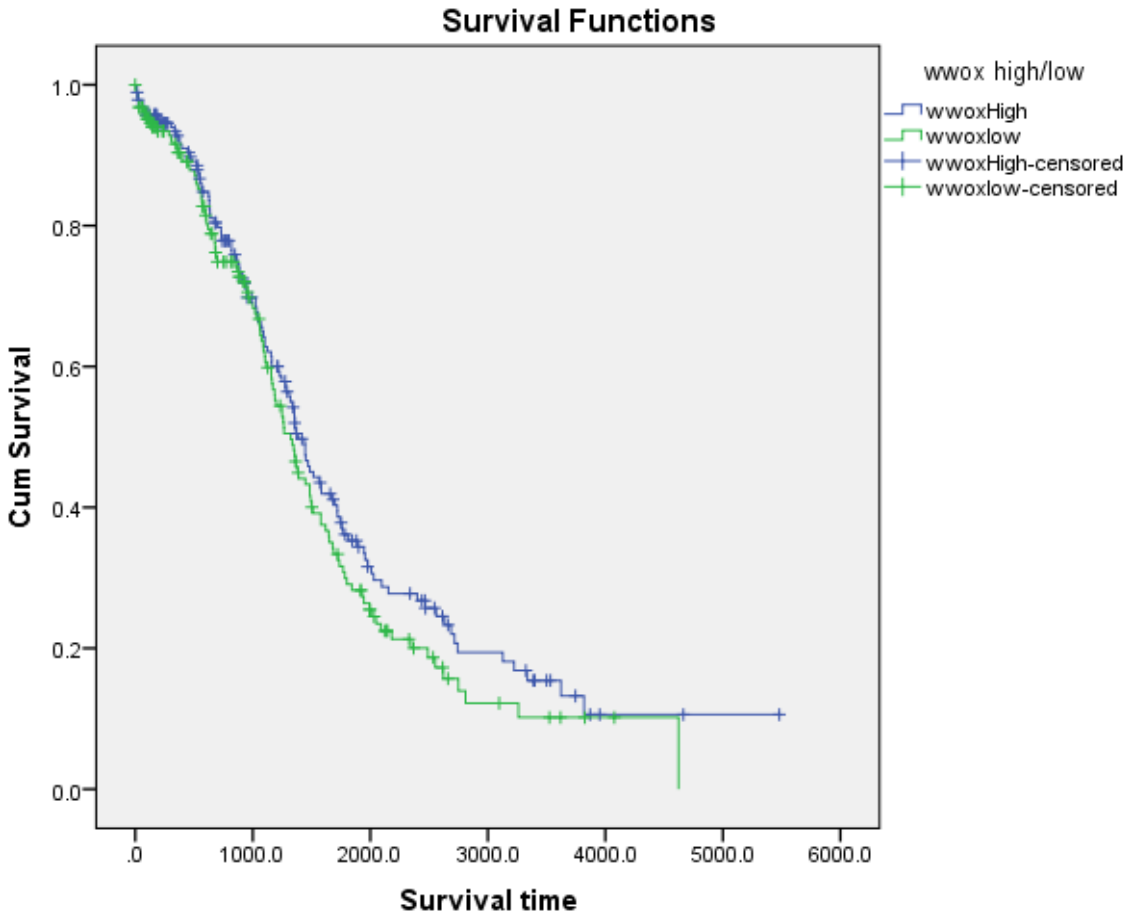


Figure 4.7: Kaplan Meier curves of ovarian cancer patients showing overall survival (OS) time. The blue curve represents patients with high WWOX expression and green curve represents patients with low WWOX expression. The vertical lines in both curve represents the censored observations.

4.2.2.2 The effect of *GRP-78* expression on overall survival (OS) time in ovarian cancer patients

Table 4.20 below shows that there were 188 patients each for both low and high GRP-78 expression groups. For high GRP-78 group 119 patients had died and 69 patients (36.7%) were still alive. In low GRP-78 group 111 patients had died and 77 patients (41%) were still alive. Figure 4.8 shows the overall survival graph plotted by Kaplan Meier analysis showing curves for both high and low GRP-78 expressing groups. Both curves (blue for high GRP-78 and green for low GRP-78) overlap each other for most part of the observation and show same trend. At the start of observation both groups show lot of events (deaths). The table 4.21 below show P value (p=0.492) confirming there was no significant difference between both groups in terms of survival time.

Table 4.20: Summary of patients in low and high GRP-78 groups

Case Processing Summary

GRP-78 H/L	Total N	Dead Patients	Patients still alive	
			N	Percent
GRP High	188	119	69	36.7%
GRP Low	188	111	77	41.0%
Overall	376	230	146	38.8%

Table 4.21: Log Rank comparison of high GRP-78 and low GRP-78 patient groups

Overall Comparisons

	Chi-Square	df	P value
Log Rank (Mantel-Cox)	0.473	1	0.492

(df) represents degree of freedom

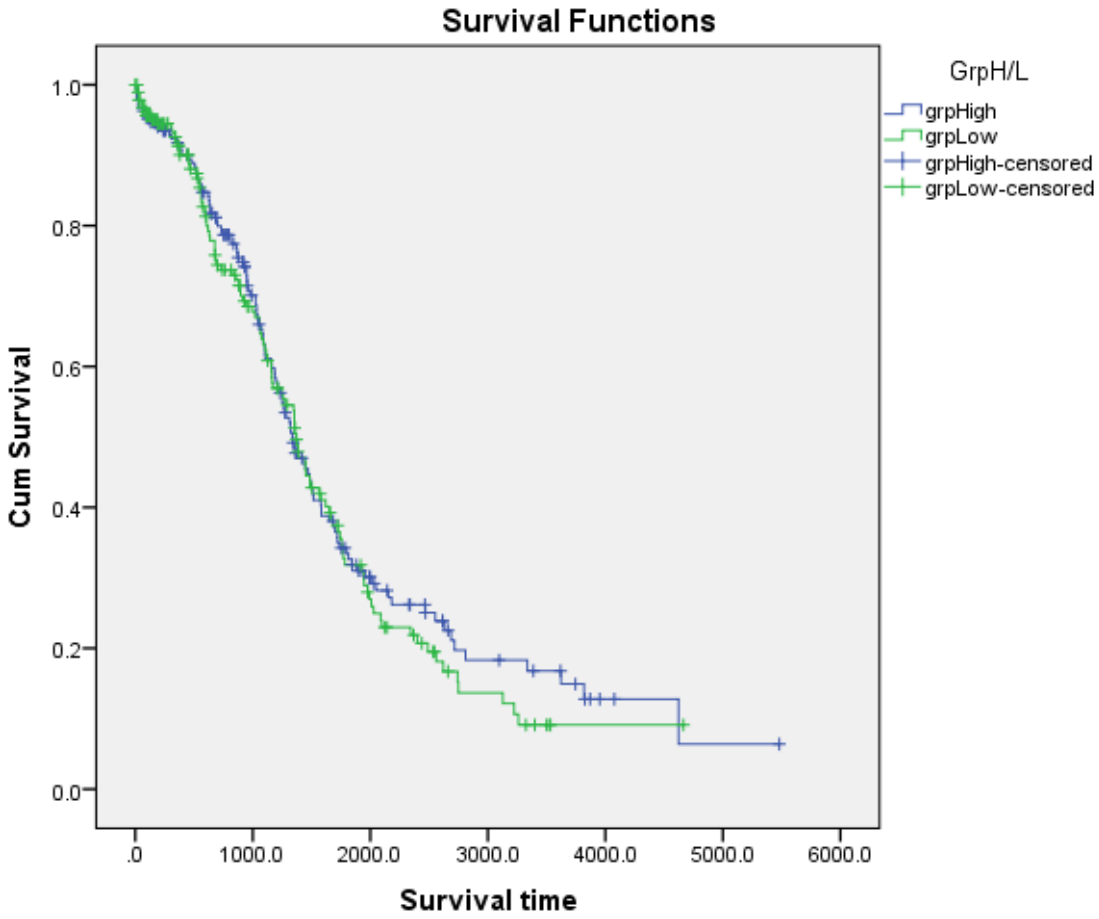


Figure 4.8: Kaplan Meier curves of ovarian cancer patients showing overall survival (OS) time. The blue curve represents patients with high GRP-78 expression and green curve represents patients with low GRP-78 expression. The vertical lines in both curve represents the censored observations.

4.2.2.3 The effect of combined *WWOX/GRP78* expression on overall survival (OS) time in ovarian cancer patients

Table 4.22 below shows that there were 104 patients each for high-WWOX/high-GRP-78 and low-WWOX/low-GRP-78 groups. Also, there were 84 patients each for high-WWOX/low-GRP-78 and low-WWOX/high-GRP-78 groups. For high-WWOX/high-GRP-78 group 70 patients had died and 34 (32.7%) were still alive, whereas for high-WWOX/low-GRP-78 group 45 patients had died and 39 (46.4%) were still alive. Similarly, for low-WWOX/high-GRP-78 group 49 patients had died and 35 (41.7%) were still alive, whereas for low-WWOX/low-GRP-78 group 66 patients had died and 38 (36.5%) were still alive. Figure 4.9 show the overall survival graph plotted by Kaplan Meier analysis showing curves for all four high or low WWOX or GRP-78 expressing groups. All four curves show lot of event (deaths) at the start of the observation. For most part of the observation all four curves overlapped each other. At the end of the observation blue (high-WWOX/high-GRP-78), green (high-WWOX/low-GRP-78) and yellow curves (low-WWOX/high-GRP-78) were overlapping each other but appear to be higher compared with purple curve representing low-WWOX/low-GRP-78. Graph shows there was no effect of high or low WWOX or GRP-78 on the survival time as show by the table 4.23 showing P value (p=0.142) which means no statistical significance.

Table 4.22: Summary of patients in low or high WWOX or GRP-78 groups

Case Processing Summary

WWOX/GRP78	Total N	Dead Patients	Patients still alive	
			N	Percent
WH/GRh	104	70	34	32.7%
WH/Grlo	84	45	39	46.4%
WLo/GRh	84	49	35	41.7%
WLo/Grlo	104	66	38	36.5%
Overall	376	230	146	38.8%

Table 4.23: Log Rank comparison of low or high WWOX or GRP-78 patient groups

Overall Comparisons

	Chi-Square	df	P value
Log Rank (Mantel-Cox)	5.436	3	0.142

(df) represents degree of freedom

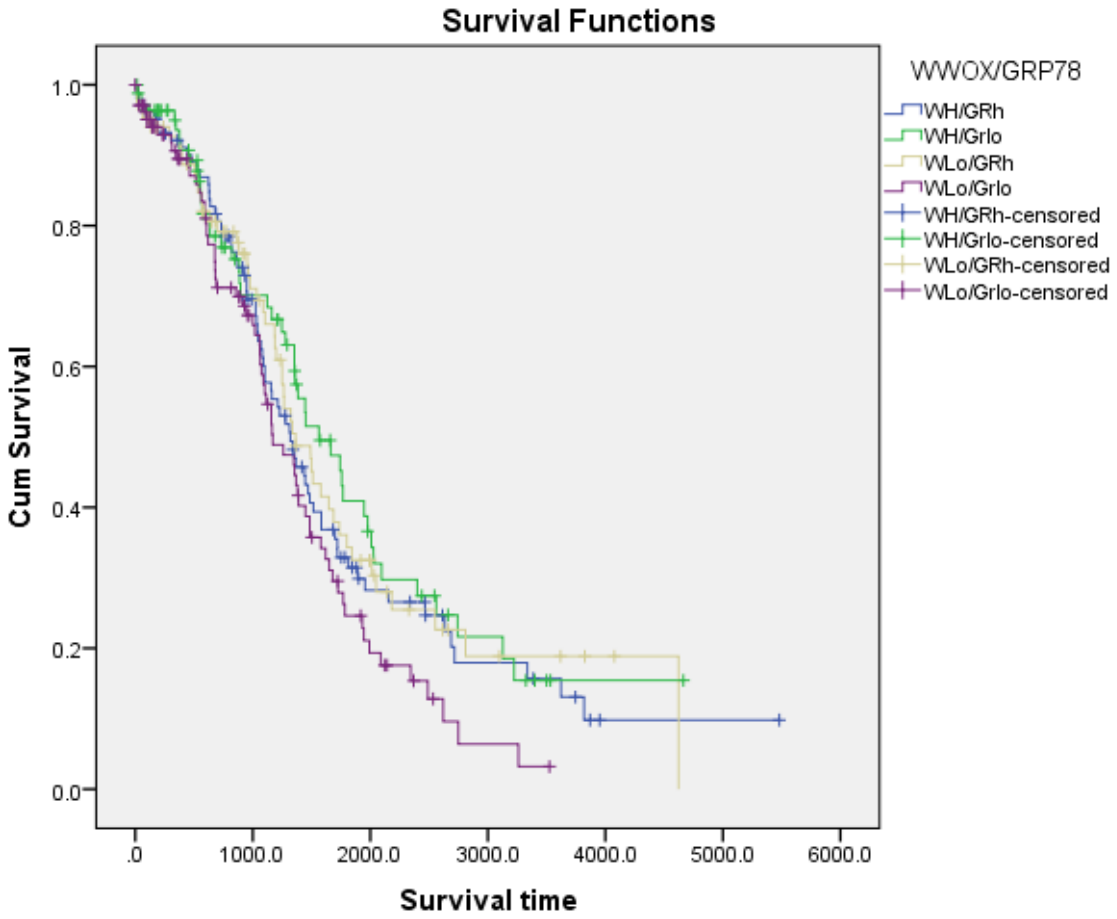


Figure 4.9: Kaplan Meier curves of ovarian cancer patients showing overall survival (OS). The curves blue, green, yellow and purple represents (high-WWOX/high-GRP-78), (high-WWOX/low-GRP-78), (low-WWOX/high-GRP-78) and (low-WWOX/low-GRP-78) respectively. The vertical lines in both curve represents the censored observations.

4.2.2.4 The effect of *WWOX* expression on overall survival (OS) time in colorectal cancer patients

The effect of WWOX expression on overall survival time in 388 colorectal cancer patients was analysed by Kaplan Meier analysis. The table 4.24 below show the case processing summary of colorectal cancer patients. There are 195 patients for high WWOX expression and 193 patients for low WWOX expression group. For high WWOX group, 46 patients had died and 149 patients (76.4%) were still alive. For low WWOX group, 46 patients had died and 147 patients (76.2%) were still alive. Figure 4.10 show the graph showing Kaplan Meier curves for high and low WWOX expressing groups. The blue curve representing high WWOX expression is overlapping green curve representing low WWOX in the start of analysis and at the end, but high WWOX expression group appears high in the middle of the observation. However, this difference was not significant and does not represents a beneficial effect of WWOX for the patients in terms of overall survival because there is no statistical difference between both groups as shown in table 4.25 (p=0.856).

Table 4.24: Summary of patients in low and high WWOX groups

Case Processing Summary

WWOX STATUS	Total N	Dead Patients	Patients still alive	
			N	Percent
WWOX-high	195	46	149	76.4%
WWOX-low	193	46	147	76.2%
Overall	388	92	296	76.3%

Table 4.25: Log Rank comparison of low and high WWOX patient groups

Overall Comparisons

	Chi-Square	df	P value
Log Rank (Mantel-Cox)	0.033	1	0.856

(df) represents degree of freedom

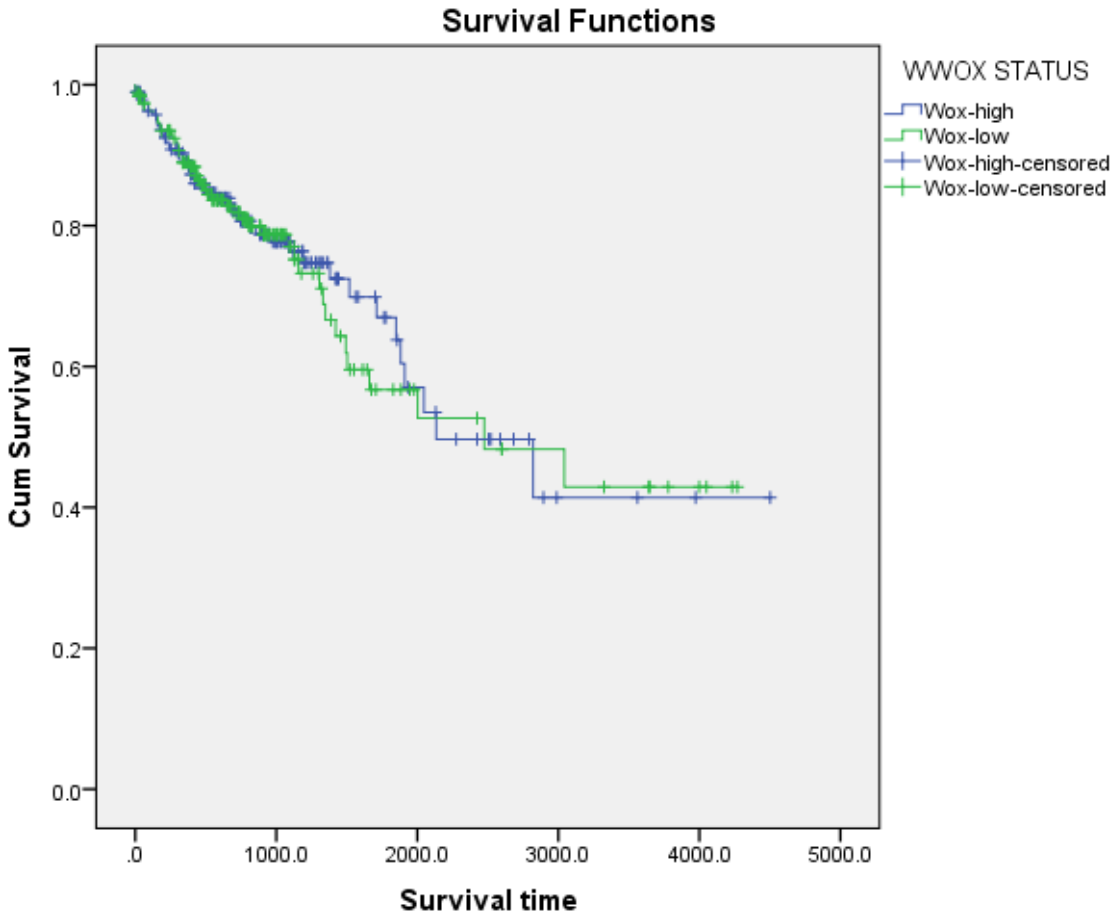


Figure 4.10: Kaplan Meier curves of colorectal cancer patients showing overall survival (OS) time. The blue curve represents patients with high WWOX expression and green curve represents patients with low WWOX expression. The vertical lines in both curve represents the censored observations.

4.2.2.5 The effect of *GRP-78* expression on overall survival (OS) time in colorectal cancer patient

Table 4.26 below shows that there are 195 patients for high GRP-78 and 193 patients for low GRP-78 expression groups. For high GRP-78 group 45 patients had died and 150 patients (76.9%) were still alive. In low GRP-78 group 47 patients had died and 146 patients (75.6%) were still alive. Figure 4.11 show the overall survival graph plotted by Kaplan Meier analysis showing curves for both high and low GRP-78 expressing groups. The blue curve representing high GRP-78 appears high at the start of observation and at the end of observation but it overlaps green curve (low GRP-78) in the middle. High GRP-78 curve gives an impression of better survival but table 4.27 show (p=0.160) that it was not significant. This result suggests a possible trend for better survival associated with high GRP-78.

Table 4.26: Summary of patients in low and high GRP-78 groups

Case Processing Summary

GRP-78 status	Total N	Dead Patients	Patients still alive	
			N	Percent
GRP-high	195	45	150	76.9%
GRP-low	193	47	146	75.6%
Overall	388	92	296	76.3%

Table 4.27: Log Rank comparison of low and high GRP-78 patient groups

Overall Comparisons

	Chi-Square	df	P value
Log Rank (Mantel-Cox)	1.978	1	0.160

(df) represents degree of freedom

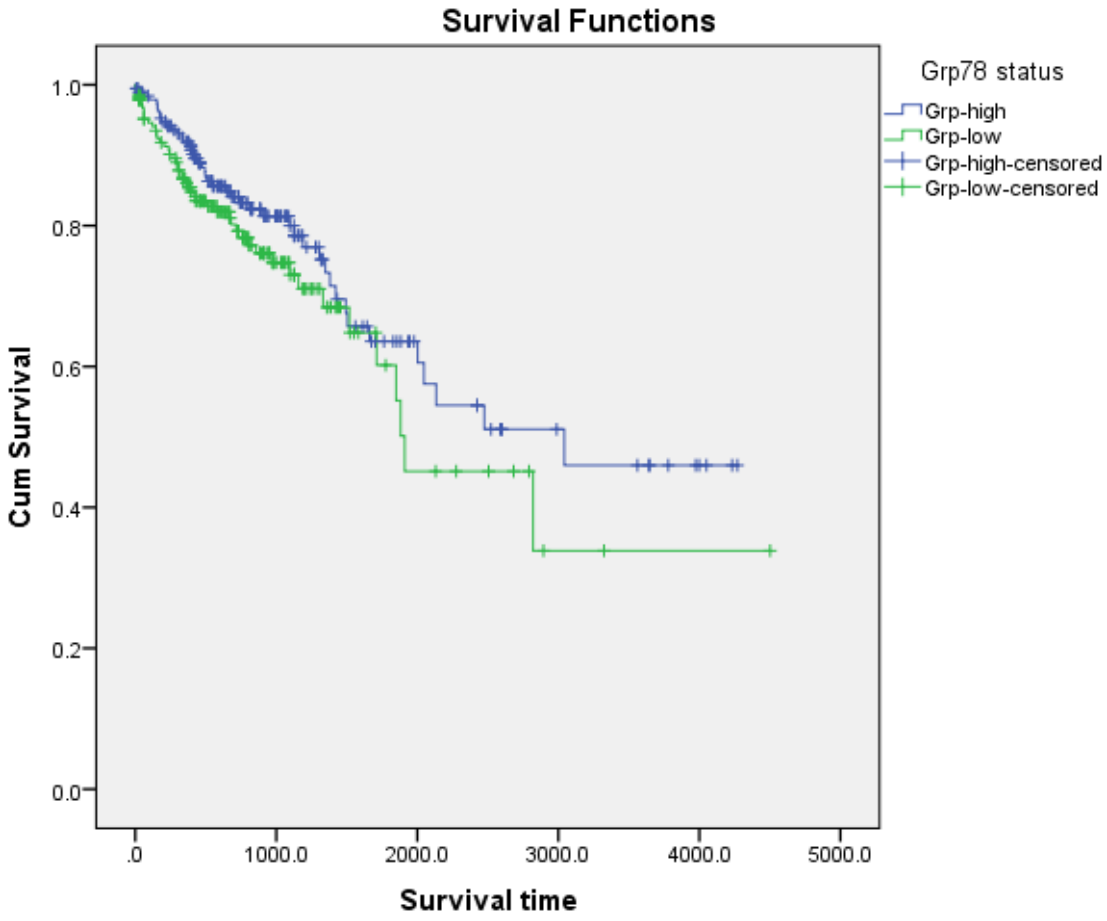


Figure 4.11: Kaplan Meier curves of colorectal cancer patients showing overall survival time. The blue curve represents patients with high GRP-78 expression and green curve represents patients with low GRP-78 expression. The vertical lines in both curve represents the censored observations.

4.2.2.6 The effect of combined *WWOX/GRP78* expression on overall survival (OS) time in colorectal cancer patients

Table 4.28 below shows that there were 106 patients each for high-WWOX/low-GRP-78 and low-WWOX/high-GRP-78 groups. Also, there were 89 patients for high-WWOX/high-GRP-78 and 87 patients for low-WWOX/low-GRP-78 groups. For high-WWOX/high-GRP-78 group 20 patients had died and 69 (77.5%) were still alive, whereas for high-WWOX/low-GRP-78 group 26 patients had died and 80 (75.5%) were still alive. Similarly, for low-WWOX/high-GRP-78 group 25 patients had died and 81 (76.4%) were still alive, whereas for low-WWOX/low-GRP-78 group 21 patients had died and 66 (75.9%) were still alive. Figure 4.12 show the overall survival graph plotted by Kaplan Meier analysis showing curves for all four high or low WWOX or GRP-78 expressing groups. All four curves show lot of event (deaths) at the start of the observation. For most part of the observation blue (high-WWOX/high-GRP-78), green (high-WWOX/low-GRP-78) and yellow curves (low-WWOX/high-GRP-78) overlap each and appear high compared with purple curve representing low-WWOX/low-GRP-78. Graph shows there was no effect of high or low WWOX or GRP-78 on the survival time as show by the table 4.29 showing P value (p=0.300) which means no statistical significance.

Table 4.28: Summary of patients in low or high WWOX or GRP-78 groups

Case Processing Summary

WWOX/GRP78	Total N	Dead Patients	Patients still alive	
			N	Percent
WH/GR h	89	20	69	77.5%
WH/GR lo	106	26	80	75.5%
W Lo/GR h	106	25	81	76.4%
W Lo/GR lo	87	21	66	75.9%
Overall	388	92	296	76.3%

Table 4.29: Log Rank comparison of low or high WWOX or GRP-78 patient groups

Overall Comparisons

	Chi-Square	df	P value
Log Rank (Mantel-Cox)	3.665	3	0.300

(df) represents degree of freedom

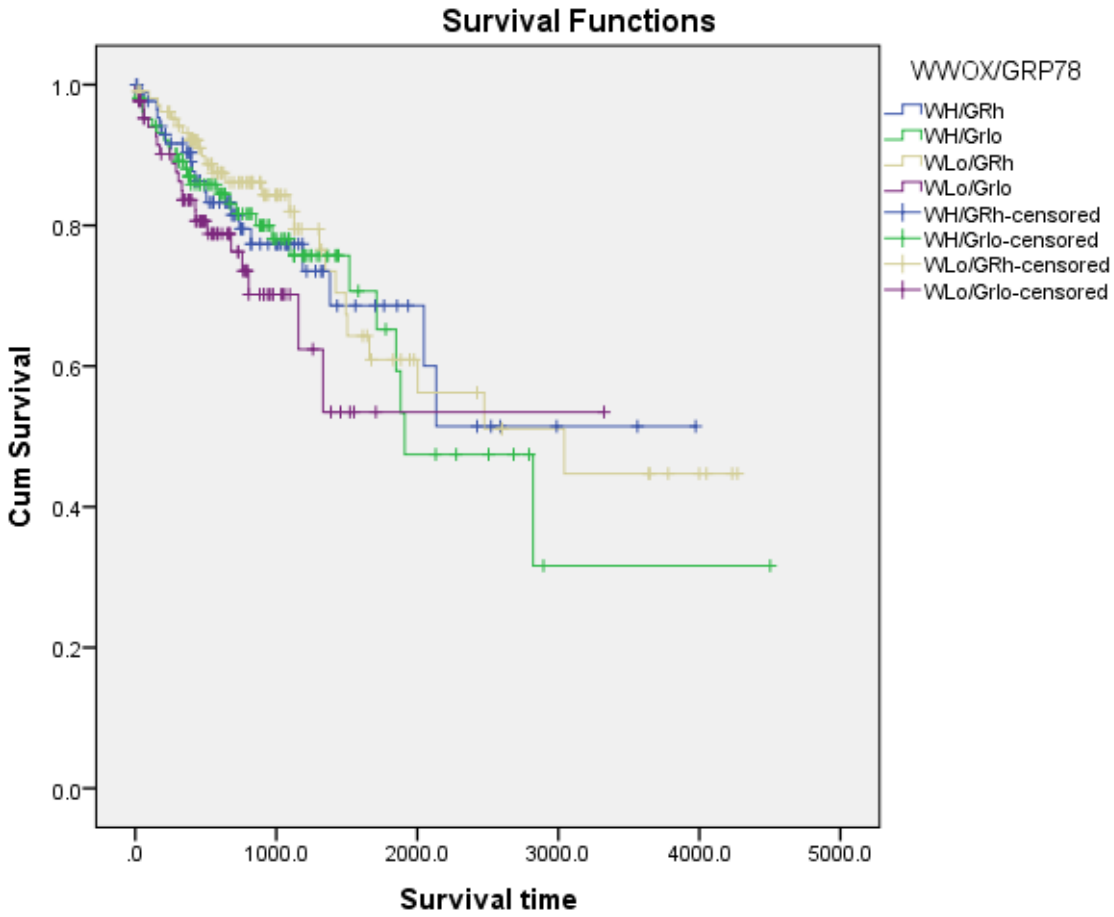


Figure 4.12: Kaplan Meier curves of colorectal cancer patients showing overall survival time. The curves blue, green, yellow and purple represents (high-WWOX/high-GRP-78), (high-WWOX/low-GRP-78), (low-WWOX/high-GRP-78) and (low-WWOX/low-GRP-78) respectively. The vertical lines in both curve represents the censored observations.

4.3 Discussion

4.3.1 Association of GRP-78 with OS or PFS

Previous studies show that paclitaxel induces ER stress response in cancer cells (Mhaidat et al., 2009). GRP-78 is considered as marker of ER stress and regulates multiple arms of unfolded response. GRP-78 is transcriptionally regulated by a conserved ER stress response element in its promoter (Kim et al., 2008). Increased expression of GRP-78 has been observed in many cancers such as breast, ovarian, lung and prostate cancer (Lee et al., 2006; Pootrakul et al., 2006; Gazit et al., 1999). GRP-78 expression is also associated with cancer progression, metastasis, anti-apoptotic effects and drug resistance (Fu and Lee, 2007; Lee, 2007). All these suggest there is a correlation between paclitaxel, ER stress and GRP-78.

In the IHC study there were 50 patients, all stage III or IV and all had received taxol treatment. The high GRP-78 patients showed better survival time compared with low GRP-78 group (p=0.08). Based on these findings it can be said that there is a possible correlation of GRP-78 protein expression with overall survival in ovarian cancer patients. In the few studies where correlation between expression of GRP-78 and over survival times have been observed, most show that low GRP-78 expression predicts longer overall survival compared to high GRP-78 expression. For example, Niu et al. (2015) reported that high protein expression of GRP-78 in pancreatic ductal adenocarcinoma showed significantly poor overall survival (p=0.0007) compared to low expression. This study also showed high GRP-78 expression in tumour tissues compared with normal tissues suggesting it has a role in progression of cancer. In a study by Yu et al., (2016) IHC analysis of 89 NSCLC tumour samples showed high GRP-78 expression predicted shorter overall survival (p=0.043). Only one published study (Thornton et al., 2013) showed that high expression of GRP-78 predicts better overall survival. In this study, Stage II colorectal cancer patients who underwent only surgery, showed improved survival rate with high GRP-78 expression compared with low GRP-78 (p=0.032). However, stage III patients did not show any difference in terms of

overall survival between high and low GRP-78 groups. Interestingly stage III patients with high GRP-78 expression after undergoing 5-FU chemotherapy (vs non-chemotherapy) showed improved 5-year survival rate (52% vs 28%) compared to low GRP-78 expression patients (28% vs 32%). Also, stage III patients showed 7 months longer median survival in high GRP-78 group compared with low GRP-78. Unlike Thornton et al., in my study it is stage III cancer patients with high GRP-78 expression who show better overall survival not stage II. Thornton et al. further showed all patients (regardless of stage) who had 5-FU chemotherapy showed increased overall survival in patients with high GRP-78 compared with low GRP-78 group. Although my study is focused on taxol whose mechanism of action is different to 5-FU, this suggests the possibility of a common mechanism of GRP-78-mediated chemo sensitization. In my study, even prior to taxol treatment, patients already have high GRP-78 showing that ER stress is inherently activated in these patients. This would suggest that it is playing a cytoprotective role (inducing chaperones and increasing the rate of protein processing) instead of apoptotic, and that the apoptotic response to prolonged ER-stress is being suppressed. Possibly, however, the further induction of ER-stress upon taxol treatment may result in it shifting from being cytoprotective to apoptotic.

Interestingly, GRP-78 expression correlates with overall survival but not progression free survival in my IHC study. A similar difference between association with OS and PFS is reported in the study of Ma et al., (2015) in which 163 peripheral blood samples from NSCLC were analysed for expression of GRP-78 and correlation between overall survival and relapse free survival was observed. In their study, Kaplan Meier analysis showed that high GRP-78 expression showed low overall survival time but, like my data, no correlation was seen between GRP-78 expression and relapse free survival time. It is not clear why GRP-78 shows better survival in OS but not in PFS, and this question needs further investigation.

I also observed the impact of expression of GRP-78 mRNA on clinical outcome of patients treated with paclitaxel in a publicly available RNAseq dataset of ovarian cancer patients (TCGA). There were 376 patients, and all received platinum plus taxane treatment. Kaplan

Meier analysis showed no correlation of GRP-78 with overall survival (p=0.492) in ovarian cancer patients, unlike the results of my IHC study of ovarian cancer. Other studies have also suggested a link between GRP-78 mRNA expression and patient survival. For example, Mozos et al. (2011) showed that high GRP-78 mRNA expression in B-cell Lymphoma showed poor overall survival compared to low GRP-78 mRNA expression (p=0.048). A possible reason for the difference between the results of my TCGA and IHC study could be because of differences within these cohorts; however, both patient groups represent high stage ovarian serous cancer patients treated with platinum plus taxane therapy. Another possible factor that might explain the difference between these two studies is that IHC is protein analysis while TCGA is mRNA analysis. Proteins are responsible for cellular mechanisms, and although mRNA translates into protein it does not necessarily mean high mRNA expression will always show high amount of protein. However, Mezos et al. do show that high GRP-78 mRNA expression predicted shorter overall survival, consistent with the GRP-78 protein correlations reported by Niu et al. (2015) and Yu et al. (2016). A third possibility is that the large number of censored patients in this study affected the statistical analysis.

Effect of GRP-78 on overall survival in colorectal cancer patients from TCGA was also analysed. There were 388 patients in this group and but not all of them received taxane treatments. Taxanes are not a standard treatment for colorectal cancer and there are multiple different drug combinations that might be used, so probably these patients received one of several different drug combinations that are not too similar to each other. So, this study investigates the role of ER stress in colorectal cancers but not as part of taxane response. The results showed there may be a possible correlation of GRP-78 with overall survival (p=0.160). Although the results were not significant the high GRP-78 curve in the graph shows a trend towards better survival. These results show similarity to the IHC results of ovarian cancer where a possible correlation of GRP-78 with OS was observed. These results hint about the possible correlation between GRP-78 and OS, independent of taxane treatment, even though it was not seen in ovarian TCGA results.

4.3.2 Association of WWOX with OS or PFS

It has been established by many previous studies that WWOX is a tumour suppressor gene and its loss promote development of tumours or cancers of various types such as endocrine or exocrine carcinomas and renal cell carcinoma (Lin et al., 2013; Iliopoulos et al., 2006). WWOX also plays an important role in regulation of cellular apoptosis. The effect of WWOX protein expression on overall survival in ovarian cancer patients in the IHC study show that there was no correlation between them (p=0.895). Similarly, there was no correlation of WWOX with PFS (p=0.181). In the analysis of RNAseq data from TCGA, no correlation of WWOX was observed with overall survival in ovarian cancer patients (p=0.218), or colorectal cancer patients (p=0.856). These results were therefore all in agreement.

Aldaz et al. (2014) showed the prognostic value of WWOX mRNA expression in breast and ovarian carcinomas. The information was extracted from a publicly available gene expression data set (Oligo-microarrays). Patients were divided into low and high WWOX groups. Kaplan Meier analysis of breast cancer patients showed that low expression of WWOX correlated with short relapse free survival and shorter progression free survival in ovarian cancer patients. Ludes et al. (2007) also observed correlation between WWOX expression and survival time in $Wwox^{+/+}$ and $Wwox^{gt/gt}$ mice (hypomorphs). The Kaplan Meier survival curves showed that $Wwox^{gt/gt}$ mice had significantly shorter survival time compared with wild type $Wwox^{+/+}$ mice. Nunez et al. (2005b) also showed correlation between WWOX expression and overall survival. Reduced expression of WWOX in ovarian cancers showed a significant correlation with FIGO stage IV (p=0.007) and shorter overall survival (p=0.03). Correlation of WWOX and overall survival was observed by using cases from MDACC TMA (n=354). A statistical significant correlation was observed between WWOX and overall survival, whereas low WWOX protein expression correlated with shorter survival (p=0.03). All three studies suggest low WWOX expression is correlated with low survival, contrary to my results of IHC or RNA expression analysis which show no effect of WWOX on survival.

WWOX is a tumour suppressor gene and its loss is associated with development of tumours. WWOX is also associated with cellular apoptosis (Iliopoulos et al., 2006). Considering these functions of WWOX I had predicted that, in my study of IHC and RNA expression, high WWOX expressing patients would show better survival time, but in fact WWOX showed no effect in terms of survival. One possibility of no effect of WWOX on survival in my studies could be due to the fact that WWOX expression is down regulated or lost in tumours and though I have divided the patients in RNA expression study into high WWOX and low WWOX expressing groups but many patients of high WWOX group have expression which apparently does not look very high compared to low WWOX group. The expression levels for 188 low WWOX group were 0.253885-1.951157, whereas for 132/189 patients from the high WWOX group expression was only slightly higher (1.951157-3.794393) whilst 57/189 patients from the high WWOX group expression was much higher (4.004566-14.0381). Therefore, the median expression value may not be the optimal threshold for defining high and low expressing groups. Also, about 76% of colorectal patients were censored (table 4.25) but only 38.8% of ovarian patients (table 4.18) were censored, which can also affect the statistical analysis and hence affected the survival curves. Similarly, in IHC study total group was of 50 patients and among that 17 were low WWOX and rest were moderate or high WWOX expressing patients. Increased number of patients may have shown a clearer survival effect.

WWOX exhibits LOH or homozygous deletion in several tumour types (Driouch et al., 1997). Apart from predominant full-length transcripts (variant 1), spliced transcripts of WWOX have also been observed in tumour sample which can potentially interfere with normal function of WWOX in dominant negative fashion, especially variant 4 (lacking exon 6-8) (Paige et al., 2001). Gourley et al., (2005) reported that ovarian tumours express significantly low expression of variant 1 compared to normal ovaries which was not associated with any particular clinical subgroup. Low levels of Variant 4 were expressed which show association with advanced stage ovarian cancer. The survival analysis showed that tumours co-

expressing variant 4 and variant 1 showed poor survival compared with variant 1 expressing tumours. Also, variant 4 was frequently present in non-malignant ovarian tissue. These results show WWOX variant 1 is tumour suppressor in ovarian cancer, but variant 4 functions are not known. Comparing this study of Gourley et al, with my results of IHC or RNA expression it can be speculated that co-expression of these variants may also be a reason that no correlation was observed in survival.

4.3.3 Association of WWOX/GRP-78 with OS or PFS

The impact of WWOX/GRP-78 together was observed on overall survival or progression free survival in both studies (IHC and TCGA). There were four groups' high-WWOX/high-GRP-78, high-WWOX/low-GRP-78, low-WWOX/high-GRP-78 and low-WWOX/low-GRP-78. The aim was to see if any of these combinations predicts high survival rate in ovarian or cancer patients. In IHC study of ovarian cancer patients no correlation between all these four groups and overall survival was observed (p=0.358), although both groups with high GRP78 showed better survival than the two low-GRP78 groups. In the same set of patients, no correlation between WWOX / GRP-78 status and progression free survival was observed (p=0.542). The ovarian and colorectal cancer patients from TCGA data set also showed similar results. No correlation between WWOX / GRP-78 status and overall survival was observed (p=0.142) in ovarian cancer patients, or in colorectal cancer patents (p=0.300). Overall both studies showed no effect of combined GRP / WWOX status together on survival time.

In a study by Janczar et al. (unpublished data) it was shown that paclitaxel triggers ER stress response in ovarian cancer cells and WWOX transfection decreases survival of cells treated with tunicamycin (an ER stress inducer). It was further shown that induction of ER stress was unaltered by WWOX, but WWOX re-expression caused a significant shift towards an apoptotic response to ER stress which could be reversed by siRNA. The clinical relevance of these findings was observed in a publicly available microarray gene expression

dataset (GEO GSE9899) of 260 ovarian cancer patents. Kaplan Meier analysis showed high WWOX mRNA expression predicted longer PFS in patients treated with paclitaxel (n=195, p=0.01) but not in patients which did not have paclitaxel treatment (n=65, p=0.66). Interestingly, WWOX affected PFS only in patients whose tumours expressed low-GRP-78 (p=0.003), whilst the high GRP-78 expressing group did not show any correlation of WWOX with PFS (p=0.469). We hypothesised that the patient samples with high GRP-78 expression (pre-treatment) may have intrinsically activated ER stress which is not dependent on paclitaxel. Cancers in which ER stress is inherently active would be expected to benefit from the cytoprotective functions of ER-stress but would presumably be resistant to ER-stress induced apoptosis. It was therefore speculated that WWOX status had no impact on these patients as paclitaxel would only be able to kill the cancer cells through its mitotic arrest function, and not through an ER-stress related mechanism. In contrast, in low-GRP-78 expressing patients, we hypothesise that ER-stress is not inherently active, and therefore could be activated by paclitaxel treatment. In these tumours, we speculated that WWOX status could influence the apoptotic outcome of ER-stress, and thus higher WWOX expression predicted longer PFS. The data from this study show a correlation between WWOX and UPR. Contrary to findings of this study the Kaplan Meier analysis data from my IHC and TCGA study did not show any correlation between WWOX and UPR. Whilst it can again be speculated that patients with high GRP-78 expression may have intrinsic ER stress activated, such that treatment with paclitaxel did not induce ER stress associated apoptosis, and hence there was no impact of WWOX status. However, surprisingly, in my analysis low GRP-78 also showed same results like high GRP-78.

4.4 Conclusion

My results do show some evidence that ER-stress is related to patient survival, and higher GRP78 (indicating inherently activated ER-stress) appeared to provide an overall survival advantage in two of the three patient cohorts studied. However, this is independent of WWOX status, and is not clearly related to paclitaxel response (since this is an association

with inherent ER-stress activation). Whilst further work is required to address the inconsistencies with earlier data from our group, these findings, like those in chapter 3, do not provide supportive evidence linking ER-stress and paclitaxel response. Therefore, for the remainder of my PhD work I instead focussed on cisplatin response in cancer cells.

Chapter 5 Role of WWOX in mediating cisplatin induced cell death

5.1 Introduction

5.1.1 Involvement of WWOX in cancer

WWOX is a WW domain containing oxidoreductase tumour suppressor gene, located at the second most commonly expressed fragile site called FRA16D. It has been established by many previous studies that WWOX is a tumour suppressor gene and its loss promote development of tumours or cancers of various types such as endocrine or exocrine carcinomas and renal cell carcinoma (Lin et al., 2013; Iliopoulos et al., 2006). Immunohistochemical staining of different histological types of ovarian carcinomas (serous, endometrioid, clear cell and mucinous) showed variable expression of WWOX compared with normal ovarian tissues which strongly expressed WWOX (Nunez et al., 2005). Another study by Qin et al., (2006) showed that prostate cancer cells (LNCap, PC3 and DU145) showed a significant reduction in WWOX mRNA and protein expression as compared to non-cancerous prostate cells (PWR-1E) and immunohistochemical staining showed down regulation of WWOX in 84% of prostate cancer tumours (Qin et al., 2006). Down regulation of WWOX expression in human cervical cancer was shown in a study by Qu et al., (2013), and when WWOX was re-expressed in HeLa cells, it inhibited their proliferation and induced apoptosis, while knockdown of WWOX in SiHa cells enhanced proliferation and inhibited apoptosis. All these studies show down regulation of WWOX is associated with cancer development. Tumour suppressor function of WWOX was shown by Bednarek et al., (2001). WWOX expressing breast cancer cells MDA-MB-435/WWOX and MDA-MB-435/vector were injected in nude mice and results showed WWOX strongly inhibited tumorigenicity. Work from my own lab showed that WWOX is disrupted by homozygous deletion (Paige et al., 2001) and its expression is decreased in ovarian cancer (Gourley et al., 2005). Transfection of WWOX in ovarian cancer cells suppressed *in vivo* tumorigenicity in nude mice and generated apoptosis in *in vitro* suspension cell culture but had no effects on *in vitro* growth rate in adherent cultures (Gourley et al., 2009). The same study showed that *WWOX* expression resulted in reduced attachment with fibronectin (an extracellular matrix involved

in peritoneal metastasis). An apoptotic mechanism behind WWOX tumour suppression was shown by another study of lung and prostate cancer cell lines lacking *WWOX* expression. Prostate cancer cells (DU145) were transfected with WWOX and then cells were injected in nude mice (5-week-old and 5 mice per group). Same number of uninfected cells was injected in control mice (10 mice). Control mice showed tumour progression whereas mice injected with transfected WWOX cells suppressed tumorigenicity (Qin et al., 2006). In a similar study, lung cancer cells lines (H460, a549 and U2020) were transfected with Ad-WWOX and injected in 5 nude mice. Five control mice were injected with Ad-GFP. After 28 days' tumours formation was checked and WWOX infected mice showed almost no tumours whereas control mice showed tumours (Fabbri et al., 2005).

p63 and p73 proteins are the family members of p53 protein. These three proteins are very important transcription factors and their structure shows that they have three domains common in them by which they perform their function. The three domains include an oligomerization domain (OD), a DNA binding domain and a transactivation domain (TA). ΔNp63 is an isoform (truncated gene) of p63 that acts as a dominant negative repressor of p63 function (Melino et al., 2002; Yang et al., 2002). The expression of ΔNp63α by various mechanisms is also involved in chemotherapy resistance (Sen et al., 2011). In a recent study by Salah et al., (2013) it was shown that WWOX can sensitize cancer cells to chemotherapy drugs. In this study the link between WWOX and ΔNp63α was observed and it showed that when cancers cells expressing only ΔNp63α were treated with cisplatin they showed low cell death rate but cancer cells expressing both WWOX and ΔNp63α on treatment with cisplatin, showed increased rate of cell death by approximately 14-fold. This clearly shows that the linkage between WWOX and ΔNp63α was responsible of increased sensitivity of cancer cells to cisplatin and it is thought that WWOX does this by restricting the ΔNp63 to the cytoplasm which results in suppression of transcription of ΔNp63 (Salah et al., 2013).

5.1.2 Involvement of *FHIT* in cancer

FRA3B is the most commonly expressed common fragile site in human genome and it was first common fragile site to be studied in detail (Smeets et al., 1986). Further studies showed that like WWOX, FHIT is a gene located at this CFS which is also a target of deletion in multiple cancers, and expression of FHIT was suppressed in many tumour types. In a study of mice by Dumon et al., (2001), human FHIT gene was expressed in heterozygous *Fhit*$^{+/-}$ knockout mice by adenoviral or adeno-associated vectors. The *Fhit*$^{+/-}$ knockout mice were vulnerable to development of tumour on treatment with carcinogens. Expression of human FHIT gene inhibited tumour development in mice. Similarly, in another study FHIT gene was re-expressed by adenoviral vector in various ovarian and lung cancer cell lines lacking FHIT expression, and this induced apoptosis in all cell lines (Roz et al., 2002).

Nishizaki et al., (2004), showed the combined effects of FHIT and p53 on NSCLC proliferation and apoptosis *in vitro* and *in vivo* using adenoviral transfer of FHIT and p53. The results showed that FHIT and p53 together suppressed the tumour growth *in vitro* and *in vivo* (xenografts in nude mice). Further investigations showed that MDM2 (Mouse double minute 2 homolog), which is a negative regulator of p53, was inactivated by FHIT but p53 being in stabilized form worked as tumour suppressor along with FHIT. Hence this study shows FHIT along with p53 can improve the therapies from treatment of lung or other cancers (Nishizaki et al 2004).

(Andriani et al., 2006) investigated the extrinsic pathways activated by FHIT and intrinsic pathways activated by cisplatin at the same time in NSCLC (H460 cells) to see if it could enhance the cell death. FHIT gene was re-expressed in FHIT negative NSCLC by adenovirus, which slightly increased the sensitivity to cisplatin. As evident by previous studies, this study also showed that wild type p53 was important for this sensitivity to cisplatin. This study shows FHIT as an important gene to overcome the drug resistance due to cisplatin therapy (Andriani et al., 2006).

Cisplatin is widely used as a chemotherapy drug for the treatment of different types of cancer. Its mechanism of action involves forming DNA-damaging adducts. The adducts initiate signalling by DNA damage recognition proteins, activating repair mechanism to fix the damaged DNA and when it fails to repair the DNA then it leads to apoptosis. Common fragile sites are sensitive to DNA damage, leading to stalled replication and chromosomal breaks that might affect the expression of nearby genes. Cancer cells show down regulated expression of WWOX and FHIT as compared to normal cells. As explained by many studies WWOX and FHIT inhibit proliferation of cancer cells and initiate apoptotic pathways to kill the cancer cells. In other words, both of these fragile site-associated genes are beneficial for the survival of cancer patients. Here in my study I originally hypothesised that WWOX would enhance cisplatin cytotoxicity and that this may be via the ER-stress pathway. However, my initial data demonstrated that this was not the case and led to a revised hypothesis that treatment of cancer cells line with cisplatin will induce DNA damage that may affect common fragile sites, leading to down regulation of nearby tumour suppressors such as WWOX and FHIT.

5.2 Experimental Approach

In this chapter the aim was to see if cisplatin can induce ER stress mediated by WWOX in ovarian, colorectal or prostate cancer cell lines. Before the start of experiments the first step was to find out a suitable dose (IC50) to be used for experiments. Cancer cells were treated with various doses of cisplatin and cells were observed under the microscope (appendices Figures S3.1, S3.2 and S3.3) and the dose which killed approximately 50% of cells was selected for use in experiments. Ovarian cancer cells A2780, colorectal cancer cells HCT116 and prostate cancer cells LNCap were treated with cisplatin and quantitative PCR was performed (Chapter 2 materials and methods). Each experiment consists of three treatments i.e. untreated, cisplatin treated and DMF treated cells. Since cisplatin was dissolved in dimethyl formamide (DMF) (appendices S6.1) so cells were also treated DMF only to see if the effect on cells was due to cisplatin or DMF. Each experiment was repeated

three times and final average values were used to plot graphs and analyse relative expression levels of WWOX (Chapter 2 material and methods). Correct PCR product was confirmed by the analysis of melt curve in Rotor gene 6000 software. Unexpectedly in these experiments, rather than showing that WWOX increased cisplatin induced cell death, I discovered that cisplatin resulted in loss of WWOX expression. Subsequent experiments were then performed to determine if the loss of WWOX expression was related to its location at a common fragile site, or to cisplatin induced transcriptional regulation.

5.3 Results

5.3.1 Expression of *WWOX* following cisplatin treatment

Following cisplatin treatment, expression of WWOX was analysed in ovarian cancer cells A2780, colorectal cancer cells HCT116 and prostate cancer cells LNCap.

5.3.1.1 Expression of *WWOX* in A2780 cells

Cisplatin treatment reduced the expression of WWOX mRNA in ovarian cancer A2780 cells by 65% after 24 hours and by 85% after 48 hours (Figure 5.1) as compared to DMF and untreated cells ($p<0.05$).

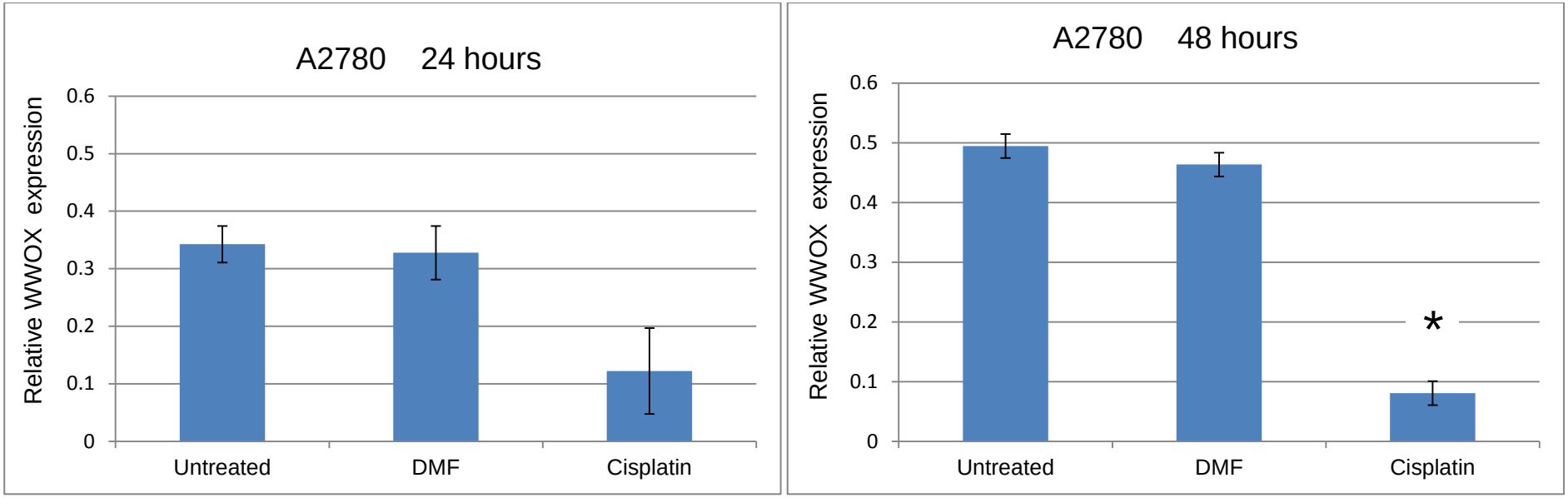


Figure 5.1

Down regulation of *WWOX* expression in A2780 cells after 24 hours and 48 hours of cisplatin treatment. Graph shows expression of WWOX mRNA relative to B-actin measured by qRT-PCR in A2780 cells following 24 hours' and 48 hours' growth in the presence of 4µg/ml cisplatin, vehicle (DMF) or no drug. Means of triplicate experiments ± SEM shown. T-test was perfomed to find out significance among the treatments. (*, $p<0.05$).

5.3.1.2 Expression of *WWOX* in HCT116 cells

Cisplatin treatment reduced the expression of WWOX mRNA in colorectal cancer cells HCT116 by 60% after 24 hours and by 75% after 48 hours (Figure 5.2) as compared to DMF and untreated cells (*, $p<0.05$).

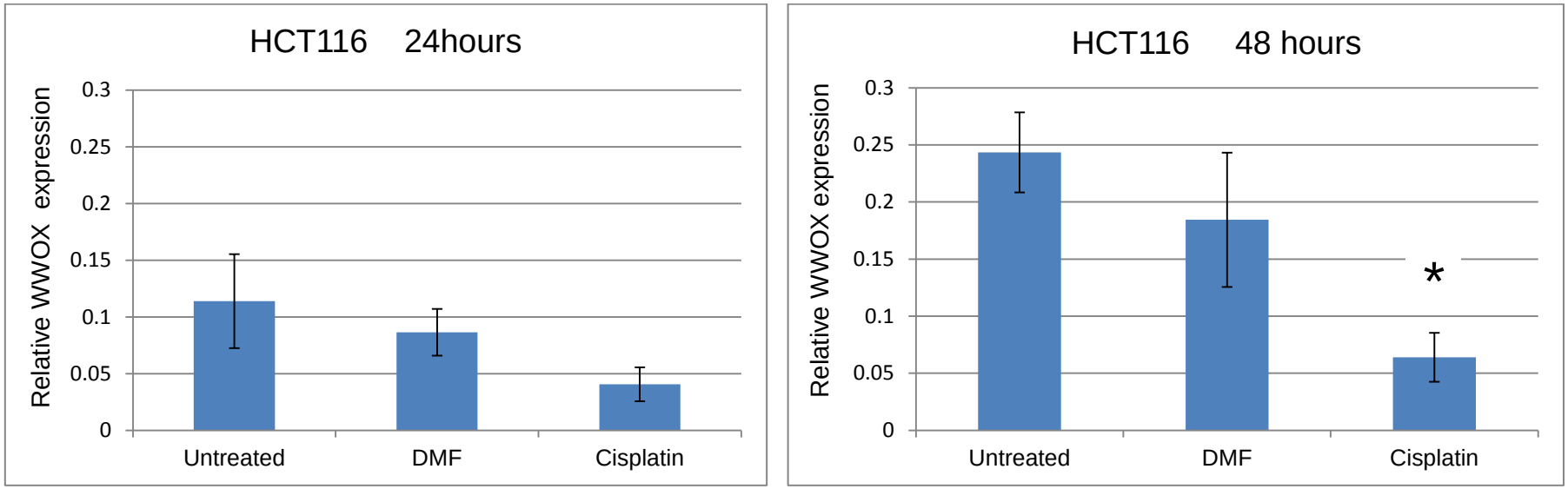


Figure 5.2

Down regulation of *WWOX* expression in HCT116 cells after 24 hours and 48 hours of cisplatin treatment. Graph shows expression of WWOX mRNA relative to B-actin measured by qRT-PCR in HCT116 cells following 24 hours' and 48 hours' growth in the presence of 5µg/ml cisplatin, vehicle (DMF) or no drug. Means of triplicate experiments ± SEM shown. T-test was performed to find out the significance among the treatments. (*, $p<0.05$)

5.3.1.3 Expression of *WWOX* in LNCap cells

No significant change in the expression of WWOX mRNA was identified in prostate cancer cells LNCap after 24 or 48 hours of cisplatin treatment compared to untreated or DMF (Figure 5.3).

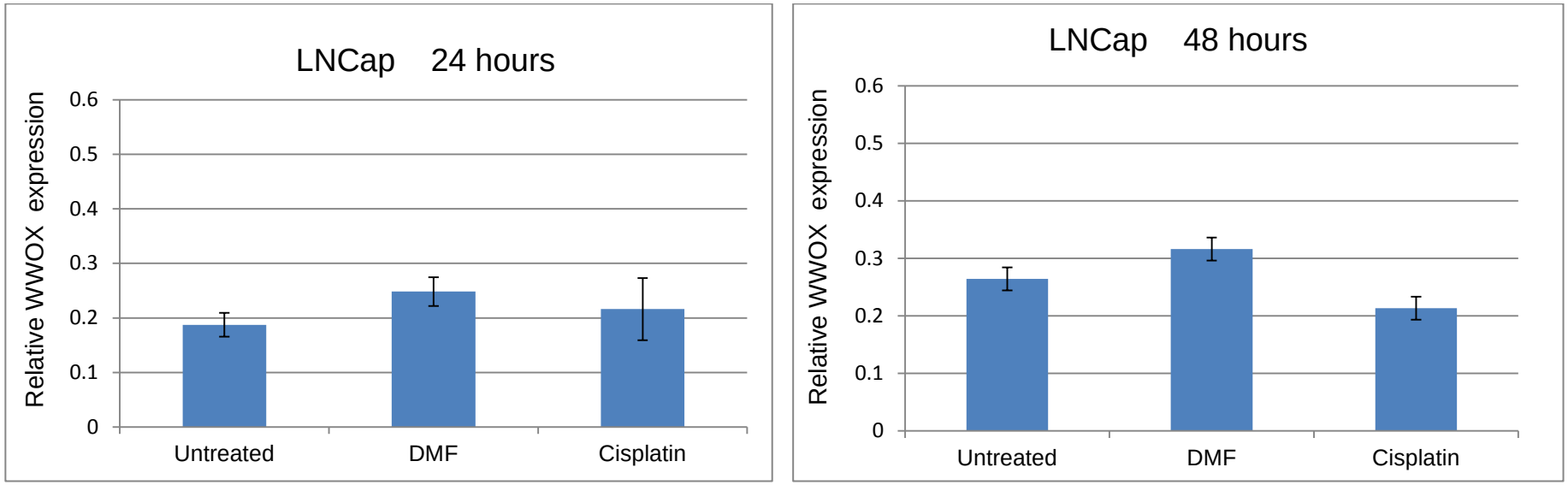


Figure 5.3

***WWOX* expression in LNCap cells after 24 hours and 48 hours of cisplatin treatment.**

Graph shows expression of WWOX mRNA relative to B-actin measured by qRT-PCR in LNCap cells following 24 hours' and 48 hours' growth in the presence of 2.5µg/ml cisplatin, vehicle (DMF) or no drug. Means of triplicate experiments ± SEM shown.

5.3.1.4 Expression of WWOX in HCT116 and HCT116-p53null cells after 24 hours of cisplatin treatment

In this experiment, I used colorectal cancer cells HCT116 along with another cell line, HCT116 lacking p53 expression, to investigate the mechanism of DNA damage. P53 is a key regulator of DNA damage response. So, if cisplatin induces DNA damage that disrupts the common fragile sites and results in reduced WWOX expression this may require p53. I intended to analyse 24 hours and 48 hours (as I did for the earlier experiments), but 24 hours experiments showed a clear loss of WWOX expression, and so the 48 hours' time point was then not needed. I wanted to do qRT-PCR, but the machine broke down, therefore I used semi quantitative PCR instead. Although it was not quantitative PCR but the concept of technique in this experiment was like the qRT-PCR performed in above experiments. I used different amounts of template to prepare six different standard dilutions (1:1, 1:5, 1:25, 1:50, 1:75, and 1:100). The template cDNA (1µg) used was from a *WWOX* transfected ovarian cancer cell line (PEO1-H8) having high amount of *WWOX* in it. Figure 5.4-A shows amount of product related to amount of template and the product formation did not reach saturation when using 1:5 dilution or less.

Next, I analysed untreated, DMF (dimethylformamide) or cisplatin (5µg/ml) treated HCT116 and HCT116-p53null cells, using 1:25 dilutions of template (Figure 5.4-B). Expression of WWOX was seen in both cell lines when untreated or treated with DMF. In cisplatin treated HCT116 cells, WWOX expression can no longer be seen (confirming the qRT-PCR results from above figure 5.6). In the HCT116-p53null cells cisplatin treatment also causes loss of WWOX expression demonstrating p53 is not required for the altered WWOX expression. No amplification of either product was seen in –RT controls (not shown) confirming the RNA samples were genomic DNA free.

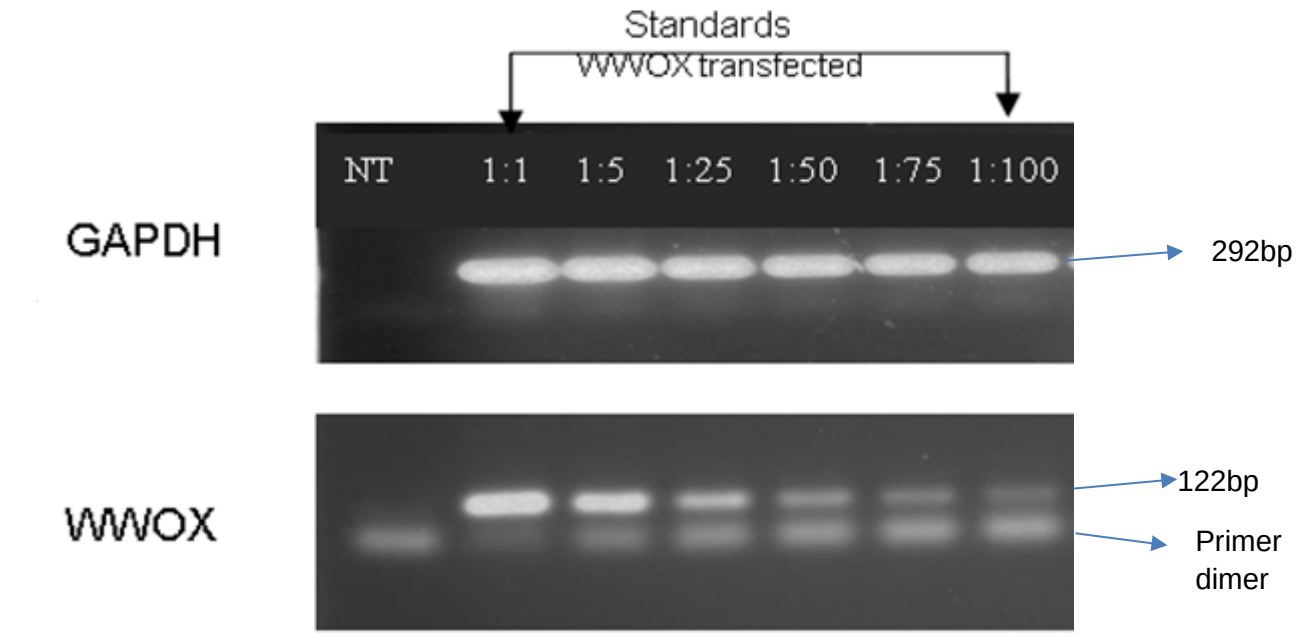


Figure: 5.4-A

WWOX (122bp) and GAPDH (292bp) expression of PEO1-WWOX transfected templates used as standards. No template (NT) show no contamination. (For detailed image see appendices S8.1)

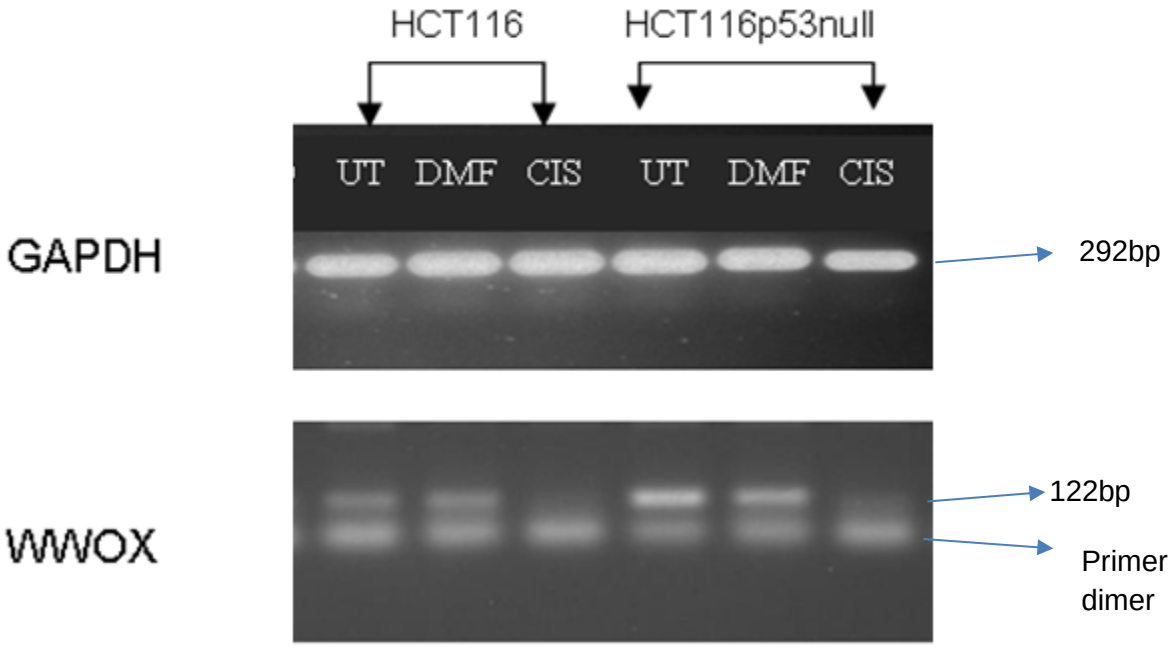


Figure 5.4-B

Gel images of HCT116 and HCT116p53null after treatment with cisplatin.

Expression of WWOX (122bp) and GAPDH (292bp) in HCT116 and HCT116-p53null cells with or without 5µg/ml cisplatin exposure. UT represents (untreated), DMF (dimethylformamide used as vehicle) and CIS represent cisplatin treatments. (For detailed image see appendices S8.1)

5.3.2 Expression of *FHIT* following cisplatin treatment

In my experiments above I observed change in expression of tumour suppressor WWOX gene after cisplatin treatment. Here in these experiments I hypothesize that if the change in WWOX expression due to cisplatin was related to this being a CFS associated gene, then I would expect other CFS associated genes to show the same effect. FHIT is another tumour suppressor gene located on a very commonly expressed CFS. So, is it also affected by cisplatin?

5.3.2.1 Expression of *FHIT* in A2780 cells

Cisplatin treatment reduced the expression of FHIT mRNA in ovarian cancer cells A2780 by 60% after 24 hours and by 80% after 48 hours (Figure 5.5) as compared to DMF and untreated.

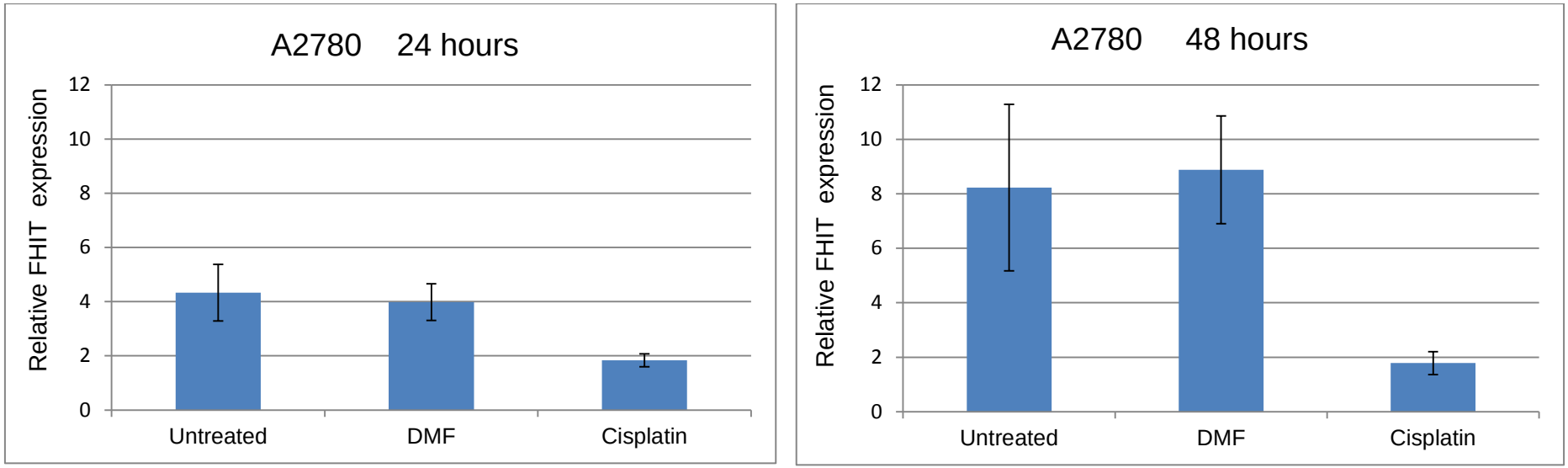


Figure 5.5
***FHIT* expression in A2780 cells after 24 hours and 48 hours of cisplatin treatment.** Graph shows expression of FHIT mRNA relative to B-actin measured by qRT-PCR in A2780 cells following 24 hours' and 48 hours' growth in the presence of 4µg/ml cisplatin, vehicle (DMF) or no drug. Means of triplicate experiments ± SEM show

5.3.2.2 Expression of *FHIT* in HCT116 cells

There was no change in the expression of FHIT mRNA in colorectal cancer cells HCT116 after 24 hours of cisplatin treatment, but 48 hours' treatment showed that cisplatin decreased the expression of FHIT by 25% as compared to DMF or untreated (Figure 5.6) but this did not reach statistical significant.

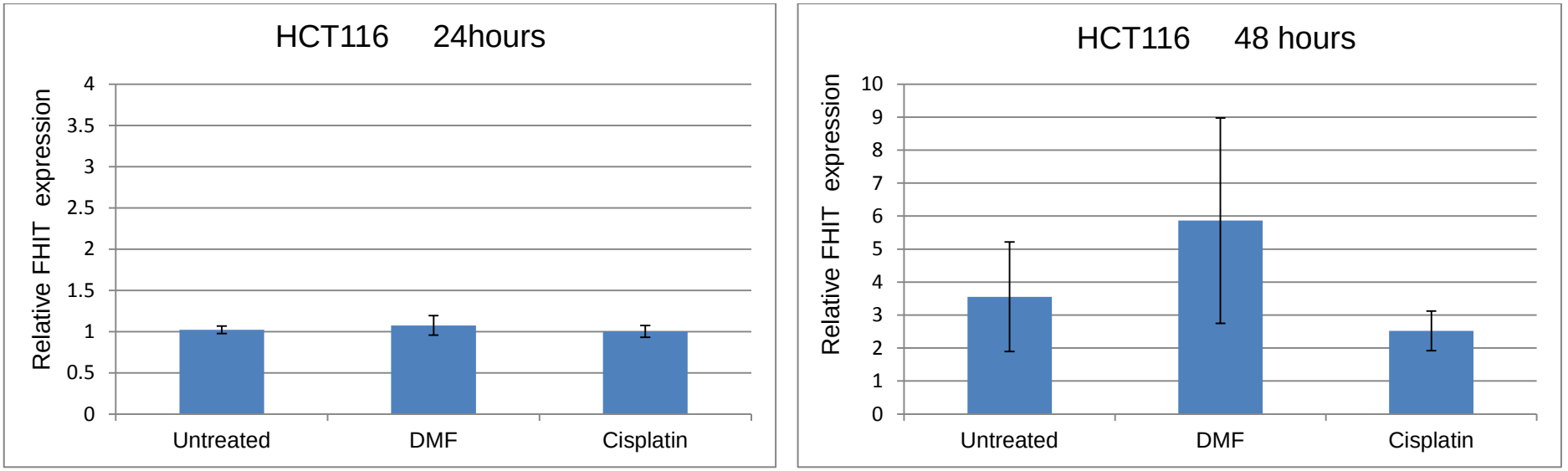


Figure 5.6
***FHIT* expression in HCT116 cells after 24 hours and 48 hours of cisplatin treatment.** Graph shows expression of FHIT mRNA relative to B-actin measured by qRT-PCR in HCT116 cells following 24 hours' and 48 hours' growth in the presence of 5µg/ml cisplatin, vehicle (DMF) or no drug. Means of triplicate experiments ± SEM shown.

5.3.3 Analysis of RNA sequencing data of ovarian and colorectal cancer tumours

5.3.3.1The effect of *FHIT* expression on OS in ovarian cancer patients

Table 5.1 below shows that there were 188 patients each for both low and high FHIT expression groups. For high FHIT group 116 patients had died and 72 patients (38.3%) were still alive. In low FHIT group 114 patients had died and 74 patients (39.4%) were still alive. Figure 5.7 shows the overall survival graph plotted by Kaplan Meier analysis showing curves for both high and low FHIT expressing groups. Both curves (blue for high FHIT and green for low FHIT) overlap each other and show same pattern. This show there was no difference in terms of effect of FHIT on survival time in both groups. The table 5.2 below show P value (p=0.623) confirming there was no significant difference between both groups.

Table 5.1: Summary of patients in low and high FHIT groups

Case Processing Summary

FHIT STATUS	Total N	Dead Patients	Patients still alive	
			N	Percent
Fhit high	188	116	72	38.3%
Fhit Low	188	114	74	39.4%
Overall	376	230	146	38.8%

Table 5.2: Log Rank comparison of high FHIT and low FHIT patient groups

Overall Comparisons

	Chi-Square	df	P value
Log Rank (Mantel-Cox)	0.242	1	0.623

(df) represents degree of freedom

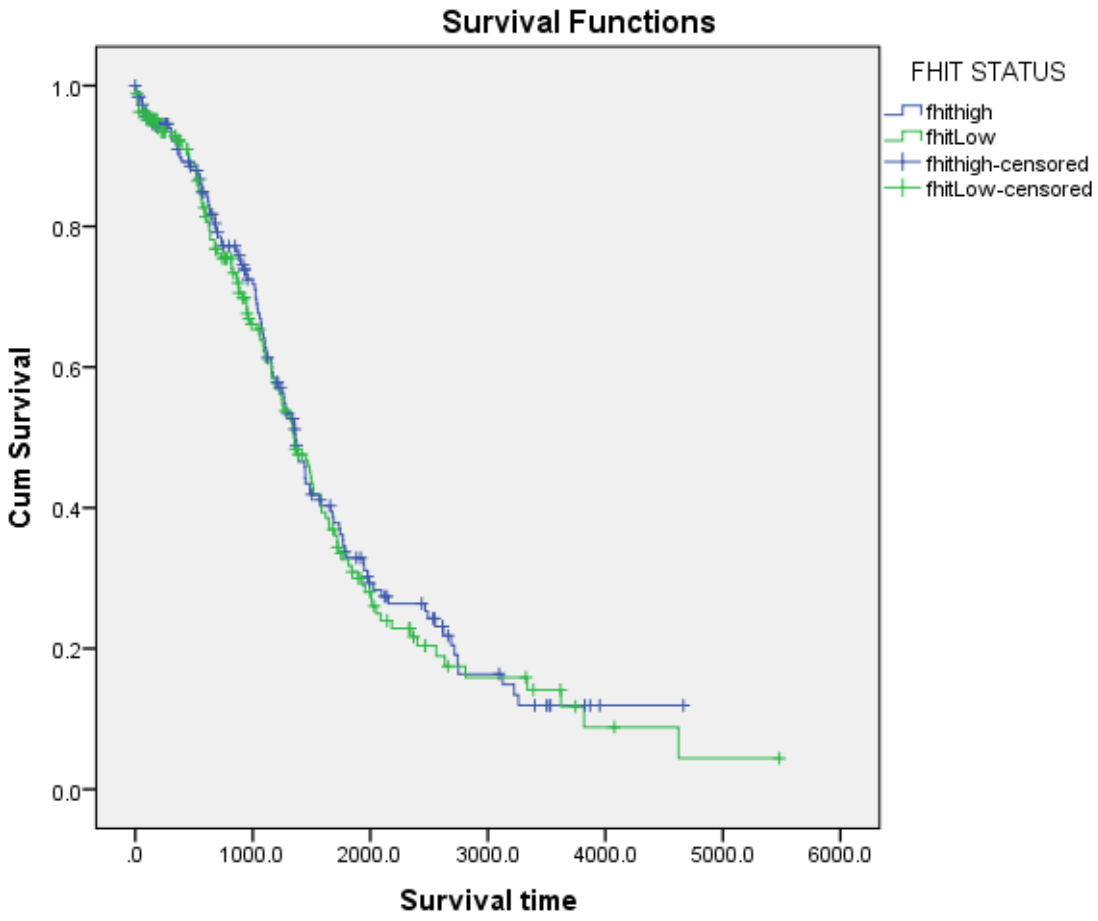


Figure 5.7

Kaplan Meier curves of ovarian cancer patients showing overall survival time. The blue curve represents patients with high FHIT expression and green curve represents patients with low FHIT expression. The vertical lines in both curve represents the censored observations.

5.3.3.2The effect of *FHIT* expression on OS time in colorectal cancer patients

Table 5.3 below shows that there are 195 patients for high FHIT and 193 patients for low FHIT expression groups. For high FHIT group 51 patients had died and 144 patients (73.8%) were still alive. In low FHIT group 41 patients had died and 152 patients (78.8%) were still alive. Figure 5.8 below shows the overall survival graph plotted by Kaplan Meier analysis showing curves for both high and low FHIT expressing groups. Green curve representing low FHIT appears high compared with blue curve representing high FHIT expressing patients. This actually suggests a trend that has not quite reached significance (p=0.077, table 5.4). However, the trend is only in the middle of the curve, and at later times (from 3000 days) there is no clear effect of FHIT expression on survival.

Table 5.3: Summary of patients in low and high FHIT groups

Case Processing Summary

FHIT Status	Total N	Dead Patients	Patients still alive	
			N	Percent
FhitHigh	195	51	144	73.8%
FhitLow	193	41	152	78.8%
Overall	388	92	296	76.3%

Table 5.4: Log Rank comparison of high FHIT and low FHIT patient groups

Overall Comparisons

	Chi-Square	df	P value
Log Rank (Mantel-Cox)	3.135	1	0.077

(df) represents degree of freedom

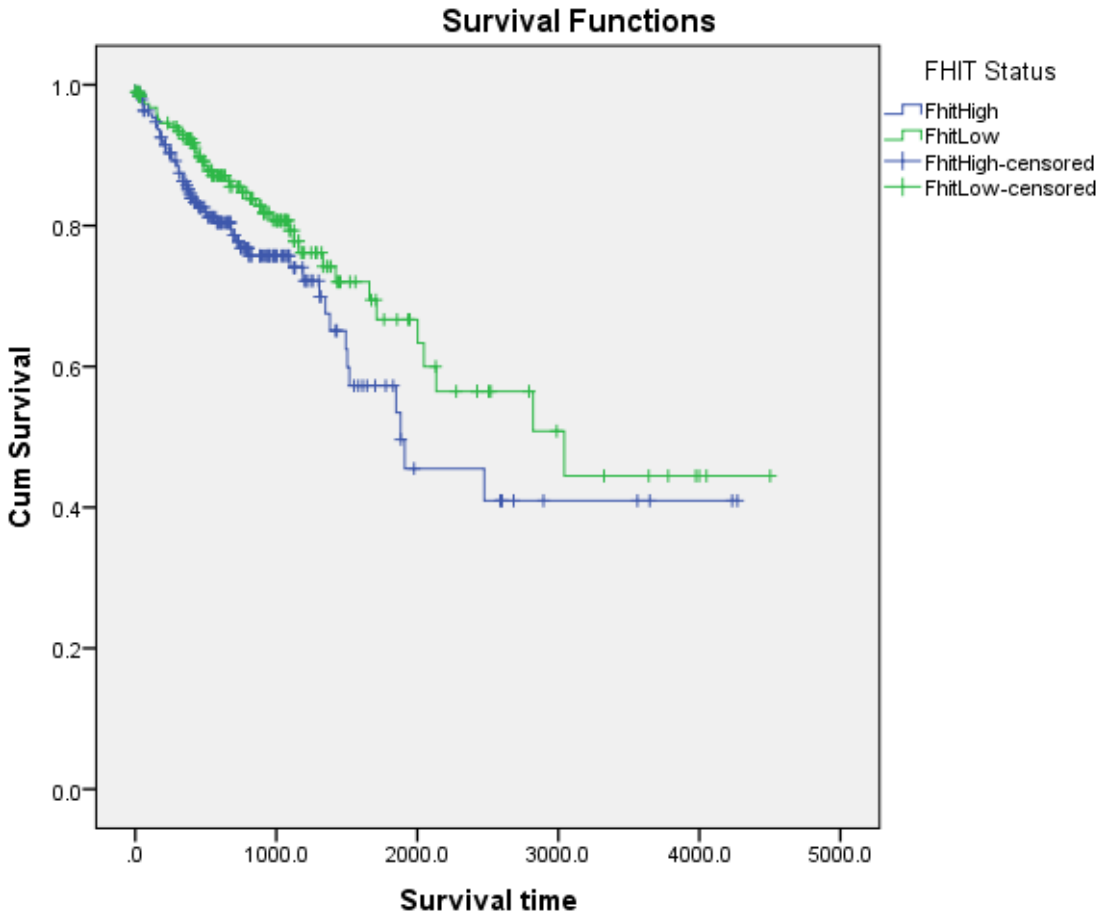


Figure 5.8

Kaplan Meier curves of colorectal cancer patients showing overall survival time. The blue curve represents patients with high FHIT expression and green curve represents patients with low FHIT expression. The vertical lines in both curve represents the censored observations.

5.4 Analysis of NF-κB2 and PAR2 Protein expression in various cancer cell lines after 24 or 72 hours of cisplatin treatment

5.4.1 Expression of NF-κB2 in A2780 cells (Western Blotting)

The decreased expression of WWOX following cisplatin treatment could be the result of altered transcriptional regulation. WWOX transcription has been reported to be regulated by NF-κB2 (the p50 isoform of NF-κB), with increased NF-κB2 levels causing decreased WWOX expression (Fu et al., 2011). I therefore investigated whether there was any increase in NF-κB2 activation following cisplatin treatment that might explain the decreased WWOX expression. Various cell lines were treated with cisplatin for 24 or 72 hours, protein was extracted and the expression of p50 (the cleaved, activated form of NF-κB2) was examined by immunoblotting.

A2780 cells show an increased expression of NF-κB2 (54KDa) in cisplatin treated cells after 24 or 72 hours as compared to untreated and DMF treated cells (figures 5.9 and 5.10). This pattern of increased NF-κB2 expression was similar in all the three independent replicates.

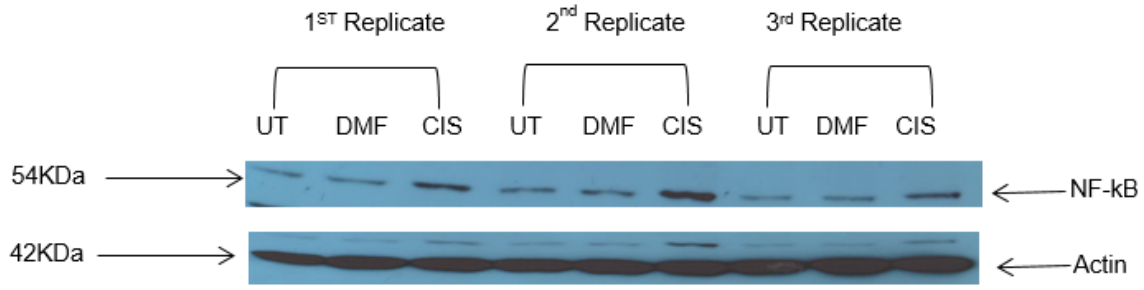


Figure 5.9
Protein expression of NF-κB2 (54KDa) in A2780 cells after 24 hours of 4μg/ml cisplatin treatment. Actin (42KDa) was used as loading control.

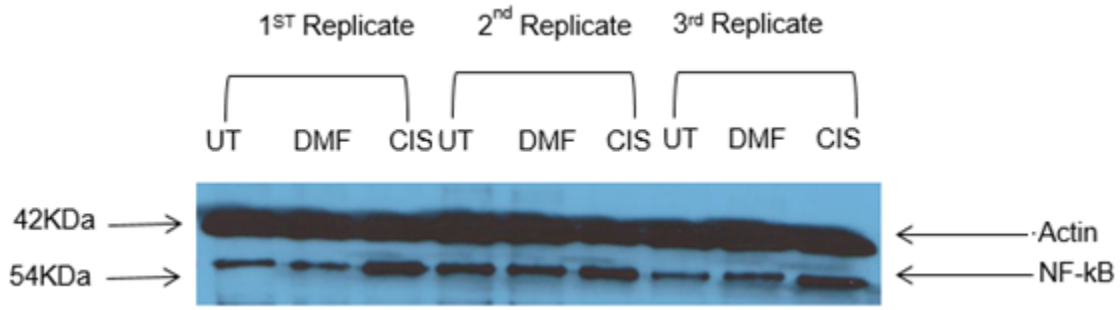


Figure 5.10
Protein expression of NF-κB2 (54KDa) in A2780 cells after 72 hours of 4μg/ml cisplatin treatment. Actin (42KDa) was used as loading control.

5.4.2 Expression of NF-kB2 in HCT116 cells (Western Blotting)

HCT116 cells show an increased expression of NF-κB2 (54KDa) in cisplatin treated cells after 24 or 72 hours as compared to untreated and DMF treated cells (figures 5.11 and 5.12). This pattern of increased NF-κB2 expression was similar in all the three independent replicates.

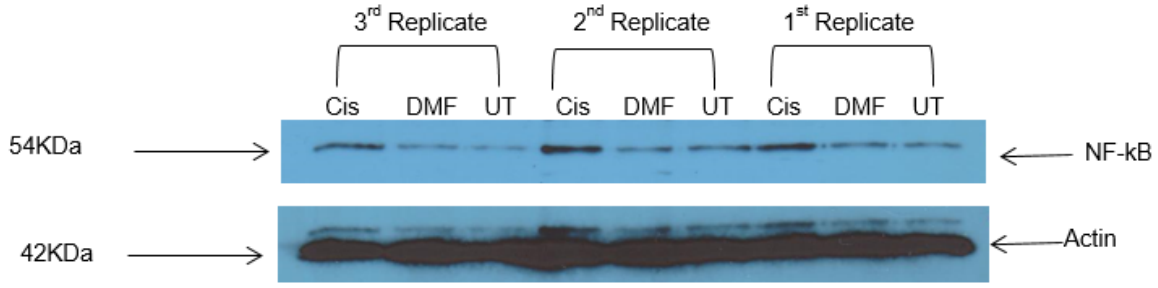


Figure 5.11
Protein expression of NF-κB2 (54KDa) in HCT116 cells after 24 hours of 5μg/ml cisplatin treatment. Actin (42KDa) was used as loading control.

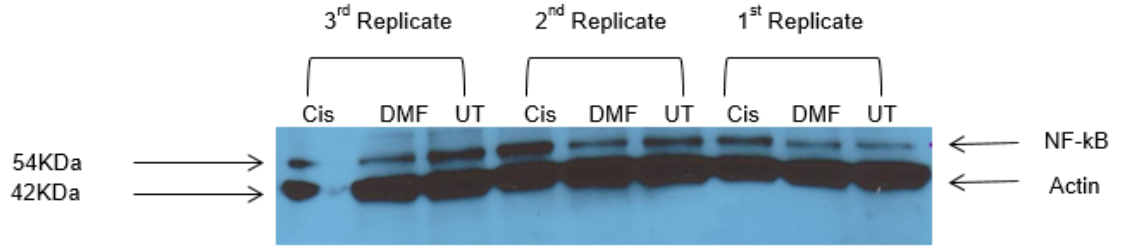


Figure 5.12
Protein expression of NF-κB2 (54KDa) in HCT116 cells after 72 hours of 5μg/ml cisplatin treatment. Actin (42KDa) was used as loading control.

5.4.3 Expression of Nf-kB2 in SK-MEL-28 cells (Western Blotting)

SK-MEL-28 cells show an increased expression of NF-κB2 (54KDa) in cisplatin treated cells after 72 hours as compared to untreated or DMF treated cells (figure 5.13). This pattern of increased NF-κB2 expression was similar in all the three independent replicates.

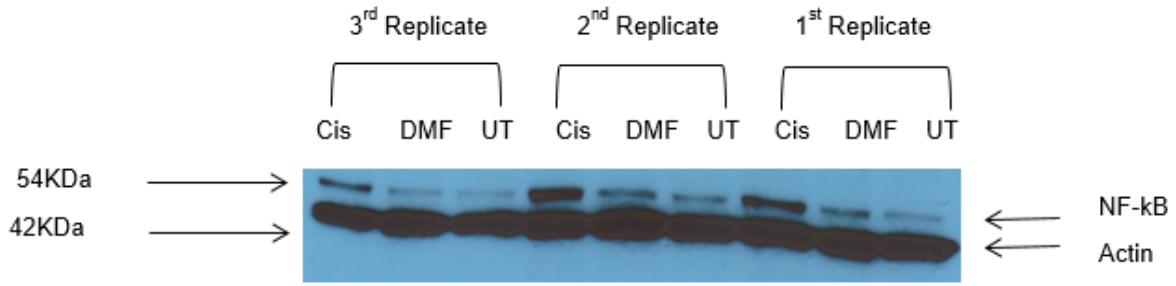


Figure 5.13
Protein expression of NF-κB2 (54KDa) in SK-MEL-28 cells after 72 hours of 3μg/ml cisplatin treatment. Actin (42KDa) was used as loading control.

5.4.4 Expression of PAR2 in HCT116 cells (Western Blotting)

Another transcription factor that has been reported to regulate WWOX transcription is PAR2, with increased PAR2 levels causing increased WWOX expression (Suen et al., 2010). I therefore investigated whether there was any decrease in PAR2 activation following cisplatin treatment that might explain the decreased WWOX expression. Various cell lines were

treated with cisplatin for 24 or 72 hours, protein was extracted and the expression of PAR2 was examined by immunoblotting.

HCT116 cells show decreased expression of PAR2 (55KDa) in cisplatin treated cells (24 and 72 hours) as compared to untreated or DMF (figures 5.14 and 5.15). This pattern of decreased PAR2 expression was similar in all the three independent replicates.

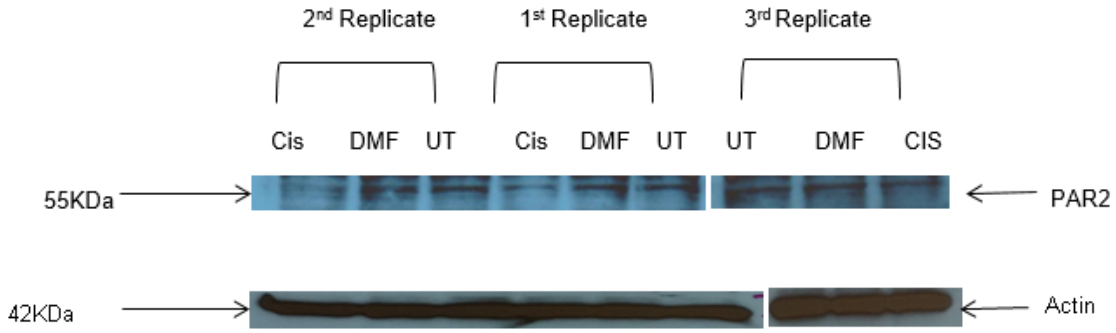


Figure 5.14
Protein expression of PAR2 (55KDa) in HCT116 cells after 24 hours of 5µg/ml cisplatin treatment. Actin (42KDa) was used as loading control.

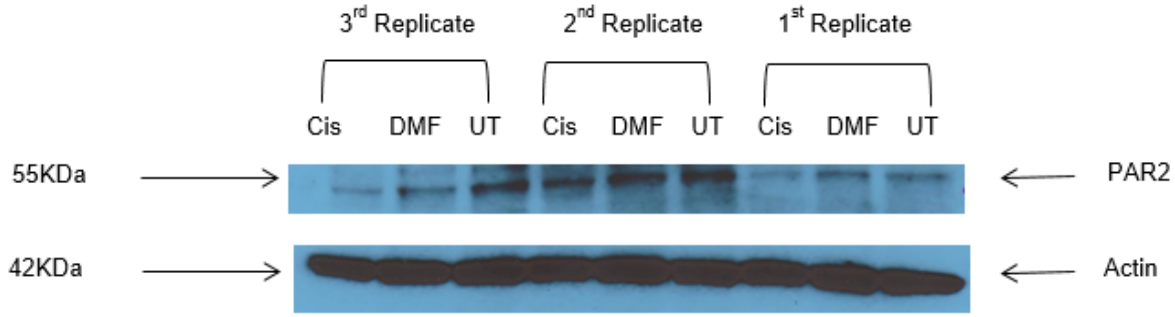


Figure 5.15

Protein expression of PAR2 (55KDa) in HCT116 cells after 72 hours of 5µg/ml cisplatin treatment. Actin (42KDa) was used as loading control.

5.4.5 Expression of PAR2 in A2780 cells (Western Blotting)

A2780 cells show decreased expression of PAR2 (55KDa) in cisplatin treated cells (24 and 72 hours) as compared to untreated or DMF (figures 5.16 and 5.17). This pattern of decreased PAR2 expression was similar in all the three independent replicates.

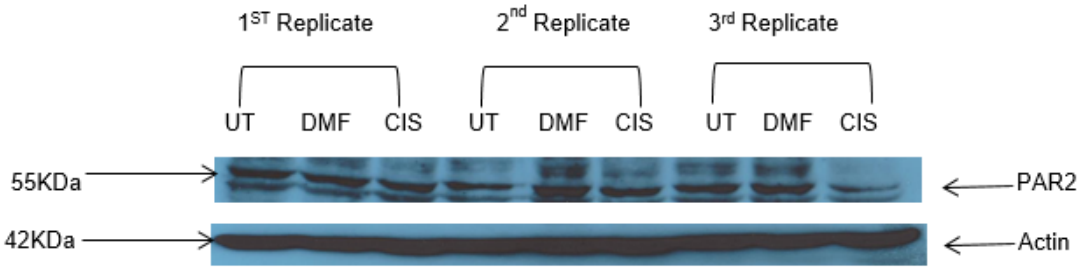


Figure 5.16
Protein expression of PAR2 (55KDa) in A2780 cells after 24 hours of 4µg/ml cisplatin treatment. Actin (42KDa) was used as loading control.

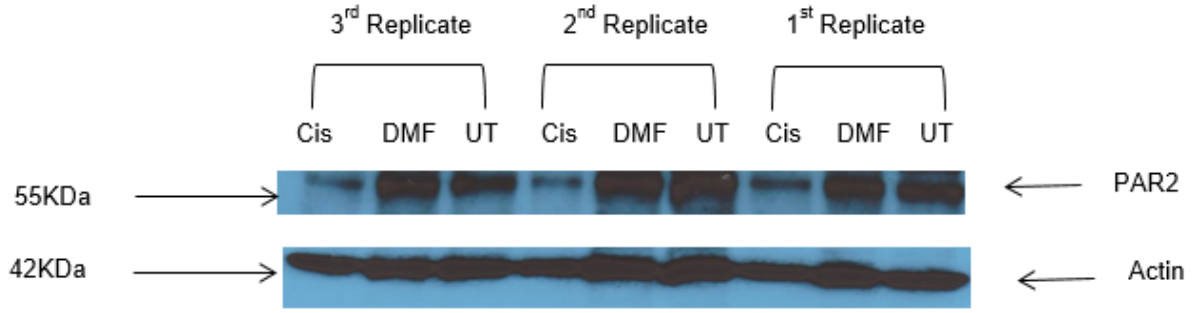


Figure 5.17
Protein expression of PAR2 (55KDa) in A2780 cells after 72 hours of 4µg/ml cisplatin treatment. Actin (42KDa) was used as loading control.

5.5 NF-κB2 knock down by esiRNA in A2780 cells after 72 hours of cisplatin treatment

5.5.1 esiRNA knockdown of NF-kB2 in A2780 and HCT116 cells

The results from western blotting showed the changes in the expression of NF-κB2 in A2780 and HCT116 cell lines following cisplatin treatment. The decrease in WWOX expression following cisplatin treatment may therefore be due to increased NF-κB2 levels. The next step was to confirm a functional link of NF-κB2 with down regulation of WWOX expression by cisplatin treatment. For this purpose, experiments were performed to knockdown the expression of NF-κB2 in A2780 and HCT116 cells. The plan was to successfully knock down NF-κB2 and repeat the experiments of QRT-PCR to examine the expression of WWOX to see if it shows different results compared with existing NF-κB2 expressing cells. The first step was to check if the NF-κB2 knockdown was successful. 700,000 log phase A2780 and HCT116 cells per well were plated in 6-well plates in serum free media (SFM) and were treated with NF-κB2 esiRNA (53.3pmol/well) in 12µl transfection reagent according to manufacturer's instructions. As a control for off-target effects, cells were also treated with GFP esiRNA (53.3pmol/well) in 12µl transfection reagent. Cells treated only with 12µl transfection reagent (TR) were used to confirm that this did not affect their behaviour. Lysates were prepared and probed for NF-κB2 and actin. Initial results showed NF-κB2 was successfully knocked down in A2780 cells but not in HCT116 cells, as compared to controls (Figure 5.18).

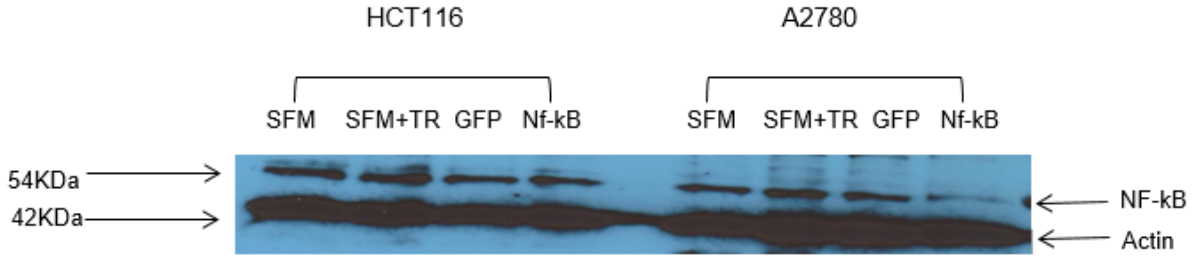


Figure 5.18
The figure shows the expression of NF-kB2 following treatment with SFM (serum free media only i.e. untreated), SFM+TR (serum free media + transfection reagent), GFP (Green Fluorescent Protein, Mission esiRNA targeting EGFP) and NF-κB2 (Mission esiRNA for human NF-κB2) in ovarian cancer cells A2780. 20µg protein for A2780 and 10µg protein for HCT116 was used.

5.5.2 esiRNA knockdown of NF-κB2 in A2780

Once the conditions were optimized for esiRNA knockdown in A2780 cells, three independent replicates were performed with the established conditions. 700,000 log phase A2780 cells per well 53.3pmol/well esiRNA (NF-κB2 or GFP) and 12µl transfection reagent. Transfected cells were then treated with cisplatin or DMF for 72 hours. Lysates were prepared and probed for NF-κB2 and actin.

As in previous experiments, cisplatin treatment resulted in increased NF-κB2 levels compared to untreated or DMF controls. This was observed in all of the transfected lines (figure 5.19). However, NF-κB2 expressions knock down by esiRNA was unsuccessful in A2780 cells as compared to controls (SFM, SFM+TR and GFP). All three independent replicates showed similar results.

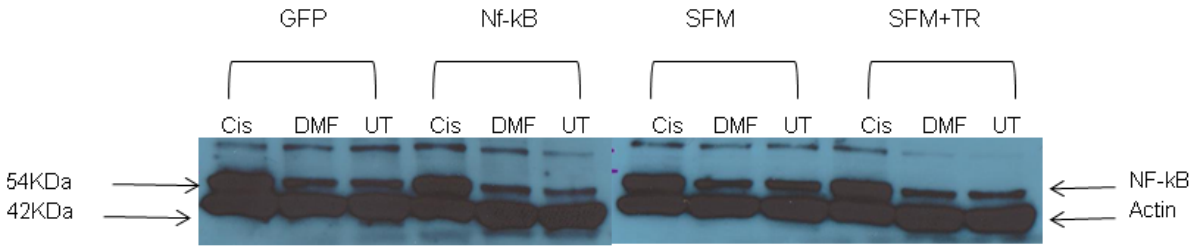


Figure 5.19

The figure shows the expression of NF-κB2 following treatment with SFM (serum free media only), SFM+TR (serum free media + transfection reagent), GFP (Green Fluorescent Protein, Mission esiRNA targeting EGFP) and NF-κB2 (Mission esiRNA for human NF-κB2) in ovarian cancer cells A2780. 20µg protein for A2780 was used. Cis = cisplatin, DMF = dimethylformamide, UT = untreated.

5.5.3 esiRNA knockdown of NF-κB2 in A2780 using different cell numbers

Although the initial results above showed that the expression of NF-κB2 was successfully knocked down in A2780 cells (figure 5.18) but when the experiments were repeated using same conditions the NF-κB2 expressions' knockdown was unsuccessful. So, I decided to try changing some conditions. Firstly, I tried varying the number of cells plated per well (500,000/well, 700,000/well and 900,000/well)

The result (figure 5.20) show, NF-κB2 expression knock down in A2780 cells was still unsuccessful as compared to controls (SFM and SFM+TR). The results were similar in all three independent replicates.

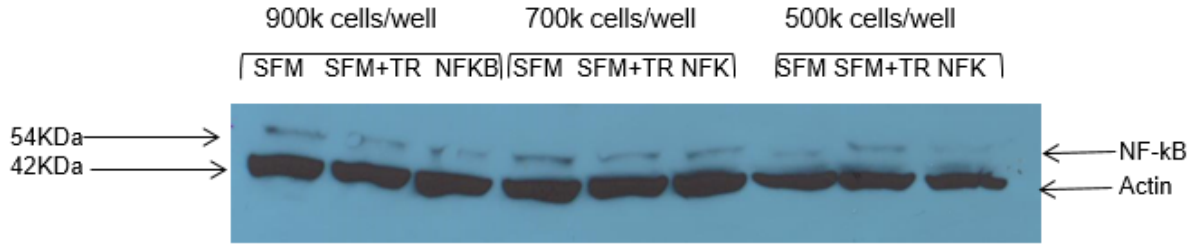


Figure 5.20

The figure shows the expression of NF-κB2 following treatment with SFM (serum free media only), SFM+TR (serum free media + transfection reagent) and NF-κB2 (Mission esiRNA for human NF-kB2) in ovarian cancer cells A2780. 20µg protein for A2780 was used.

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5.5.4 esiRNA knockdown of NF-κB2 in A2780 using concentration of esiRNA for human NF-κB2

The experiment with different cell numbers also proved unsuccessful in knocking down expression of NF-κB2. Next, I tried using different concentrations of NF-κB2 esiRNA to see if it could knockdown successfully NF-κB2. 3µl (40pmol), 4µl (53.3pmol) and 5µl (66.62pmol) of (Mission esiRNA for human NF-κB2) were used for 700,000 cells per well. Lysates were prepared and probed for NF-κB2 and actin. The results (figure 5.21) showed the cells treated with 3µl of esiRNA (40pmol) had better knockdown of NF-κB2 as compared to 4µl (53.3pmol) and 5µl (66.62pmol) of esiRNA, although this treatment did not completely suppress NF-κB2 levels.

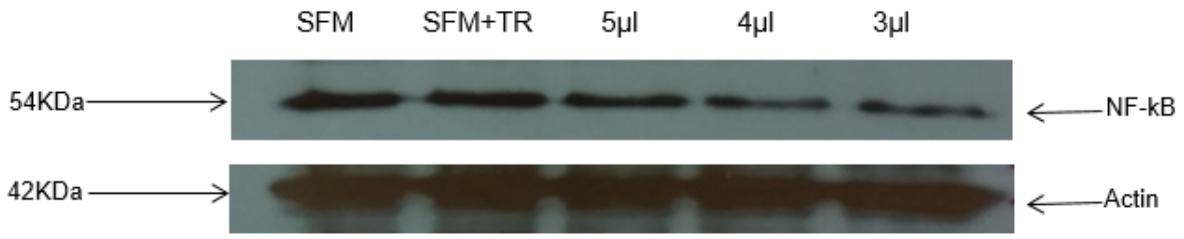

Figure 5.21

The figure shows the expression of NF-κB2 following treatment with SFM (serum free media only), SFM+TR (serum free media + transfection reagent) and 3µl (40pmol), 4µl (53.3pmol) and 5µl (66.62pmol) NF-κB (Mission esiRNA for human NF-κB2) in ovarian cancer cells A2780. 20µg protein for A2780 was used.

5.5.5 esiRNA knockdown of NF-κB2 in A2780 using low concentration of esiRNA

The results above show that esiRNA was better knocked down when low concentration of esiRNA for human NF-κB2 was used as compared two high concentrations. So, in this experiment I repeated the transfection using 40pmol of NF-κB2 esiRNA, and then treated the transfected cells with cisplatin or DMF. Lysates were prepared and probed for NF-κB2 and actin. Figure 5.22 show that siRNA transfection produced only a small decrease in NF-κB2 compared to cells with siRNA (SFM and SFM-TR). This knockdown was not sufficient to prevent cisplatin treatment from inducing NF-κB2. This experiment was done twice, and it showed similar results.

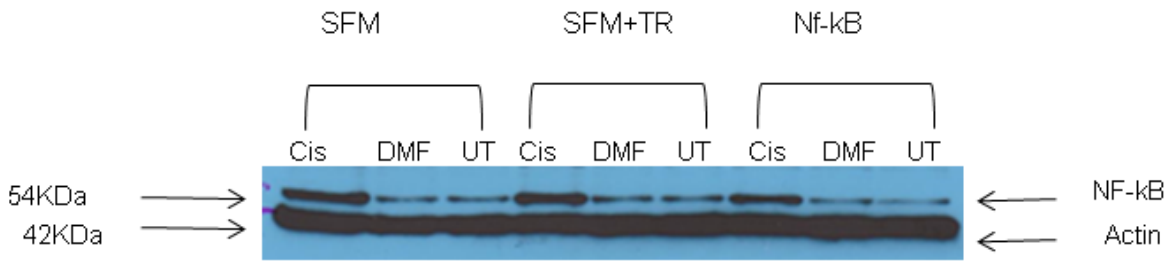

Figure 5.22

The figure shows the expression of NF-κB2 following treatment with SFM (serum free media only), SFM+TR (serum free media + transfection reagent) and NF-κB2 (Mission esiRNA for human NF-κB2) expression in ovarian cancer cells A2780. 20µg protein for A2780 was used. Cis = cisplatin, DMF = dimethylformamide, UT = untreated.

5.5.6 esiRNA knockdown of NF-κB2 in A2780 using low concentration of esiRNA and low concentration of transfection reagent

The above results show that the transfection conditions used were still insufficient to prevent cisplatin from inducing NF-κB2 expression. So, here in this experiment I decided to repeat the transfection using low concentration of NF-κB2 esiRNA (40pmol) and low concentration of transfection reagent (9µl) to see if changing concentration of transfection reagent may help in a successful knock down of NF-κB2.

Figure 5.23 shows that NF-κB2 levels appear unchanged following siRNA treatment as compared to controls (SFM and SFM-TR). Interestingly, however, the induction of NF-κB2 following cisplatin treatment appears to be reduced in the siRNA treated cells compared to the controls.

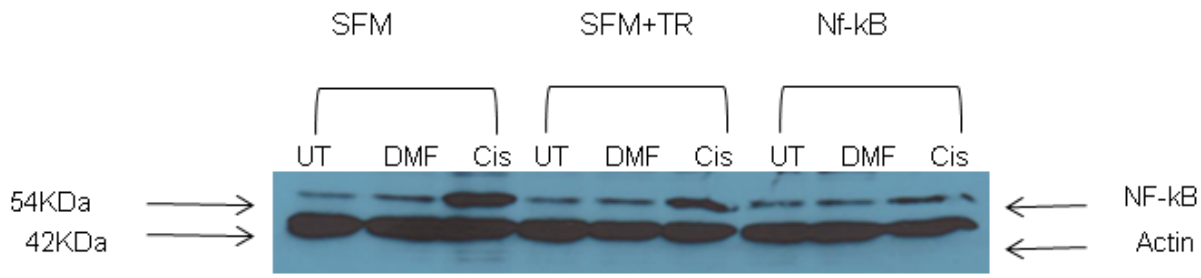

Figure 5.23

The figure shows the expression of NF-κB2 following treatment with SFM (serum free media only), SFM+TR (serum free media + transfection reagent) and NF-κB2 (Mission esiRNA for human NF-κB2) in ovarian cancer cells A2780. 20µg protein for A2780 was used. Cis = cisplatin, DMF = dimethylformamide, UT = untreated.

5.6 SRB analysis of esiRNA transfected cells

Cells treated with NF-κB2 showed low confluence when observed under microscope as compared to controls. To determine whether the siRNA treatment affected cell number

compared to controls the cells were stained with SRB 72 hours following transfection, and the SRB absorbance was measured by spectrophotometer using wavelength of 562nm.

The results show there was no significant difference of cell survival between NF-κB2 treatments compared with other three treatments (figure 5.24). A preliminary analysis of cell cycle distribution in the transfected cells using FACS also demonstrated no differences between the treatments (Figures S1 and S2 in appendix).

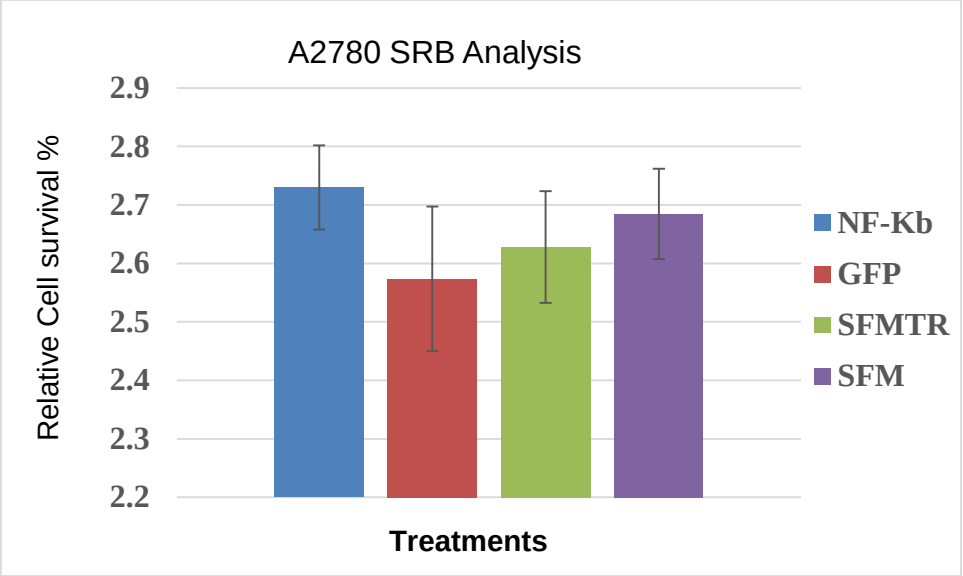

Figure 5.24

Relative A2780 cell survival of NF-κB2 siRNA treated, GFP siRNA treated, and transfection reagent treated (SFMTR) cells compared with untreated cells in serum free media (SFM). Means of triplicate experiments ± SEM shown.

Had the knockdown of NF-κB2 worked, I would then have treated these cells with cisplatin and DMF and analysed WWOX mRNA expression by qRT-PCR to determine whether cisplatin would no longer cause loss of WWOX expression in the absence of NF-κB2.

5.7 Discussion

5.7.1 Does cisplatin cause disruption of common fragile sites leading to dysregulation of nearby genes?

Normally CFS do not exhibit vast sequence similarities and their specific sequences do not seem to be accountable for their fragile nature (Huebner and Croce, 2001), but CFS-associated genes (e.g. WWOX and FHIT) share some structural and functional similarities such as; both span in size more than 1Mb and exhibit fragile regions, both are tumour suppressor genes and show frequently reduced expression levels in various types of cancers (Huebner and Croce, 2001; Ludes et al., 2003). The coordination in expression of both genes was also showed in a study by Ishii et al. (2003), where both WWOX and FHIT showed loss of expression by epigenetic mechanisms in hematopoietic tumours. Another study of breast cancers showed that both WWOX and FHIT were inactivated concordantly and showed loss of expression (Guler et al., 2004). Apart from commonalities in the structure of WWOX and FHIT, these studies suggest together these two genes can show a co-ordinated change of expression during tumorigenesis in various types of cancer.

Here in my study the results again show co-ordinated expression of these two genes, with greatly reduced expression of mRNA for both WWOX and FHIT in A2780 and HCT116 cells due to a DNA damaging chemotherapy drug cisplatin. Cisplatin's mechanism of action is by forming DNA-adducts and distorting the structure of DNA. Fragile sites like FRA16D and FRA3B (which contain the WWOX and FHIT genes respectively) are present on the DNA and they are very susceptible to DNA damage. Therefore, from these data, it can be speculated that treatment of A2780 and HCT116 with cisplatin down regulated the expression of WWOX and FHIT due to DNA damage disrupting the common fragile sites containing these genes.

Other studies also support this hypothesis, showing similar responses of WWOX and FHIT expression following a number of different DNA damaging agents. A study by Thavathiru et al. (2005) used MCF-7 (breast cancer) cells which were irradiated with UV, and the results by northern blotting showed a dramatic decrease in the mRNA levels of WWOX and FHIT after 24 hours as compared to control. Similarly, in this study, treatment of MCF-7 cells with BPDE (Benzo[a]pyrene diol epoxide, a DNA-adduct forming chemical) also showed a dramatic decrease in WWOX and FHIT expression levels. Reduced mRNA expression of WWOX was seen after 24 hours' exposure to these agents, but western blotting analysis showed there wasn't significant decrease in protein expression of WWOX until 48 or 72 hours. This suggests that the expression changes observed are due to transcriptional regulation of WWOX and FHIT.

BPDE is a carcinogenic agent and it forms bulky adducts with the DNA and blocks replication (Pietrasanta et al., 2000) which is similar to the way cisplatin form adducts with the DNA. Similarly, lesions in the DNA created by UV are like lesions produced by cisplatin and both follow NER mechanism pathway for DNA repair (Marteijn et al., 2014). NER is the most common mechanism of repairing DNA damage due to cisplatin or carcinogens (Marteijn et al., 2014; Hanawalt et al., 2002). If cells with highly damaged DNA fail to repair themselves by NER mechanism, mechanisms of cell cycle arrest or apoptosis are induced, often involving the Tumour suppressor protein P53 (Kastan et al., 1991). The nature of the DNA damaging agents and the cell types involved determine which genes are activated or which of their products participate in the DNA repair, cell cycle arrest or apoptotic pathways (Ko and Prives, 1996; Fans et al., 1998; Khan and Dipple, 2000). Given its importance in these pathways, p53 could be potentially involved in the regulation of WWOX and FHIT by cisplatin. In the study by Thavathiru et al. (2005) the involvement of p53 in the down regulation of these fragile site-associated genes was checked by using Saos-2 cells lacking p53. The results showed that UV still down regulated the expression of WWOX and FHIT suggesting it is not dependent on p53. My findings are consistent with this, showing that cisplatin suppressed WWOX expression equally in both HCT116 and HCT116p53null cells.

Despite the lack of evidence for involvement of p53 in the down-regulation of WWOX and FHIT, Thavathiru (2005) did provide evidence that S-phase checkpoint mechanism may be responsible for down regulation of WWOX and FHIT. To check the involvement of S-phase, caffeine was used to delay entry of cells into S-phase, and this prevented UV exposure from causing down regulation of WWOX and FHIT. Similar to this study, another study by Ishii et al. (2005) also showed that UV exposure on MEF (murine embryonic fibroblast) down regulated the expression of WWOX and FHIT genes in time dependent manner as compared to control. Again, G_1-S phase was partially responsible for the down regulation of WWOX and FHIT. The authors also showed that the down regulation of WWOX and FHIT was partially dependent upon the ATR pathway. This is intriguing as there is some evidence to suggest that S-phase check point arrest due to UV or BPDE could be due to activation of ATR pathway (Guo et al., 2002), and previous studies show the key involvement of ATR in regulating the stability of CFS (Casper et al., 2002). Based on the results it can be speculated that the down regulation of WWOX and FHIT by cisplatin may be due to ATR pathway recognising CFS related DNA damage during an S-phase arrest.

Concordant, down regulation of Wwox and Fhit gene expression was also shown in an *in vivo* study by Lida et al. (2005) in mice livers. The results showed that mice exposed to *o*-nitrotoluene (an industrial chemical) and methyleugenol (found in oils and food products) for two weeks, showed loss of expression of Wwox and Fhit only in livers in a dose dependent manner (analysed by qRT-PCR, micro-array and western blotting). The loss of expression of Wwox and Fhit by these carcinogens was very prominent in livers and may represent the onset of early carcinogenic process. Further analysis showed significant upregulation of Gadd45b (growth arrest and DNA damage inducible 45β) which may be responsible for early onset of carcinogenic process. Mice treated with *o*-nitrotoluene and methyleugenol also showed significant upregulation of cyclin G1 (cell cycle gene) and p21 in livers. The aim of this study was to investigate early onset of carcinogenesis due to carcinogens but strikingly loss of cancer related genes (Wwox and Fhit) was observed.

Tobacco smoke contains carcinogens linking it strongly with lung cancer. A study of Sozzi et al., (1997) showed that tobacco smoke caused lesions in the DNA and molecular analysis showed loss of heterozygosity (LOH) of FHIT genes. Microsatellite alterations of FHIT gene were observed in a group of 51 lung tumours from smokers, and among them 41 (80%) showed LOH which affected at least one locus of the FHIT gene. In another group of 40 lung tumours from non-smokers, only 9 showed LOH (22%). Comparison of smokers with non-smokers showed the difference between both groups was highly significant (p=0.0001). These results clearly suggest that FHIT is a molecular target of carcinogens. This study did not further investigate the mechanisms which may have been affected by LOH of FHIT but in a study by Cirombella et al., (2010) pre-neopalstic lesions adjacent to NSCLC were examined to see the alterations in expression of FHIT and activated check point proteins. Check point proteins chk1 and phosphoChk1 were examined in FHIT positive and negative lesions. Most of the check point proteins were visible in FHIT positive lesions but FHIT negative lesions did not showed any expression of Chk and phosphoChk1. This suggests that there is a connection between DNA damage (FHIT loss) and check point proteins, and loss of expression of FHIT is linked with loss of activated Chk2 in pre-neoplasia. From this study, it can be speculated that loss of expression of FHIT due to cisplatin in my study may be due to absence of check point proteins Chk1 and phoshphoChk1.

These studies support a possible mechanism for down-regulated WWOX and FHIT expression that involves cisplatin induced DNA adducts promoting DNA damage disrupting the highly sensitive CFS regions and causing loss of nearby gene expression through an ATR and S-phase arrest dependent pathway. However, it remains possible that cisplatin instead causes alterations in specific transcription factors that directly regulate WWOX and FHIT expression. Recent studies have shown that PAR2 and NF-κB2 are two transcription factors which could possibly regulate WWOX expression.

5.7.2 Association of FHIT with OS in ovarian and colorectal cancer patients

FHIT is a tumour suppressor protein and like WWOX, FHIT is also a target of deletion in multiple cancers and expression of FHIT was supressed in many tumour types (Mishra et al., 2010). Apoptosis induced by FHIT has been shown in different types of cancer cells (Roz et al., 2002; Sevignani et al., 2003). Because of the role of FHIT in apoptosis I expect to see a link between higher FHIT expression and better survival rate.

In a study by Ishii et al., (2001), immunohistochemical study of human primary tumours was done to observe the expression of FHIT in malignant or pre-malignant stages. FHIT protein was absent or strikingly low in 60% (*n=1948*) of primary tumours. In pre-neoplastic lesions 31% samples showed reduced or no FHIT (n=408). Preclinical studies showed that reintroduction of FHIT in 26 cancer cell lines (lung, head, neck, gastric, pancreatic etc.) showed inhibition of tumorigenicity *in vitro* and 17 cell lines out of 30 showed inhibition of in vivo tumorigenicity. This data shows FHIT expression is either loss or downregulated in cancers and its re-expression can inhibit tumorigenicity.

Rohr et al., (2005) showed the correlation between FHIT in SCLC tumours and survival. Kaplan Meier analysis showed that patients with complete lack of FHIT or with less than 25% FHIT expressing cells showed worst survival. However, survival time improved with the increase in percentage of FHIT expressing cells. Another study by Toledo et al., (2004) showed that loss of FHIT expression in NSCLC primary cells was correlated with poor survival of patients. Segawa et al., (2000) also showed that low expression of FHIT in endometrial cancer patients was associated with poor survival. Otero et al., (2004) also showed a multivariate analysis between FHIT and survival but showed contrary results to cited studies described above. They showed that tumours from advanced osopharyngeal squamous cell carcinoma patients with no reduction in FHIT expression levels showed significantly lower overall or disease-free survival suggesting no reduction in FHIT levels predicts poor survival outcome. Different again, Geradts et al. (2000) showed loss of FHIT in NSCLC tissues by immunohistochemistry analysis but Kaplan Meier analysis showed no

correlation between FHIT expression and patients' survival. Nonetheless, most of the studies show a correlation between low FHIT levels and poor survival.

My results show that cisplatin causes loss of WWOX and FHIT expression. When I looked for evidence of correlation between patient survival and FHIT expression in ovarian and colorectal cancer patients from TCGA data set, the results showed there was no correlation of FHIT with overall survival time (p=0.623) in ovarian cancer patients. Given that ovarian cancer patients would have been treated with cisplatin or carboplatin it is perhaps not surprising that no association of WWOX or FHIT expression with survival is seen. However, a possible trend towards correlation of overall survival with FHIT expression was observed (p=0.077) in colorectal cancer patients, who are unlikely to have received cisplatin, although some may have been treated with FOLFOX which includes oxaliplatin.

5.7.3 Does cisplatin affect WWOX and FHIT expression via NF-κB2 regulated transcription?

NF-κB2 is produced as an inactive p100 isoform which is activated by cleavage into the p52 isoform. My results of western blotting show activation of NF-κB2 (p52) in A2780, HCT116 and SK-MEL-28 cells after cisplatin treatment compared with untreated or DMF. This change in protein expression in western blotting experiments could be verified and quantified by pixel analysis. The other results from qRT-PCR experiments show that this correlates with WWOX expression being down regulated due to cisplatin treatment, suggesting a possible link.

The nuclear factor (NF-Kappa-B) is a pleotropic family of transcription factors which are present is almost all cell types and regulates diverse biological processes including immune response, cell growth and cell survival (Xiao et al., 2006). NF-κB can be activated by cytokines, reactive oxygen species, bacterial cell wall products, viral infection and DNA damage (Brasier, 2006). NF-κB is not just a single gene but a family of transcription factors which are closely related to each other and are five genes which includes proteolytically

processed DNA binding subunits termed as NF-κB1 (p50/p105), NF-κB2 (p52/p100) and transcriptional activators termed as RelA (p65), c-Rel and RelB (Siebenlist et al., 1994; Ghosh et al., 1998). Seven proteins arise from these five genes that share Rel Homology Domain (RHD) in their sequence, and their DNA binding, dimerization and interaction with their specific inhibitors is mediated by RHD. RelA (p65), c-Rel and RelB are synthesized in their mature forms and interact with the transcriptional apparatus by their transactivation domain. The synthesized precursor forms of NF-κB1 (p105), and NF-κB2 (p100) contain C-terminal ankyrin repeats and the production of mature (p50 and p52 respectively) proteins results when proteolysis of C-terminal ankryin repeats takes place by the proteasome (Dolcet et al., 2005).

Activation of NF-κB is important for inflammatory response and carcinogenesis and it could be activated by (TNF-α) canonical and (LTβR and CD40) noncanonical pathways (Figure 5.36). Activation of NF-κB has been repeatedly shown in cancer cells and causes cancer cell growth, progression and metastasis. In a study of lung cancer and chronic obstructive pulmonary disease (COPD), proteomic analyses have revealed the involvement and linkage of NF-κB pathway with inflammatory signalling, oxidative stress response, and glycolysis and gluconeogenesis pathways (Pastor et al., 2013). Although some studies show strong evidence of involvement of NF-κB as a mediator of inflammation and promoter of carcinogenesis (Batra et al., 2011; Chen et al., 2011; Shen and Hahn, 2011) but largely it is unknown. A recent study in the *Gprc5a* gene knockout mice showed that NF-κB was activated in airway epithelium and it lead to lung inflammation and tumorigenesis (Deng et al., 2010). Some animal models also show role of NF-κB in cancer. For example, the oncoprotein for reticuloendotheliosis (REV-T) retrovirus is v-REL (a viral homologue of c-Rel) is the transforming gene of an avian retro virus which causes leukaemia and aggressive lymphomas because of its oncogenic nature (Gilmore, 1999). The locus of NF-κB2 on chromosome 10q24 is affected by deletion or rearrangements of chromosomes, which leads

to the loss of ankyrin repeats of p100 and processing of active p52 protein which is linked with B-Cell and T-cell lymphomas and multiple myelomas (Migilazza et al., 1994; Neri et al., 1991; Neri et al., 1995). The locus of Bcl-3 on human chromosome is 19q13 which is near chromosomal break point (Ohno et al., 1990). The translocation of B-Cell lymphocytic leukemias at (14; 19) (q32; q13) results in increased expression of Bcl-3 which is a transcriptional co-activator of p50 or p52 homodimers. The high expression of Bcl-3 increases NF-κB activity (Ohno et al., 1990; Caamano et al., 1996) and up-regulates its transcriptional target i.e. cyclin D1 (Westerheide et al., 2001). Another study by (Cogswell et al., 2000) shows that Bcl-3, p50 and p52 are selectively active in breast carcinoma as compared with normal breast tissue and it is linked with increased expression of cyclin D1 (Cogswell et al., 2000). Squamous cell carcinomas of the head and neck shows activated NF-κB and when its activation was inhibited in these carcinomas, it inhibited cell survival and tumour growth (Thornburg et al., 2003).

Fu and colleagues (2011) studied adult T-cell leukaemia (ATL) which is caused by human T-cell leukaemia virus type I (HTLV-I), a process known to involve activation of NF-kB pathways (Xiao et al., 2006). For this purpose, Fu and colleagues used Jurkat cells in which they induced expression of the Tax oncoprotein from HTLV1. This suppressed the *WWOX* expression in a dose dependent manner. Further analysis showed suppression of WWOX gene due to Tax was dependent on p100/p52, as Tax expression could suppress WWOX in cells from p100-positive mice, but not p100-negative mice and this shows WWOX is a negative target of the non-canonical activation of NF-κB2. These findings were relevant with a previous study by Qing et al., (2007) which showed that constitutive processing of p100 into active p52 resulted in supressed expression of WWOX. The down regulation of WWOX due to HTLV-I was checked in primary ATL cells recovered from 9 ATL patients and the results showed that 7 out of 9 showed reduction in in WWOX expression as compared to normal T cells, suggesting it is a pathophysiologic event due to HTLV-I. Further analysis also showed that WWOX co-expression resulted in prevention of cellular transformation and tumorigenesis by Tax, while knockdown of *WWOX* increased tax mediated tumorigenesis.

This mechanism was shown to relate to activation of canonical NF-kB signalling by WWOX and could be prevented using a Y33R WWOX mutant. The principle findings of this study are, tumour suppressor gene WWOX is a negative target of the non-canonical NF-κB (p100/p52) pathway and Tax mediated tumorigenesis involves NF-κB2 suppression, WWOX upregulation, and subsequent activation of the canonical NF-κB pathway.

These results from the study of Fu et al., (2011) provide a concept to analyse the results of qRT-PCR and western blotting from my investigations. My results of qRT-PCR showed cisplatin induced down regulation of *WWOX* tumour suppressor while western blotting showed cisplatin induced activation of non-canonical NF-κB2 as compared to controls. From the findings of Fu and colleagues, it can therefore be speculated that the down regulation of WWOX by cisplatin may be because of activation of non-canonical NF-κB2 because as shown by Fu et al., WWOX is the negative target gene for non-canonical NF-κB2. WWOX is a tumour suppressor gene and in cancer cells its expression is reduced which enhances tumorigenicity, similarly activation of NF-κB2 represents cancer cell growth, progression and metastasis (Pastor et al., 2013).

This data therefore provides an alternative explanation to how cisplatin regulates WWOX expression. Instead of damage to CFS regions, it may be directly related to induction of a negative transcriptional regulator NF-κB2. I performed siRNA experiments in which I tried to confirm whether NF-κB2 was essential for cisplatin to alter WWOX expression. I optimized conditions to check first if the knock down of NF-κB2 is successful? The results suggested it was successful but when I performed actual experiments to test the effect of knockdown on WWOX expression, the knockdown was unsuccessful. I further tried to optimize by changing concentrations of siRNA reagents and cells numbers. Although optimization attempts produced some degree of NF-κB2 knockdown, this appeared to be insufficient to affect the suppression of WWOX by cisplatin.

More work needs to be done to confirm the role of NF-κB2 in mediating the cisplatin induced suppression of WWOX. Further optimisation needs to be done to address this problem of

unsuccessful siRNA knockdown of NF-κB2, perhaps using different siRNA oligos. Another technique that could be used for silencing non-canonical NF-κB2 signalling is by inhibiting its nuclear translocation. This can be done by using cell permeable peptides that contain the nuclear localizing sequence of p52. This peptide inhibits nuclear translocation of p52-containing dimers by binding to, and saturating, the nuclear import machinery responsible for the uptake of NF-κB dimers containing p52 (XU et al., 2008; ST J Nicholas; 2001; Lin et al., 2005). Another approach that could be used would be to isolate mouse embryonic fibroblasts from $p100^{+/+}$ and $p100^{-/-}$ mice (as done by Fu et al, 2011), and compare WWOX expression in these following cisplatin treatments. In each of these approaches a failure of cisplatin to suppress WWOX expression in the absence of NF-κB2 signalling would indicate that NF-κB2 is responsible for the WWOX down-regulation.

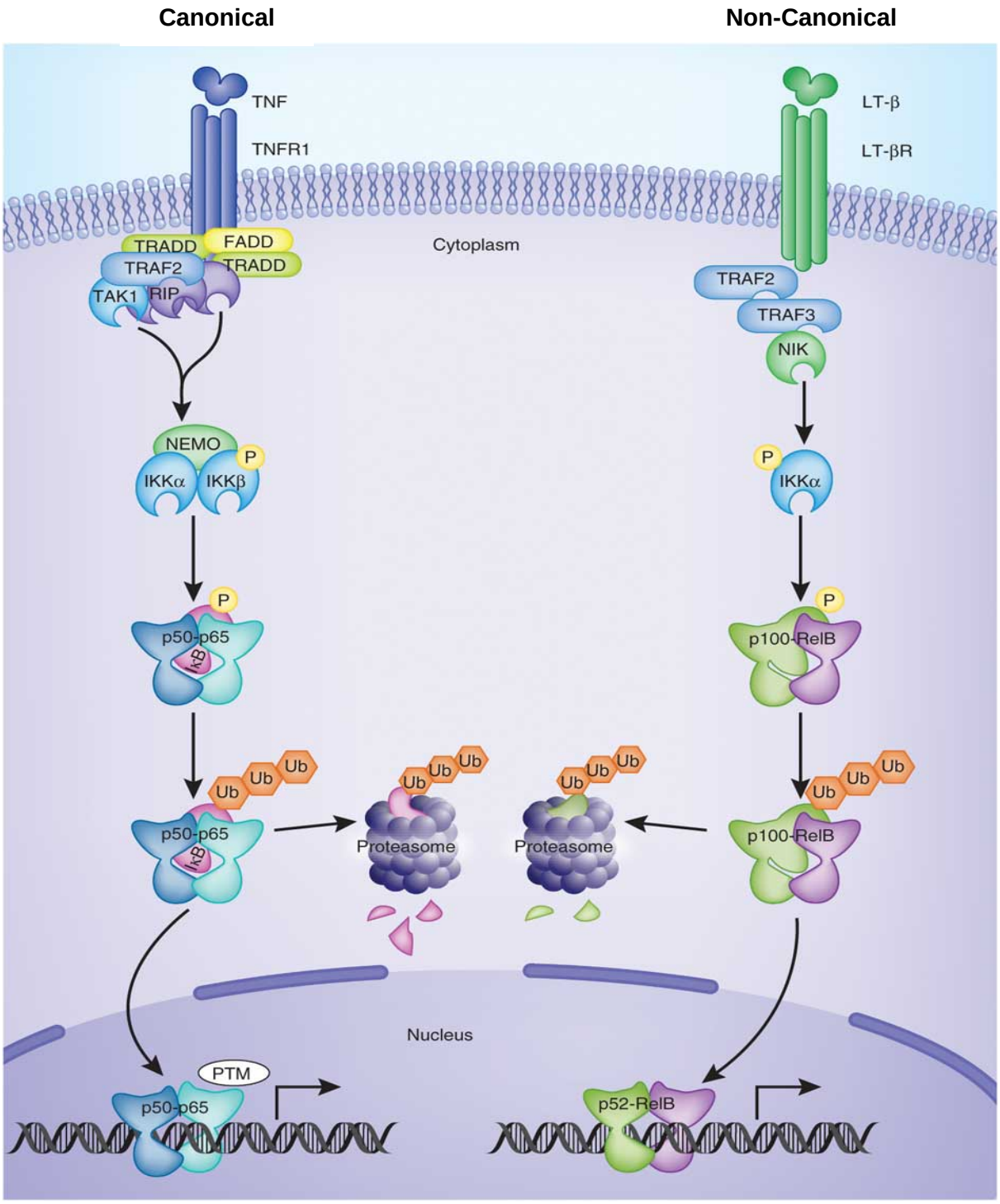

Figure 5.34: **Activation of NF-κB by canonical (left) and noncanonical pathways (right)**

During resting conditions, inactive NF-κB complexes are sequestered in the cytoplasm due to binding of inhibitory IkB protein with NF-κB dimers. Induction of the canonical pathway takes place by NF-κB stimuli represented in this figure as stimulation of TNFR1 signalling followed by binding of TRADD which further recruits FADD and TRAF2. In next step phosphorylation of IKKα takes place in an IKKβ- and NEMO-dependent manner. Following phohorylation and ubiquitylation of IkBα, heterodimers containing p65 translocates to the nucleus. On the other side non-canonical pathway is induced by TNF family cytokines such as lymphotoxin-β (LT-β) by recruiting TRAF3, TRAF2 and NIK. In next step IKKα-mediated phosphorylation of p100-ReIB complex takes pace and after ubiquitylation leads to partial processing of p100 and formation of transcriptionally active p52-RelB complexes followed by its translocation to the nucleus (Morgan and Liu, 2011)

5.7.4 Does cisplatin affect WWOX and FHIT expression via PAR2 regulated transcription?

The results of my western blotting also showed decreased PAR2 expression in A2780 and HCT116 cells treated with cisplatin. PAR2 along with related family member proteins are highly expressed in vascular and extravascular tissues, pancreas, liver, prostate, ovary, lung, eye and uterus (Macfarlane et al., 2001; Bohm et al., 1996; D'Andrea et al., 1998). Also, these proteins are involved in regulation of inflammation and responses to vascular injury (Coughlin, 2000; Ruf et al., 2003).

There are approximately ~900 human G protein coupled receptors (GPCRs) and this family of membrane spanning cell surface proteins make up about > 2% of the human genome (Elefsinioti et al., 2004; Schiöth and Fredriksson, 2005). Protease activated receptors (PARs) are exceptional GPCRs (Barry et al., 2006) with as yet no known endogenous extracellular ligands (Suen et al., 2010). Protease activated receptors (PARs) is a family of four 7-transmembrane G-protein coupled receptors which are activated by serine proteases (Trejo, 2003).

Activation of PAR is a unique mechanism and takes place indirectly by proteases which cleave the N-terminus of four isoforms of PAR and as a result new N-terminus is exposed which folds back and intramolecularly self-activates PAR (Steinhoff et al., 2005).

Research from the last few years has shown that cancer metastasis and invasion are highly dependent on elements of blood clotting (coagulation) cascade. Thromboplastin (TF) is a 47-KDa transmembrane glycoprotein found on the outside of the cells and it is the main protein involved in initiation of the extrinsic coagulation cascade (Versteeg et al., 2004). Previous studies suggest TF/VIIa activates another receptor, PAR2 on cells and induces signal transduction pathway (Schaffner and Ruf, 2009; Hjortoe et al., 2004; Rao and Pendurthi, 2005). Hjortoe et al., (2004) suggested that binding of FVIIa to TF on breast carcinoma cells results in upregulation of interleukin-8 (IL-8) via PAR2-dependent mechanism, and initiate cell migration and invasion. In another study of colon cancer cell line SW260, it was shown that PAR2 is highly expressed and PAR2-AP or TF/VIIa complex activates PAR2 and

regulates the expression of IL-8, TF, caspase-7 and hence promotes cell proliferation and migration (Zhou et al., 2008a; Zhou et al., 2010b). Activation of PAR2 causes relaxation of blood vessels, increases vascular permeability and leukocytes adhesion (Jacob et al., 2005). It was also reported that activation of PAR2 causes release of pro-inflammatory cytokines (e.g.IL-1b, IL-6, IL-8, TNF-a) and Intracellular Cell Adhesion Molecules -1 (ICAM-1) from human blood monocytes (Hjortoe et al., 2004; Johansson et al., 2005).

Darmoul et al., (2001) showed that low concentration of trypsin mediated by PAR2 activated proliferation of human colon cancer cells in culture. Gastric cancer cells (MKN-1) showed trypsin activated an integrin α5β1-dependent adhesion to fibronectin and proliferation via PAR2 (Miyata et al., 2000). In a study of lung cancer, it was reported that PAR2 m-RNA expression was up regulated to 16-fold in pulmonary tumour alveolar walls as compared to normal alveolar tissues (Jin et al., 2003). Increased PAR2 expression was observed in proliferating stromal fibroblasts surrounding the carcinoma cells in breast cancers (D'Andrea et al., 2001). Proliferation of tumour cells is promoted by PAR2 in colon (Darmoul et al., 2001; Darmoul et al., 2004), gastrium (Caruso et al., 2006; Miyata et al., 2000), pancreas (Tetsuoohta et al., 2003; Shimamoto et al., 2004), and glioblastoma (Okamoto et al., 2001). Jahan et al., (2007) showed that distribution of PAR2 was dominant in cancer cells and faint in stromal cells of the tumour. Both mRNA levels and PAR2 histoscores in cancer cells significantly increased in ovarian cancers with clinical stages ($I < II < III < IV$, $P < 0.05$) irrespective of histopathological type. The 36 months' survival rate of patients with high PAR2 was poor as compared to patients with low PAR2. This shows PAR2 is a promoter of tumour growth with dissemination of ovarian cancers through angiogenesis and leads to low survival rates (Jahan et al., 2007). Another study in breast cancer by (Ryden et al., 2010) shows that clinical biopsies of breast cancer showed up regulated expression of TF and PAR2 in invasive cancer as compared to carcinoma in situ and the patients with newly diagnosed breast cancer suffered relapse due to TF phosphorylation. This study correlates with another study by (Schaffner et al., 2010) which also showed the evidence of cross talk between TF and PAR2. All these studies show the importance of PAR2 in cancer studies.

Suen et al. (2010) showed that PAR2 is an important transcription factor which on activation strongly increases the expression of WWOX. In this study, human embryonic kidney cells (HEK293) were used and almost 19,000 human genes were compared for intersecting up or down regulation by both trypsin (an endogenous protease that activates PAR2) and a hexapeptide (2-furoyl-LIGRLO-NH_2) which activates PAR2. Both PAR1 and PAR2 activated many genes which are important in cancer. This study showed that activation of PAR2 caused up-regulated expression of *WWOX* gene more than 8-fold in human kidney cells (Suen et al., 2010). These results were interesting and provide a concept to link with my results of western blotting. The results of A2780 and HCT116 cells show PAR2 was highly expressed in untreated and DMF treated cells as compared to cisplatin treated cells. Therefore, another possible mechanism for down regulation of WWOX is decreased PAR2 following cisplatin.

To confirm whether PAR2 regulates WWOX expression or not, knock down of PAR2 by siRNA could be performed. Analysis of WWOX expression by QRT-PCR following cisplatin treatment in PAR2 knockdown and GFP knockdown control cells would determine whether PAR2 expression is required for suppression of WWOX. Another method to inactive PAR2 and study its role in mediating cisplatin-induced suppression of WWOX would be to use commercially available PAR2 inhibitors such as; ENMD 547 (sc-207618) and GB83 (Axon 1622). Alternatively, mouse embryonic fibroblasts could be isolated from $PAR2^{+/+}$ and $PAR2^{-/-}$ mice (Matej et al. 2012; de Boer et al., 2014), and used to compare WWOX expression following cisplatin treatment. In each of these approaches a failure of cisplatin to suppress WWOX expression in the absence of PAR2 signalling would indicate that PAR2 is responsible for the WWOX down-regulation.

5.8 Conclusion

I have shown that cisplatin causes loss of WWOX and FHIT expression. This altered expression is due to transcriptional repression of these genes. This is likely due to cisplatin induced damage effecting CFS, but this response is independent of p53 regulated repair. Transcriptional regulators of WWOX (e.g. NF-κB2 and PAR2 are up and own regulated respectively by cisplatin and these may be the causes of the altered WWOX and FHIT expression. Cisplatin (and potentially other DNA damaging chemotherapy agents) may therefore cause loss of tumour suppressor gene expression which may be detrimental to the desired effect of the treatments and this is discussed further in chapter 6.

Chapter 6. Discussion

6.1 Major findings of research

The hypotheses behind this study were that chemotherapy drug paclitaxel induces apoptosis via ER-stress and WWOX may sensitize ovarian or colorectal cancer cells to paclitaxel induced apoptosis. Also, that the WWOX tumour suppressor would enhance cisplatin induced cell death, possibly through an ER-stress related mechanism.

It has been shown by previous studies that paclitaxel induces apoptosis via ER-stress. The mechanism behind this activation of apoptotic pathway by paclitaxel is unfolded protein response and not the anti-mitotic action of taxanes. To understand the mechanism of how ER stress affects paclitaxel response I used inhibitors of several key ER stress proteins, but none of these resulted in altered paclitaxel chemo-response. To characterise the paclitaxel response further, I used GRP-78 and XBP-1 as markers of ER-stress, but I found no evidence of induction of ER-stress by paclitaxel in any of our cell line models.

Next, I tried to determine any association of ER-stress with clinical outcome in patient tumour samples that might support a role of ER-stress in paclitaxel response. High expression of GRP-78 protein in ovarian cancer patients treated with taxol predicted longer overall survival, consistent with a role of ER-stress in taxane response, but no correlation between GRP-78 and progression free survival was observed. Furthermore, no correlation of GRP-78 mRNA expression was observed in terms of survival in either ovarian or colorectal cancer patients from the TCGA dataset. Unpublished data from Dr Paige's group had suggested that WWOX regulates this ER stress pathway response to paclitaxel. However, in my study, WWOX expression in both IHC and RNAseq TCGA study showed no association with survival. There was no evidence that WWOX status could modulate the correlation of ER stress with survival. Overall the data therefore suggest that ER stress may have some relevance in paclitaxel response but WWOX did not seem to modulate that.

Cisplatin is another chemotherapy drug used for the treatment of tumours. Cisplatin damages the DNA by forming adducts with the structure of DNA. I show that treatment of ovarian and colorectal cancer cells with cisplatin down regulated WWOX expression. This demonstrated that my hypothesis that WWOX would enhance cisplatin response was wrong and led us to propose an alternate hypothesis that cisplatin-mediated DNA damage would disrupt expression of fragile site associated genes. Another fragile site associated gene FHIT also showed down regulation by cisplatin treatment in my experiments. This regulation of fragile site gene expression was independent of the p53 DNA damage response pathway but correlated with activation of transcription factors NF-κB2 and PAR2.

6.2 Paclitaxel and ER-stress

Ovarian cancer is the sixth most common cancer in the UK. Each year in UK 7400 women are diagnosed with ovarian cancer. The 5-year survival rate has improved in last few years and the current 5-year survival rate is 50% in UK (Cancer research UK, 2017). One of the most common problems which cancer patients face is frequent occurrence of acquired chemo resistance. Paclitaxel is used as first line therapy for treatment of ovarian cancers. Paclitaxel stabilizes the microtubules and research show that paclitaxel activates signalling molecules and transcription of various genes which could be responsible for apoptosis (Wang et al., 2000). In addition to its role in mitotic arrest, several studies suggest that apoptosis induced by Paclitaxel is due to endoplasmic reticulum (ER) stress also known as unfolded protein response (UPR) (Liao et al., 2008; Weigel et al., 2000; Pan et al., 2014).

I looked at the involvement of apoptotic arms linked with UPR target genes such as PERK, JNK and caspases, but my analysis failed to find evidence of ER stress after paclitaxel treatment. There could be a few reasons why my in vitro studies failed to show any evidence of ER stress and one of them could be that paclitaxel does not cause ER stress. Based on the studies cited above, however, it is difficult to assume that paclitaxel does not cause ER stress in some cell lines at least. But despite using a range of different cell lines from different tumour types in my experiments I saw no evidence of this. The second reason that

my study could not see any evidence of ER stress due to paclitaxel could be possibly that GRP-78 and XBP-1 may not be the best markers to indicate ER-stress induction. However, published studies show that activated ER stress does result in increased GRP-78 transcription and increased splicing of XBP-1 (Shuda et al., 2003; Thuerauf et al., 2006) Another possible explanation could be the paclitaxel used in experiments may be old or not in good quality, or its function may have been affected by the formulation of the drug. I used paclitaxel dissolved in DMSO and RPMI media, whereas the paclitaxel which is used in clinics, and was used by Dr Paige's group to generate the preliminary data about ER-stress and paclitaxel response, is supplied as a non-aqueous viscous fluid containing polyoxyethylated castor oil and dehydrated alcohol, which was then diluted into 0.9% sodium chloride or 5% dextrose injection. But in my experiments, I observed cell death in paclitaxel treated cells which show that paclitaxel was functional and had cytotoxic effect on the cells but did not induce ER stress. Overall it is not clear why there was no ER stress in my experiments. But despite my model not working based upon published data there is likely to truly be a link between paclitaxel and ER stress.

At the start of taxane therapy patients respond to paclitaxel but with time they acquire resistance to taxanes which is a big problem (Raguz and Yague, 2008). Taxanes show different levels of resistance in cancer patients with same class of drugs. For examples taxanes and epithilones have same mechanisms of action but mutation in β-tubulin show resistance to taxanes but not to epithilones (Lee et al., 2001). Similarly, patients with over expression of p-glycoprotein show resistance to taxanes but not ixabepilone although both share same mechanism of action (Mandhare et al., 2016; Hasanpourghadi et al., 2017). So, it depends on patients and their sensitivity to chemotherapy drugs and this makes treatment of cancer patients difficult because mutations vary from patient to patient and so is the response towards chemotherapy drugs. Although paclitaxel kill cancer cell by its anti-mitotic action the discovery of other pathways linked with paclitaxel response can improve the response of patients towards paclitaxel, for example, activation of ER stress. However, some cancer patients already have activated GRP-78 in their tumour cells, which means they have

inherently activated ER stress. This may be an advantage to the cancer cells because enhanced GRP-78 is cytoprotective by increasing the folding capacity of the cells to tackle misfolded proteins and maintenance of cellular homeostasis, but only if there is a failure in the ER-stress associated apoptotic response. These patients may therefore not be expected to respond to paclitaxel effectively because the inherently activated ER stress did not caused apoptosis but was limited to its cytoprotective role. In contrast, when those patients without inherently activated ER stress are given paclitaxel they will respond to the drug by activating ER stress induced apoptosis and enhancing the chemo-response to paclitaxel. Screening tumours for markers of activated ER-stress could therefore potentially identify patient groups who will respond less effectively to paclitaxel and could be used to guide treatment decisions. Furthermore, development of novel drugs which can change the cytoprotective mode of ER-stress into apoptotic response, could potentially be used to increase the efficacy of paclitaxel.

The unpublished data from Dr Szymon Janczar in Dr Paige's group suggests that WWOX functions in this way. Dr Janczar's data shows that paclitaxel induces ER response in ovarian cancer cells which was unaltered by WWOX, but WWOX re-expression caused a significant shift in this response towards apoptosis. This study also showed the clinical relevance of these findings by using a publicly available microarray gene expression dataset (GEO GSE9899) of 260 ovarian cancer patents. The survival analysis showed that high WWOX mRNA expression predicted longer PFS in patients treated with paclitaxel but not in patients which did not have paclitaxel treatment. Interestingly, WWOX affected PFS only in patients whose tumours expressed low-GRP-78, whilst the high GRP-78 expressing group did not show any correlation of WWOX with PFS. It can be hypothesized from this study that patients with high GRP-78 expression in their tumour cells may have inherently activated ER stress independent of paclitaxel treatment. It is therefore speculated that WWOX status show no impact in high GRP-78 patients and the only mechanism of cell death is anti-mitotic action of paclitaxel but not ER stress. Whereas, in patients with low GRP-78 it is hypothesized that ER-stress is not inherently active, and therefore could be activated by

paclitaxel treatment. In these tumours, we speculated that WWOX status could influence the apoptotic outcome of ER-stress, and thus higher WWOX expression predicted longer PFS.

The involvement of WWOX in mediating the ER-stress apoptotic response could also potentially be useful clinically. Tumours can be screened for WWOX expression and taxanes could be given only to those patients who have high WWOX expression. Another option for patients with low WWOX is to find a treatment that could increase WWOX expression and given that together with taxanes to improve outcomes.

6.3 Cisplatin and WWOX

My results show that cisplatin causes loss of fragile site associated tumour suppressor genes WWOX and FHIT. However, Salah et al., (2013) showed that WWOX can sensitize cancer cells to cisplatin by linking with ΔNp63. WWOX significantly enhanced the cell death (14 fold) in cancer cells expressing both WWOX and ΔNp63, whereas cancer cells expressing only ΔNp63 showed lower cell death. It was further showed WWOX does this by restricting the ΔNp63 to the cytoplasm which results in suppression of transcription of ΔNp63. The likely reason for the difference in the two studies is that Salah et al used virally transduced WWOX and not endogenous WWOX which is expressed by its normal promotor. This was the reason cisplatin could not suppress WWOX expression in their study. However, this shows that WWOX can sensitize cancer cells to cisplatin induced cell death if it were not for the fact that cisplatin instead switched off WWOX expression. The clinical significance of this finding is if WWOX is prevented from switching off, it can help cisplatin in killing cancer cells. Therefore, therapies designed to increase WWOX expression would be expected to enhance cisplatin response, as well as paclitaxel response.

Interestingly, in ovarian cancer cisplatin is combination with paclitaxel as standard treatment. Based on my results, if cisplatin switches off WWOX in these tumours it means WWOX will be unable to sensitize the cancer cells to paclitaxel induced cell death. Thus, the one drug may be producing an effect that partially counteracts the other drug. The clinical significance

for these findings is, it will be helpful if WWOX remain expressed in cancer patients during the chemotherapy treatment. Potentially this might be achieved by changing the timing of the administration of drugs. Cisplatin down regulates WWOX, so it should not be given first, instead patients should be given paclitaxel first, so it can kill cancer cells by the help of WWOX and then later cisplatin should be given and in this way, there will be no interference. Another suggestion is if WWOX could be activated by some type of therapy in combination with cisplatin/paclitaxel which means WWOX stays active and these drugs will kill cancer cells more effectively. Recently, Wang et al., (2017) show that pre-treatment with kanglaite can sensitize colorectal cancer cells to taxol. This study further showed pre-treatment with kanglaite inhibited the expression of canonical pathway of NF-κB (p65) and increased the effect of taxol. Non-canonical pathway of NF-κB2 (p52) has not been examined in this study but, given my data potentially linking p52 with WWOX suppression, this raises the question of whether there is a possibility kanglaite may also inhibit p52 (non-canonical) as well as p65 (canonical). Based on my findings, inhibition of non-canonical pathway by kanglaite would cause increased expression of WWOX which will ultimately increase the taxol response as they report. If this is indeed the mechanism happening in this study, then maybe combination of kanglaite with paclitaxel and cisplatin can give better patient outcome by helping to maintain high WWOX levels.

6.4 Concluding remarks

- My *in vitro* ER stress study could not see any evidence of generation of ER stress by paclitaxel in ovarian and colorectal cancer cells. Although in some experiments paclitaxel treatment did show the reduction in the number of cells compared to controls, this effect of paclitaxel was does not appear to be due to ER stress.

- Clinical studies showed a correlation between GRP-78 and overall survival time but it was independent of action of WWOX. The data used in these analyses was from paclitaxel treated patients, so there is *in vivo* correlation between paclitaxel and ER stress marker GRP-78.

- I have shown novel data that Cisplatin down regulates the expression of fragile sites associated genes; WWOX and FHIT. The mechanism for this is independent of the p53 DNA damage response pathway but may result from transcriptional regulation by NF-kB2 and PAR2.

- Further investigation into role of NF-kB2 is needed to confirm the link with regulatory mechanisms of WWOX, but this would suggest potential therapeutic strategies for enhancing cisplatin and paclitaxel response in cancer patients through upregulated expression of WWOX tumour suppressor.

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(www.rndsystems.com/resources/articles/dna-damage-response)

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Significant loss or reduction of Fragile histidine triad expression was noted in 18 of the 52 (34.6%) advanced colorectal carcinomas: 2 (10.5%) well-differentiated, 3 (17.6%) moderately-differentiated, 13 (81.3%) poorly-differentiated carcinomas, the frequency being significantly higher in the latter than that in the former two (*P*<0.0001).

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Appendices

S1.1 SRB various dilution and linearity test

In my experiments, I am using 0.4% SRB solution to stain cells and determine the cell number present. There is reported to be good linearity between the amount of SRB absorbance measured and the number of cells present. To confirm this, spectrophotometer readings of various dilution of SRB were performed (ranging from 0.02% to 0.4%) to determine if there was a maximum SRB amount that saturated the ability of the spectrophotometer to measure. Absorbance was measured at a wavelength of 570nm by using a spectrophotometer. Figure S1.1 shows the absorbance of various percentages of SRB dilutions.

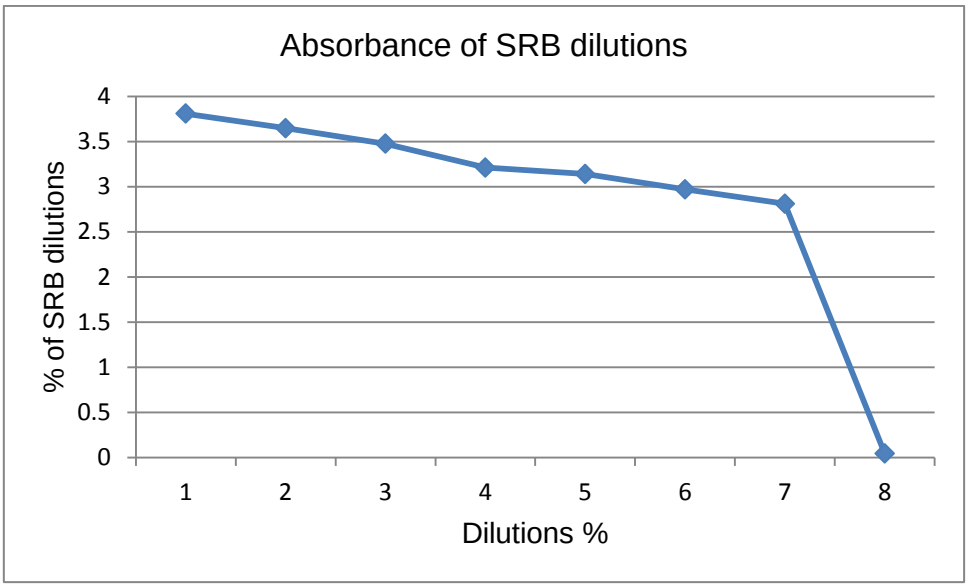


Figure S1.1: Absorbance of various percentages of SRB dilutions

I also performed another experiment in which cell numbers ranging from 5000/well to 100,000 per well were plated in 96-well plates (in triplicates) and placed in incubator for 6 hours. The plates were removed from the incubator and cells were fixed with trichloro acetic acid (TCA) followed by SRB staining. Figure S1.2 shows the SRB absorbance compared to the cell number plated and confirms the linearity relationship between number of cells and SRB dye.

Table S1.1 Representations of Dilutions % of Figure S1.1

1	0.4%
2	0.3%
3	0.2%
4	0.1%
5	0.08%
6	0.04%
7	0.02%
8	0.0%

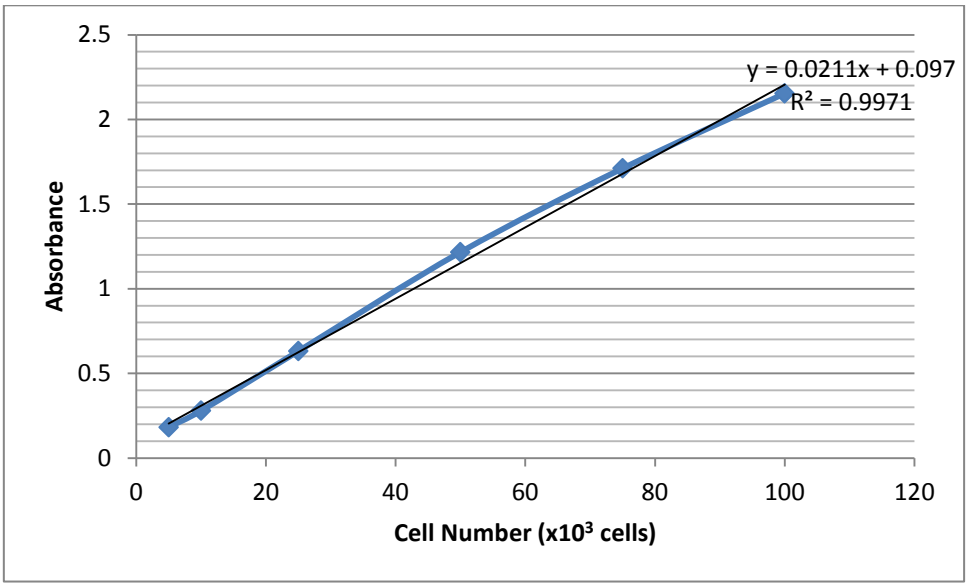


Figure S1.2: Plated Cell numbers plotted against absorbance

S 2.1 Determine the optimal concentration of Paclitaxel, tunicamycin or salubrinal to use in HCT116 isogenic cell lines

Before the start of experiment, the first step was to find out the optimal drug dose concentrations for the ER stress inhibition experiments. For this purpose, the HCT116 isogenic cancer cells were treated with various doses of paclitaxel, salubrinal or tunicamycin for 48, 72 or 96 hours and surviving cells measured by SRB staining.

S 2.1.1 Determine the optimal concentration of paclitaxel to be used in HCT116 isogenic cell lines experiments for 48, 72 or 96 hours

Paclitaxel caused reduction of cell number in all four cell lines which was dose and time dependent. The different cell lines showed similar responses to paclitaxel, although the p21-null cells exhibited a slightly greater reduction in cell number compared to the other cell lines. The aim of the experiment was to determine a paclitaxel dose producing approximately 50% reduction in cell number after 72 hours in all four cell lines. Based on the results of these experiments (Figures S 2.1, S 2.2 and S 2.3) even a 2nM dose was slightly too high (producing 60-70% reduction in cell number) and therefore a 1 nM dose was selected for subsequent experiments in these cells.

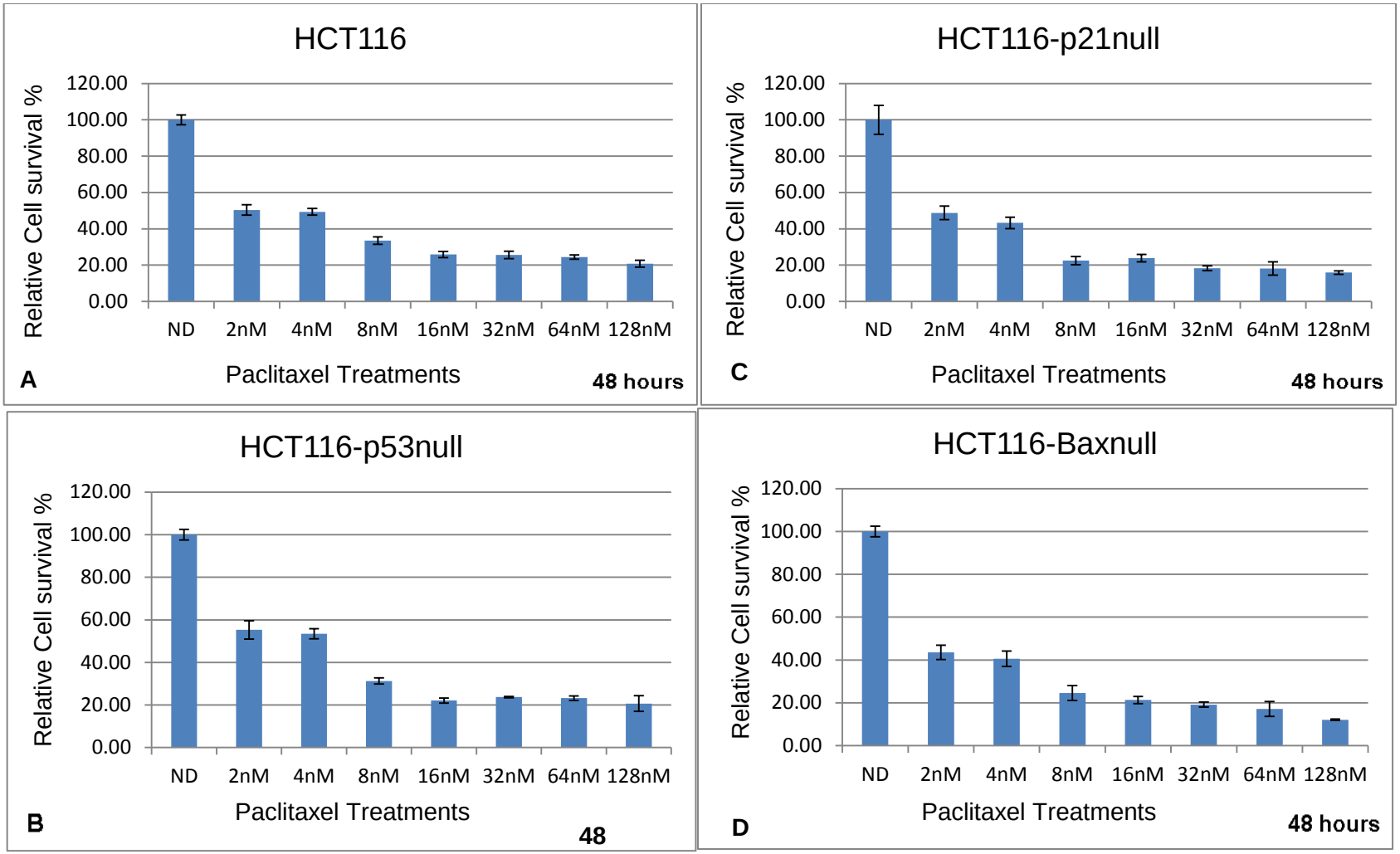


Figure S2.1: Relative cell survival in HCT116 isogenic cell after treatment with paclitaxel doses for 48 hours. Experiments were repeated three times. Means ± SEM are shown.

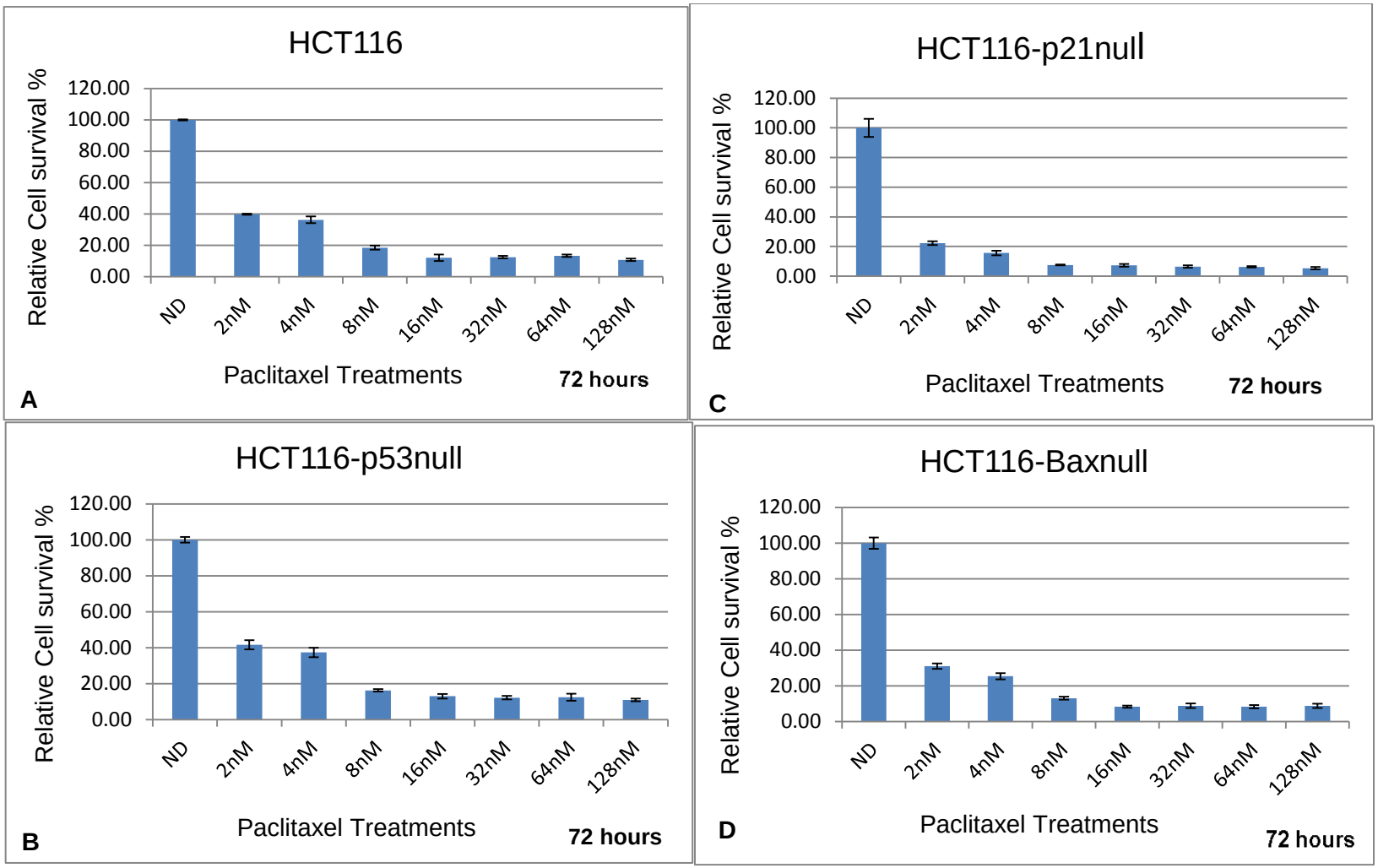


Figure S2.2: Relative cell survival in HCT116 isogenic cells after treatment with paclitaxel doses for 72 hours. Experiments were repeated three times Means ± SEM are shown.

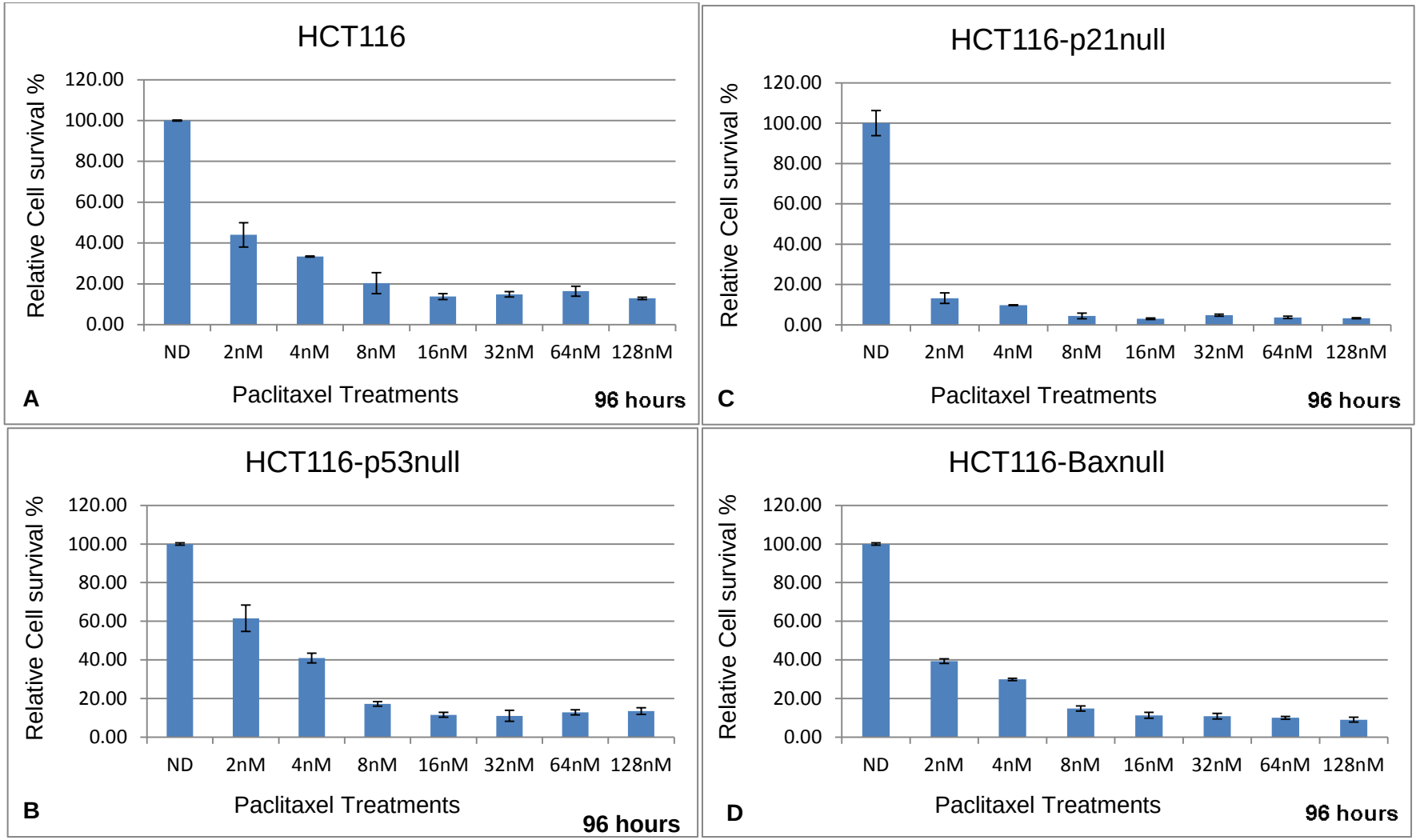


Figure S2.3: Relative cell survival in HCT116 isogenic cells after treatment with paclitaxel doses for 96 hours. Experiments were repeated three times Means ± SEM are shown.

S.2.1.2 Determine the optimal concentration of tunicamycin to be used in HCT116 isogenic cell lines experiments for 48, 72 or 96 hours

The reason for this type of result could be the increase in number of cells after 96 hours. I started my experiments with 6000 cells numbers/well and after 96 hours, cells increased their numbers and the low concentrations of drugs became ineffective on higher number of cells

The aim of the experiment was to identify a tunicamycin dose producing approximately 50% reduction in cell number after 72 hours in all four cell lines. Based on the results, I selected 75ng/ml tunicamycin doses for subsequent experiments.

Figures (S 2.4, S 2.5 and S 2.6) show that tunicamycin causes a reduction in cell number in a dose dependent manner. Curiously the apparent effect of tunicamycin on cell number decreased over time.

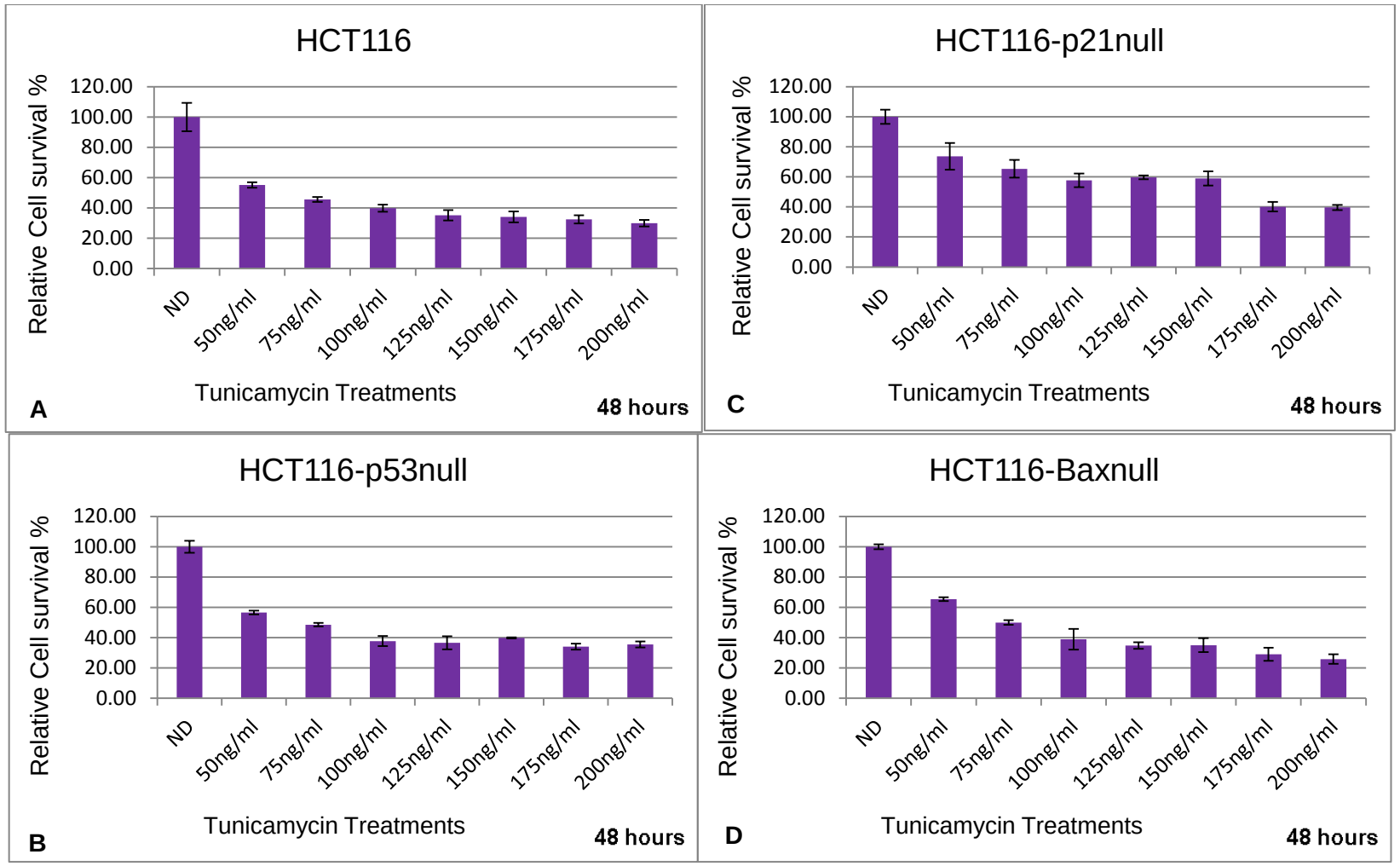


Figure S2.4: Relative cell survival in HCT116 isogenic cells after treatment with tunicamycin doses for 48 hours. Experiments were repeated three times. Means ± SEM are shown.

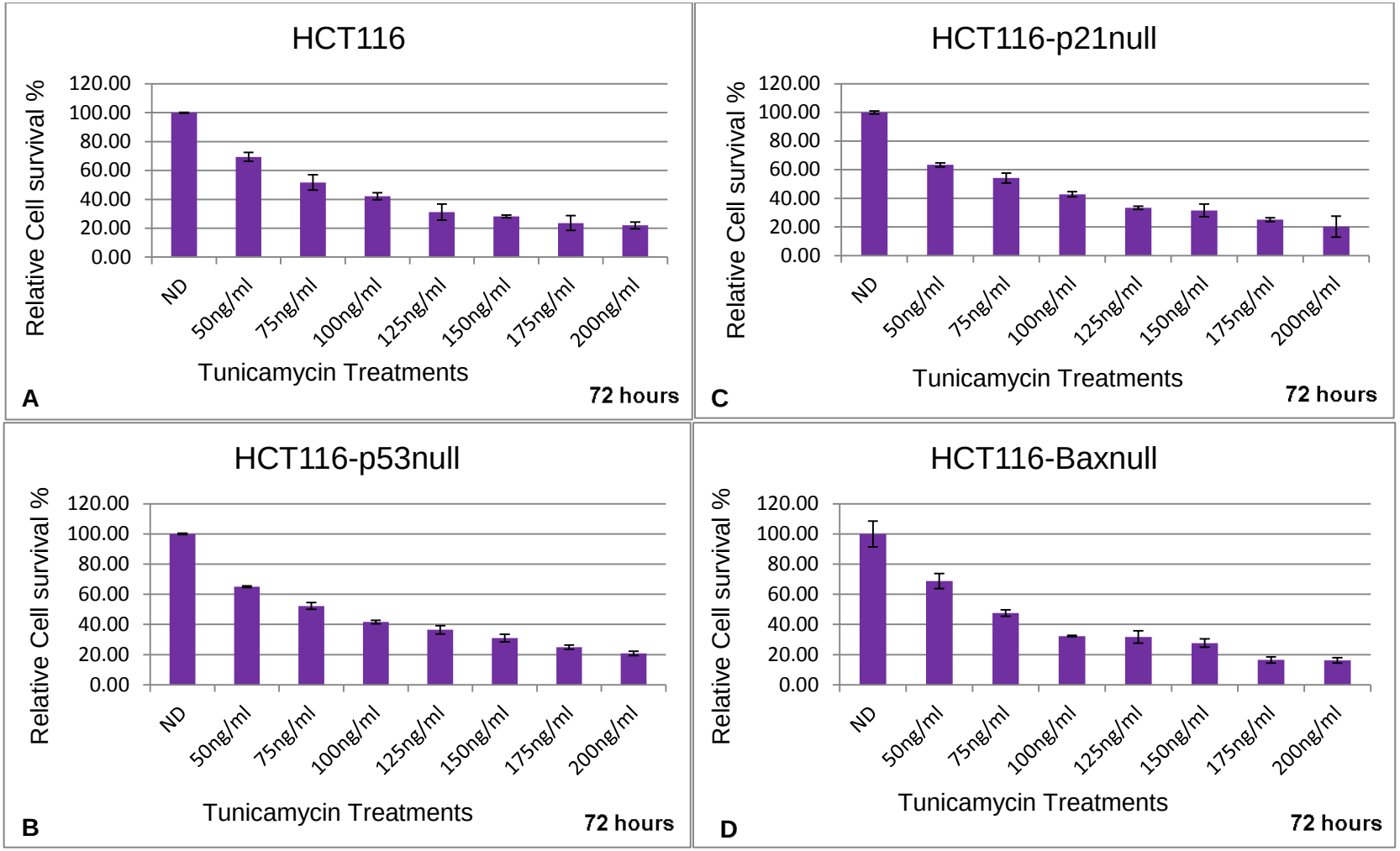


Figure S2.5: Relative cell survival in HCT116 isogenic cells after treatment with tunicamycin doses for 72 hours. Experiments were repeated three times.

Means ± SEM are shown.

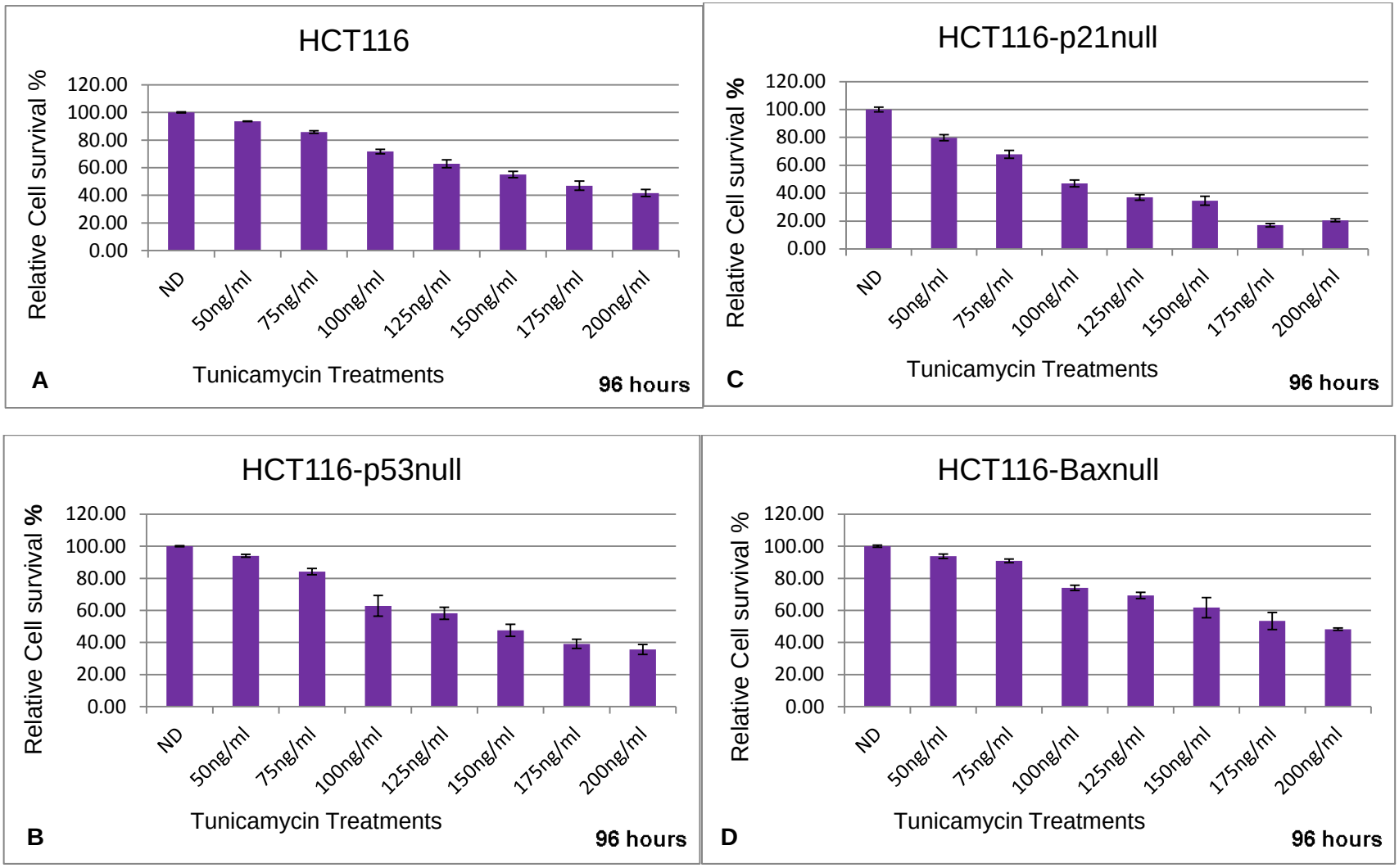


Figure S2.6: Relative cell survival in HCT116 isogenic cells after treatment with tunicamycin doses for 96 hours. Experiments were repeated three times.

Means ± SEM are shown.

S 2.2 Determine the optimal concentration of Paclitaxel, tunicamycin or salubrinal to use in in PEO1-H8 and PEO1-FP2 cell lines experiments

Before the start of experiment, the first step was to find out the optimal drug dose concentrations for the ER stress inhibition experiments. For this purpose, ovarian cancer cells PEO1-H8 and PEO1-FP2 were treated with various doses of paclitaxel, salubrinal or tunicamycin for 48, 72 or 96 hours and surviving cells measured by SRB staining.

S2.2.1 Determine the optimal concentration of paclitaxel to use in PEO1-H8 and PEO1-FP2 cell lines experiments

PEO1-H8 and PEO1-FP2 cells were treated with 2nM, 4nM, 8nM and 16nM concentrations of paclitaxel, and percentage surviving cells determined by SRB staining after 48 and 72 hours. The results (S2.7) showed that paclitaxel reduced surviving cells in a dose dependent manner, with minimal effect seen below 8 nM. The results at 48 hours and 72 hours show little difference. Paclitaxel response in the PEO1-FP2 cells was slightly greater than in PEO1-H8 cells but this did not reach statistical significance. From these data, 16nM paclitaxel was chosen as an optimal dose to be used in subsequent experiments as it caused approximately 50% reduction in surviving cells after 72 hours.

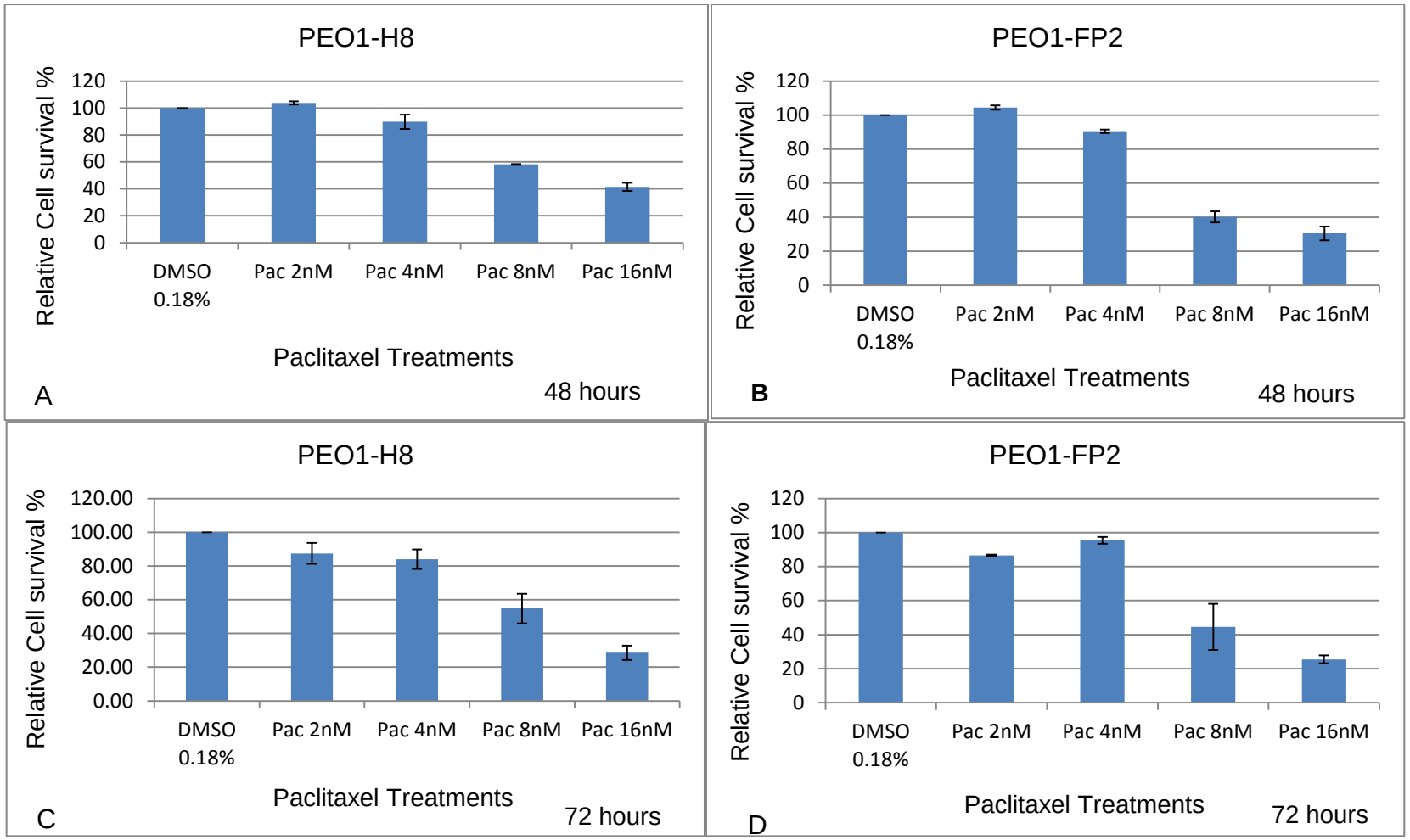


Figure S2.7 shows the cell survival in PEO1-H8 and PEO1-FP2 cells after treatment with 2nM, 4nM, 8nM and 16nM paclitaxel. Cells were also treated with 0.18% DMSO. After 48 or 72 hours, spectrophotometric analysis was used (post SRB staining) to observe cell survival. Experiments were repeated twice. Means ± SEM are shown.

S2.2.2 Determine the optimal concentration of salubrinal to use in PEO1-H8 and PEO1-FP2 cell lines experiments

PEO1-H8 and PEO1-FP2 cells were treated with 10µM, 20µM, 30µM and 60µM concentrations of salubrinal. After 48 or 72 hours of salubrinal treatment, surviving cells were measured with SRB staining. Salubrinal alone did cause some reduction in surviving cells (20% reduction at 48 hours and 30% reduction at 72 hours), which was not dose dependent (Figure S2.8). Therefore, doses of 30µM and 60µM were chosen for use with PEO1 cells, consistent with the experiments in A2780, SKOV3 and HCT116 cells above.

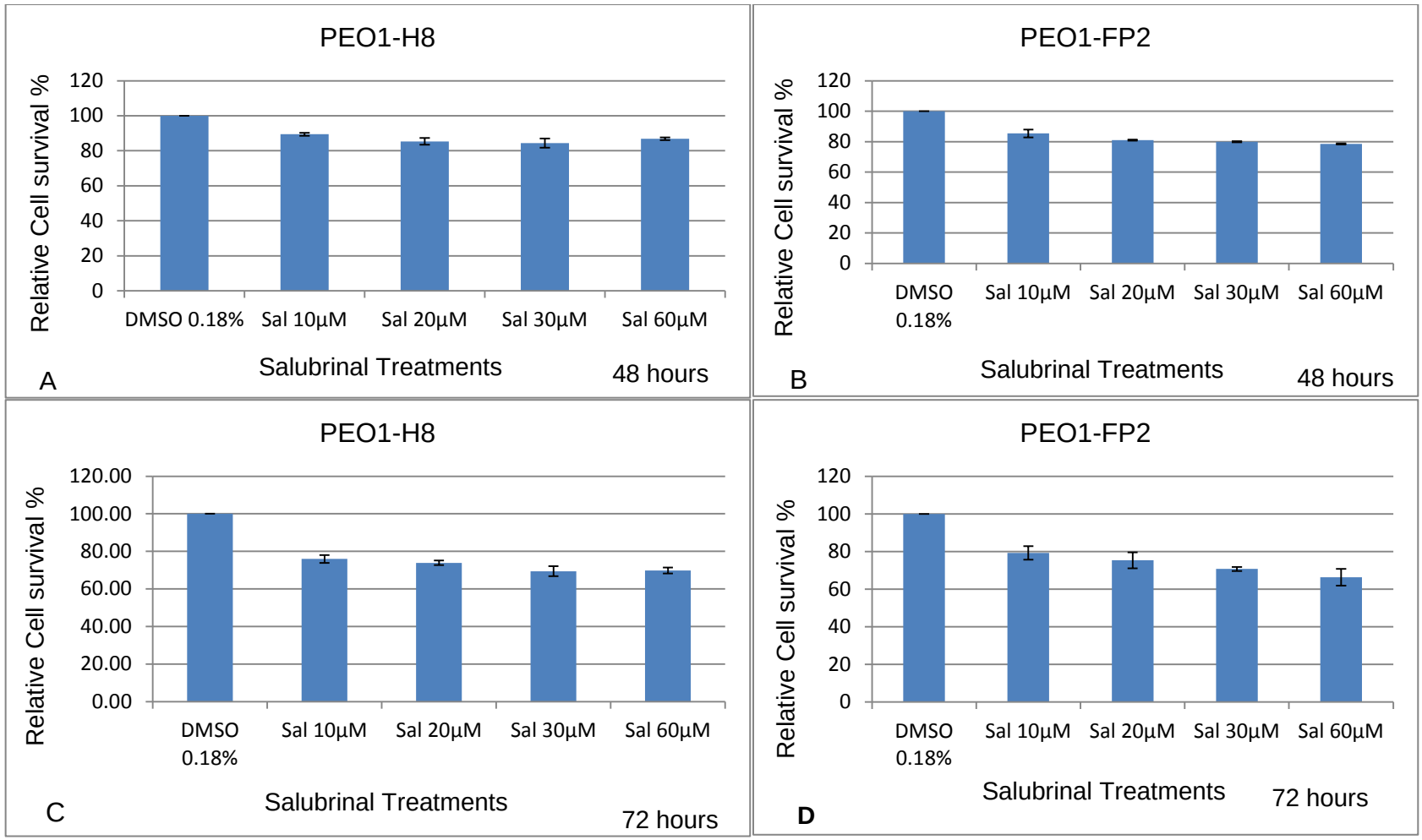


Figure S2.8 shows the cell survival in PEO1-H8 and PEO1-FP2 cells after treatment with 10µM, 20µM, 30µM and 60µM concentrations of salubrinal. Cells were also treated with 0.18% DMSO. After 48 or 72 hours, spectrophotometric analysis was used (post SRB staining) to observe cell survival. Experiments were repeated twice. Means ± SEM are shown.

S2.2.3 Determine the optimal concentration of tunicamycin to use in PEO1-H8 and PEO1-FP2 cell lines experiments

PEO1-H8 and PEO1-FP2 cells were treated with 50ng/ml, 100ng/ml, 150ng/ml and 200ng/ml concentrations of tunicamycin, and surviving cells measured by SRB staining. The results (Figure S2.9) show that tunicamycin produced a reduction in surviving cells in a dose dependent manner. PEO1-FP2 cells appear to be less sensitive to tunicamycin than the PEO1-H8 cells. For subsequent experiments, we wanted to use the same tunicamycin dose in both of these lines to allow comparison. Therefore, we chose 200 ng/ml as this produced approximately 50% reduction in surviving cells after 72 hours in the PEO1-H8 cells and was consistent with doses used in other cell lines previously.

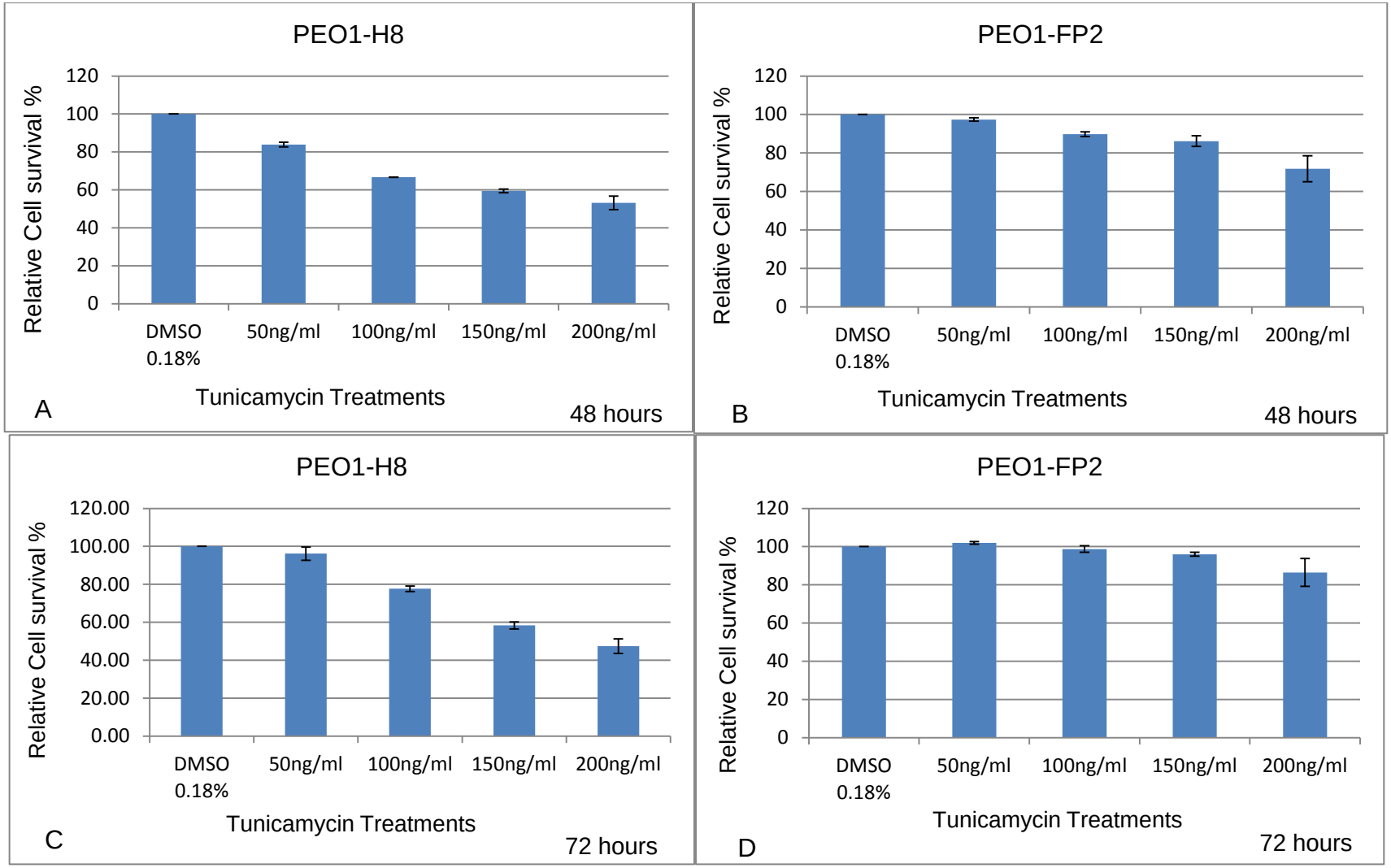


Figure S2.9 shows the cell survival in PEO1-H8 and PEO1-FP2 cells after treatment with 50ng/ml, 100ng/ml, 150ng/ml and 200ng/ml concentrations of tunicamycin (positive control for ER stress). Cells were also treated with 0.18% DMSO. After 48 or 72 hours, spectrophotometric analysis was used (post SRB staining) to observe cell survival. Experiments were repeated twice. Means ± SEM are shown.

S3.1 Appearance of cells following exposure to various treatments

Pictures of cells taken after 48 hours of cisplatin treatment. S3.1 represents untreated cells, S3.2 represents DMF treated cells and S3.3 represent cisplatin treated cells.

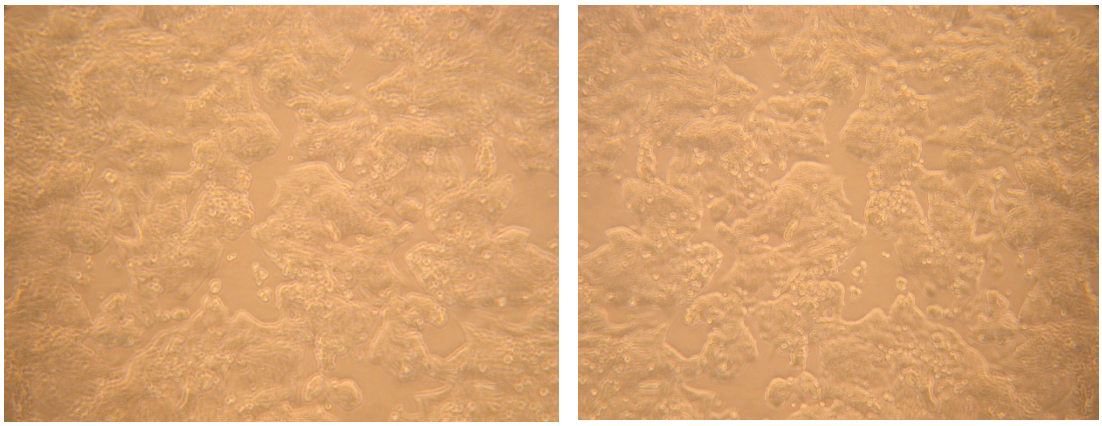


Figure S3.1: A2780 cells after 48 hours (Untreated) 100x

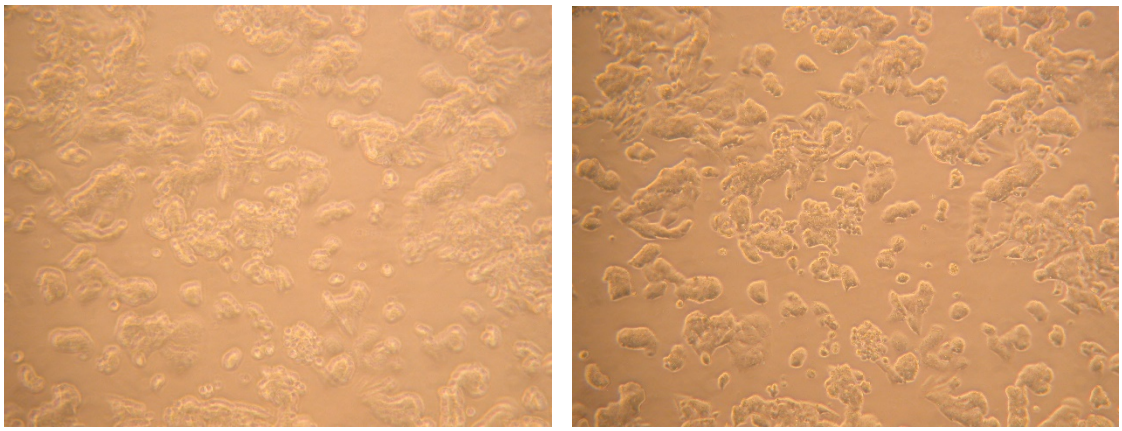


Figure S3.2: A2780 cells after 48 hours of DMF treatment 100x

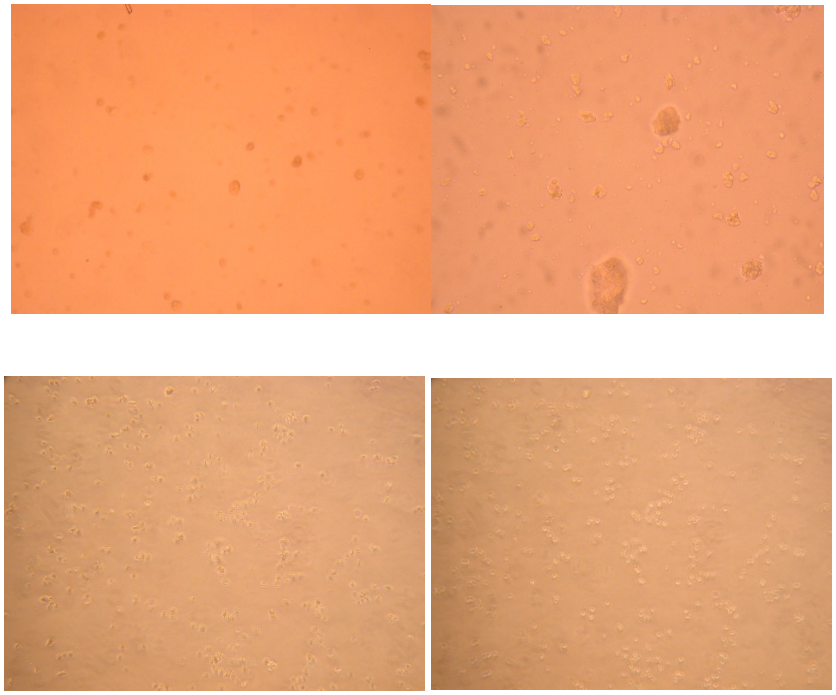


Figure S3.3: A2780 cells after 48 hours of cisplatin treatment (100x)

S4.1 PCR product sizes

Table S3.1: WWOX and FHIT primers used for QRT-PCR

Primers	**Product size (bp)**
WWOX	100
FHIT	166
B-Actin	385

S5.1 Antibodies used in project

Table S4.1 Antibodies used for western blotting and their dilutions

Antibodies	Dilutions	Product codes
Anti-PAR2 antibody [EPR13675]	1:10000	ab180953

Anti-NF-kB p100 / p52 antibody [0.T.173]	1:2000	ab32859
Goat Anti-Rabbit IgG H&L (HRP)	1:1000	ab6721
Anti-Actin antibody [ACTN05 (C4)]	1:1000	ab3280
Polyclonal Goat Anti-Mouse Immunoglobulins HRP	1:5000	Dako P0447

S6.1 Preparation of cisplatin solution

Cisplatin (C2210000-100mg) is a yellow powder which was dissolved in dimethyl formamide (DMF) to prepare the primary stock solution of concentration 15mg/ml. This primary stock solution was used to prepare secondary stock solution having concentration 1mg/ml. For experimental drug solution preparation, 1mg/ml cisplatin stock solution was used. The first step was to find out the suitable cell numbers and drug dose for the experiments. This was achieved by treating various cell numbers with various drug concentrations and selecting the one which is an IC50 dose. All the experiments were repeated three times and were independent of each other.

S7.1 Analysis of DNA content by flow cytometer analysis

The aim of this experiment was to see the different stages of cell cycle following esiRNA NF-kB2 knockdown along with GFP, SFM and SFM+TR treatments. This experiment was just to see if these different treatments disturb cell cycle at any stage during the experimental time period of 72-96 hours. A2780 cells were plated in 6-well plates and transfected as described in materials and methods. DNA content of the cell was measured by flow cytometer following propidium iodide (PI) staining.

The results of the Flow cytometer analysis show that most of the cells were at G_O/G_1 stage in all the treatments (figure S5.2 panels C-F). All treatments that included transfection reagent show a slightly increased number of apoptotic cells (indicated by the Sub-G0 fraction in the Flow cytometer data, Figure S6.3 panel A, however this did not reach significance, and no difference in cell cycle distribution was detected between the different treatments (Figure S6.3 panel B).

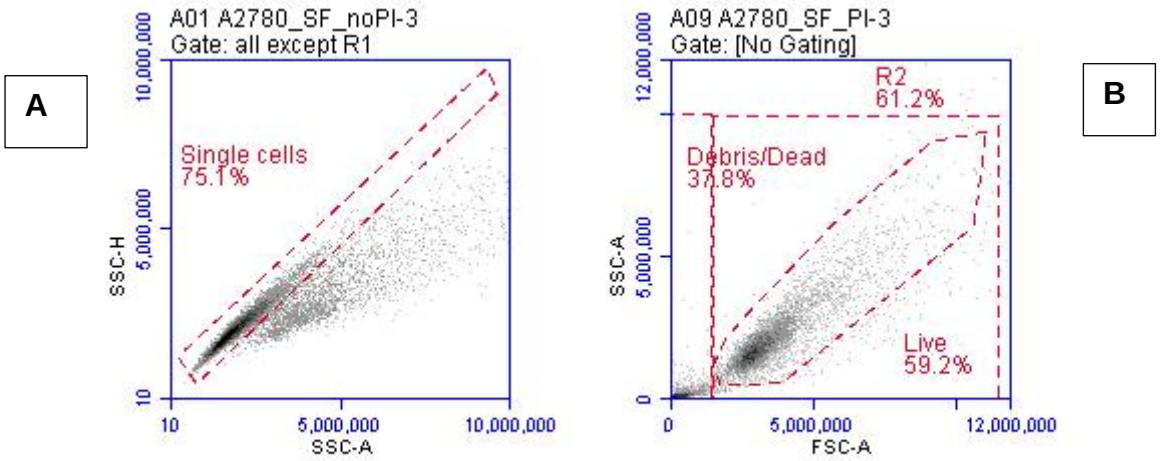


Figure S7.1 (A-B): Side scattered graphs showing single cells and doublets. Cells in the red dotted box in (A) are the single cells used for the analysis (Figure S7.2, C-F). (B) shows the alive cells in the red dotted area including single cells and doublets.

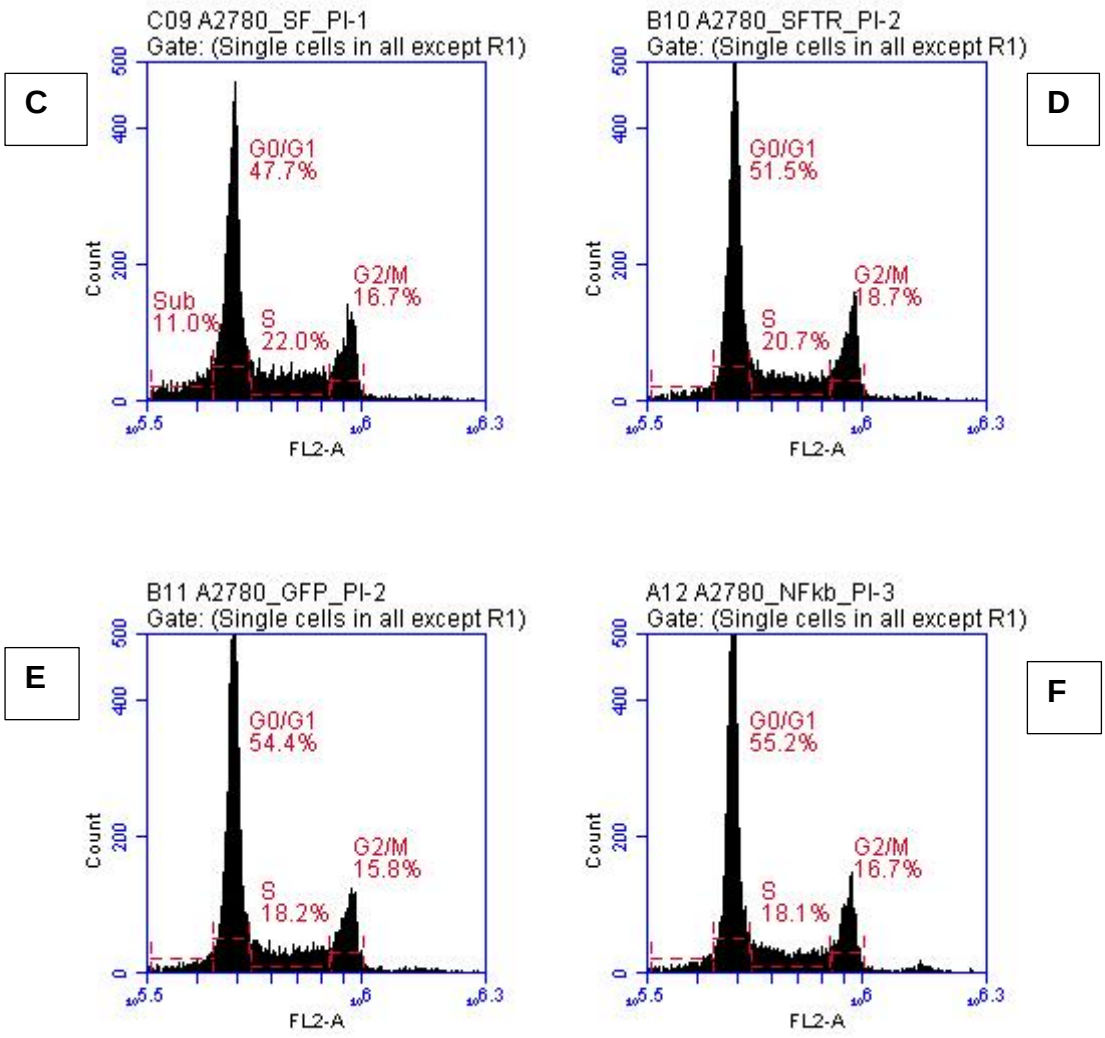
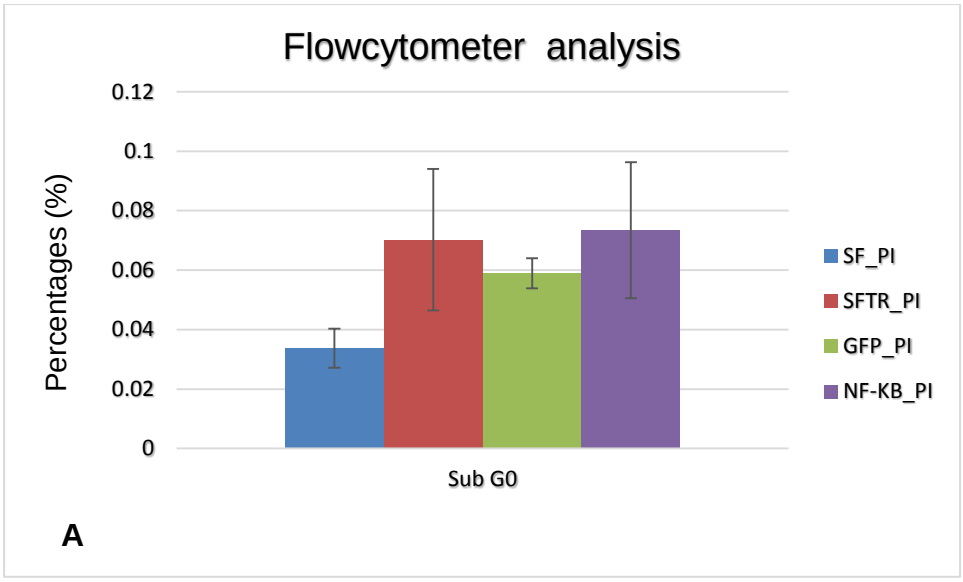


Figure S5.2 (C-F): Cell cycle analysis plot. Only single cells were used to make these plots. Cell cycle analysis of A2780 cells after 72 hours treatment with Nf-kB2, GFP, SFM+TR and SFM. A2780 cells were fixed with ethanol



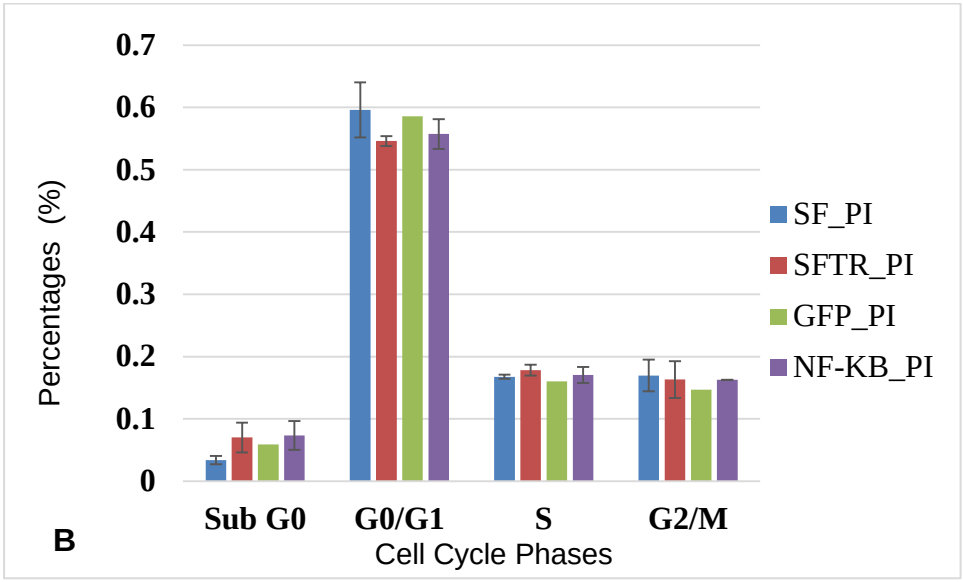


Figure S7.3: Flow cytometer analysis of cell cycle phases.

S8.1 Expression of WWOX in HCT116 and HCT116-p53null cells after 24 hours of cisplatin treatment

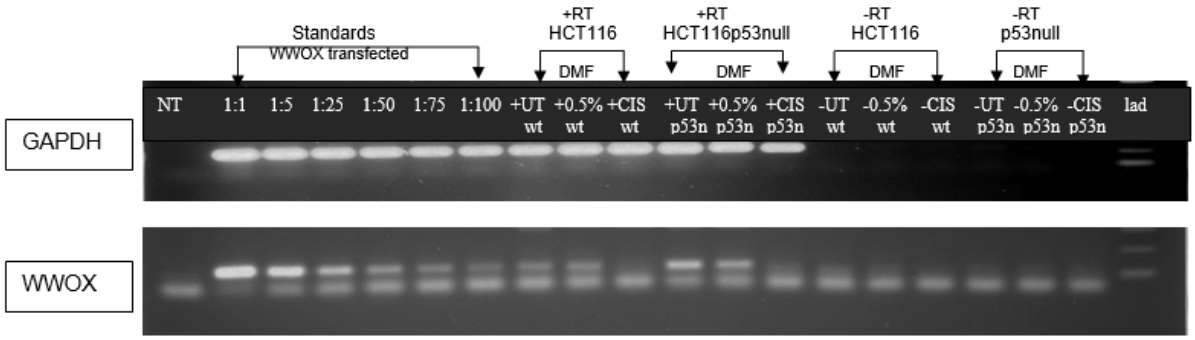


Figure S8.1: Gel images of HCT116 and HCT116p53null after treatment with cisplatin.

Figure shows WWOX expression in HCT116 and HCT116p53null cells with and without cisplatin exposure. NT represents no template control. Six different standards dilutions were used (1:1, 1:5, 1:25, 1:50, 1:75, 1:100) from a *WWOX* transfected cell line having high amount of *WWOX* in it. UT represents untreated cell. Dimethylformamide (DMF) (0.5%) shows the effect of vehicle alone. cDNA samples are indicated by +, - RT samples are indicated by -.

S9.1 Paclitaxel treatment and ER stress induction in HCT116 cells

In this experiment colorectal cancer cells HCT116 were treated with doses of paclitaxel. The results (Figure S9.1) show that no amplification was observed in the 'no reverse transcriptase' controls showing no genomic DNA contamination was present, except for the DMSO and untreated dose of GRP-78. Similar intensity of beta actin expression was seen in all samples showing equal loading of template (except DMSO and untreated). These results showed there was no increase in expression levels of GRP-78 or XBP-1(S). It shows there was no induction of ER-stress.

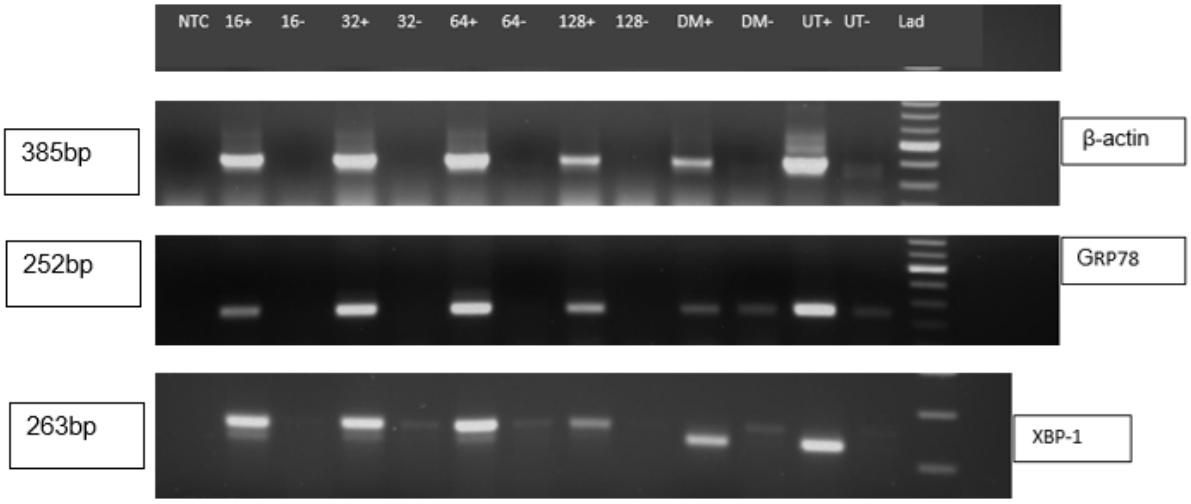


Figure S9.1: Gel images of HCT116 after treatment with paclitaxel for 72 hours.

Samples include NTC (no template control), UT (untreated), DM (DMSO 0.18%) and paclitaxel (16nM, 32nM, 64nM and 128nM) treated samples. (+) represents cDNA samples from the different treatments and (-) indicates no reverse transcriptase controls to confirm that no genomic DNA was present.