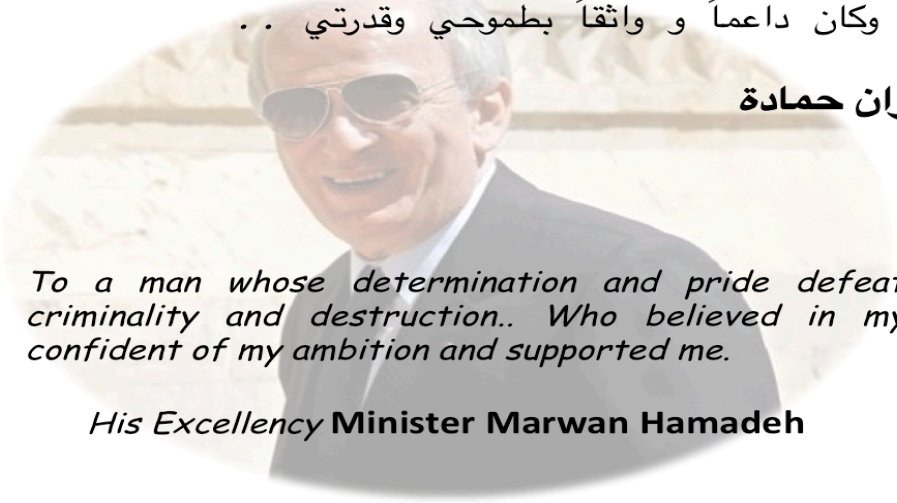
This work is dedicated to ……

إلى رجل لم تستطع حتى يد الإجرام أن تنال من عزيمته وعنفوانه. . .
إلى من مد لي يده وكان داعماً و واثقاً بطموحي وقدرتي . .

معالي الوزير مروان حمادة



To a man whose determination and pride defeated the hands of criminality and destruction.. Who believed in my ability and was confident of my ambition and supported me.

His Excellency **Minister Marwan Hamadeh**

إلى من ابعدني عنه القدر.. لكن رغم مرور السنين لا زال حبه قوتي ورعايته تحميني، وصوته يناديني دكتورة يرافقني.. فحققت أمله!!

To a man who was estranged by destiny and despite the years of separation, his love and care are still accompanying me, and his voice calling me Dr. Rola is a chime throughout my days … I made your wish come true… **My FATHER**

إلى من صوتها كان دائماً يبعث في الامل، وضحكتها تبدد كل قسوة وألم . .

مياااا

To a voice who was always giving me hope, and a smile was my driving force through out the days of pain and hardship .. To the sweet Angel..
My MIAAAAAAA!

Pro-survival Pathways in Triple Negative Breast Cancer

Focus on the roles of RIP2 and HSPB1

A thesis submitted to the National University of Ireland Galway in fulfillment of the requirement for the degree of

Doctor of Philosophy

by

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2016

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Acknowledgements

I would first like to thank my supervisor Dr. Adrienne Gorman for supporting me for getting the Irish Research Council scholarship, and giving me the chance to join her lab for my PhD. I greatly appreciate her constant support, guidance and encouragement throughout my 4 years. I would also like to sincerely thank Prof. Afshin Samali for his helpful suggestions and ideas in lab meetings.

A huge “Thank You” goes to the Apoptosis Research Center supervisors and lab members for the lovely and professional environment, co-operation, encouragement and support. It is a great place to work and learn, enjoy and celebrate. These will be part of my best memories form Galway.

I would also like to thank my family, especially my mother who was always supporting and encouraging me despite all circumstances and difficulties; in addition to my friends in Lebanon for their support and understanding and helping me get through the hard times, without them my achievement would not have been possible.

List of abbreviations

AIF	Apoptosis inducing factor
APAF-1	Apoptotic peptidase activating factor 1
AE	Adverse event
BAD	BCL-2-antagonist of cell death
BAK	BCL-2 homologous antagonist/killer
BAX	BCL-2 associated protein X
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma 2- extra large
BIM	BCL-2-interacting mediator of cell death
c-FLIP	Cellular FLICE inhibitory protein
CARD	Caspase activation and recruitment domain
caspase	Cysteine-dependent aspartate-directed proteases
CHOP	C/EBP-homologous protein
CSF	Colony stimulating factor
DNA	Deoxyribonucleic acid
eIF2α	Eukaryotic translation initiation factor 2 α
FBS	Fetal bovine serum
HEPES	2-hydroxyethyl-piperazine-N-2-ethanesulphonic acid
HER-2	Human epidermal growth factor receptor-2
HRP	Horseradish peroxidase
HS	Horse serum
HSF	Heat shock factor
HSP	Heat shock protein
HSPA1	Heat shock protein 72
HSPB1	Heat shock protein 27
HSR	Heat shock response
IAP	Inhibitor of apoptosis proteins
IL	Interleukin
Ικκ	Inhibitor of NF-κb kinase
JNK	c-Jun N-terminal kinase
LN	Lymph node
MAPK	Mitogen activated protein kinase
MOMP	Mitochondrial outer membrane permeabilization
MTT	3-(4,5-dimethylthiazol-,2-yl)-2,5-diphenyl tetrazolium bromide
NF-κB	Nuclear factor-κb
NGF	Nerve growth factor
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline containing Tween
PCR	Polymerase chain reaction

PERK	Double-stranded RNA-activated protein kinase (PKR)-like kinase
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PMSF	Phenylmethylsulfonyl fluoride
PR	Progesterone receptor
PS	Phosphatidylserine
PTEN	Phosphate/tensin homologue deleted on chromosome 10
RIP	Receptor interacting protein
RLU	Relative light unit
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
sHSP	Small heat shock protein
siRNA	Small interfering RNA
Smac/DIABLO	Second mitochondria-derived activator of caspases/direct
TAK1	Transforming factor-β-activated kinase 1
TBS	Tris buffer saline
Tg	Thapsigargin
Tm	Tunicamycin
TMRE	Tetramethylrhodamine, Ethyl Ester, Perchlorate
TNBC	Triple negative BC
TNF	Tumour necrosis factor
TNF	Tumour necrosis factor
TNFR	TNF receptor
TRADD	TNF-receptor associated death domain
TRAF	TNFR-associated factor
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
XBP1	X-box binding protein 1
XBP1s	Spliced X-box binding protein 1
XIAP	X-linked IAP
Δψm	Mitochondrial membrane potential

Abstract

Triple negative breast cancers (TNBC) lack estrogen and progesterone receptors, and HER-2 markers rendering them resistant to current targeted therapies. Most TNBCs show basal-like characteristics and the terms TNBC and basal-like breast cancer (BC) are used interchangeably. Both have high histological grade, characterized by exceptionally high mitotic indices, and the presence of central necrotic or fibrotic zones. Also, they have been reported to overexpress anti-apoptotic proteins, such as inhibitors of apoptosis proteins (IAPs), and release growth factors and cytokines that are important for pro-survival signaling and enhance carcinogenesis. Understanding the mechanism and role of these proteins in breast cancer would help develop effective and targeted therapy. In Chapter 1 of this thesis, proteins essential for the induction of survival pathways were identified and their relative messenger ribonucleic acid (mRNA) expression was analyzed among different (BC) patient samples obtained from online GEO NCBI. This is the first study that comprises expression analyses of different isoforms of mRNA expression of genes important in pro-survival pathways regulation, to understand basal-like BC tumourigenesis, and determine main proteins involved in BC prognosis and identify potential therapy targets. mRNA expression of genes involved in the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and the heat shock response (HSR) pathways were found to be most associated with TNBC subtype, and the mRNA expression of genes involved in these pathways were found to be correlated with tumour size, tumour grade and worse prognosis. In addition, the genes involved in these pathways were highly expressed in patient samples of basal-like or TNBC subtypes and positively correlated with mRNA gene expression of *TP53* and the proliferation marker *MKI-67*. Interestingly, the NF-κB pathway related genes' mRNA expression was highly correlated with *TP53* and *MKI-67* mRNA expression in the basal-like BC subtype. Therefore, MDA-MB-231 cell line was used in order to understand the involvement of the NF-κB pathway and HSR in TNBC.

In Chapter 2 of this thesis, the role of receptor interacting serine/threonine protein kinase 2 (RIP2) in enhancing BC development was studied. RIP2 protein has been reported to increase NF-κB activation and have a role in increasing cell proliferation and survival. It has been reported to associate with TNBC enhancing metastasis. Therefore, the role of RIP2 protein in TNBC was studied. An anti-apoptotic role of RIP2 in TNBC via activation of NF-κB was demonstrated. It is well known that NF-κB signaling mediates cancer cell proliferation and protects breast cancer cells from apoptosis. Therefore, targeting RIP2 could potentially sensitize cells to therapeutic agents. In this study, inactive mutant forms of RIP2 or shRNA against RIP2 sensitized TNBC cells to apoptosis. Anti-apoptotic proteins

expression levels, such as, B-cell lymphoma 2 (Bcl-2), B-cell lymphoma 2-extra large (Bcl-xL), and IAPs, increased upon RIP2 overexpression in MDA-MB-231 cells. This overexpression of anti-apoptitic proteins might explain the resistance of TNBC to drug induced cell death. Upon inhibition of NF-κB, the expression of anti-apoptotic proteins enhanced by RIP2 was reduced, and the cells were sensitized to drug treatment. RIP2 also mediated the release of cytokines and growth factors from these cells. Released factors include interleukin (IL) IL-6, IL-8, vascular endothelial growth factor (VEGF) and (C-X-C motif) ligand 1 (CXCL-1). These results were confirmed by data analysis of patients' breast cancer databases, where high RIP2 expression was associated with poor prognosis, and decreased survival of patients. RIP2 expression was also associated with TNBC subtype; in addition, it was correlated with high mRNA expression of Bcl-xL, X-linked inhibitor of apoptosis protein (XIAP) and cellular inhibitor of apoptosis protein (cIAP) anti-apoptotic proteins and IL-6, tumour growth factor (TGF) and VEGF released factors.

The last section of the thesis focused on the role of intracellular chaperone heat shock proteins (HSPs), HSPB1 and HSPA1in TNBC pro-survival signaling. HSPB1 belongs to the family of small HSPs and is a potent regulator of apoptosis. However, the role of HSPB1 in endoplasmic reticulum (ER) stress induced apoptosis has not been defined. Previous work in the lab showed that heat shock could protect cells from ER stress induced cell death. Treating cells with ER stress inducing drugs did not affect HSPB1 or HSPA1 expression, and neither phosphorylation level of HSPB1 in different TNBC cell lines. Therefore, a crosstalk between ER stress and HSR pathways in TNBC was not observed. An interesting finding was the release of non-phosphorylated form on HSPB1 into culture media of MDA-MB-231 and MDA-MB-453 cells. Neutralizing HSPB1 by treating culture media with anti-HSPB1 antibody did not show any effect on cell survival, resistance to drug treatments or cell migration.

Chapter 1: Introduction

1.1. Breast Cancer

According to World Health Organization, cancer is one of the primary reasons of mortality and morbidity, accounting for almost 8,000,000 casualties in 2012 (World Cancer Report 2014). Breast cancer (BC) is of the most common forms of cancer in the United Kingdom with 51,000 cases detected in 2012, and it is estimated that 1 out of every 8 females would be diagnosed with BC (Cancer Research UK 2012). In Ireland, BC is the most common cancer form in females (30.2%) with approximately 3000 cases diagnosed per year (30% of all female cancers), with average 5-year survival rate of BC patients between 2008 and 2012 was 81.4% (www.ncri.ie).

1.1.1. BC Classification

BC is vastly heterogeneous originating from diverse cells types and cause distinctive clinical consequences. There are several ways to classify BC but the most recent classification is based on deoxyribonucleic acid (DNA) microarrays that distinguish gene-expression profiles (Kwei et al., 2010, Hennessy et al., 2009, Sorlie et al., 2003). Accordingly, five distinct molecular subtypes of BC have been recognized, the luminal (Lum) subtypes (Lum A and Lum B) that express normal breast luminal epithelium markers; the HER-2 subtype, identified by overexpression of human epidermal growth factor receptor 2 (HER-2); the basal-like subtype which lacks any differentiation markers (Weigelt et al., 2010) and the normal-like breast cancer subtype (Perou et al., 2000; Sorlie et al., 2001). BC is also classified based on histological properties, what determines tumour grade and type indicating the tumour growth pattern its phenotype (Weigelt et al., 2010, Elston and Ellis, 2002).

According to the Bloom-Richardson grade scale, grade 1 tumours are well differentiated resembling normal tissue and are associated with good prognosis; grade 2 tumours are associated with intermediate prognosis and grade 3 tumours are poorly differentiated and associated with worst prognosis (Bansal et al., 2014). Moreover, all BCs are now examined for the expression of the Estrogen Receptor (ER), Progesterone Receptor (PR) and HER-2 (Makki, 2015). This protein profiling allows identification and ultimate prognosis of BC and helps define optimum treatment. The ER-positive BC overexpresses ER and constitutes around 70% of patient incidents. ER is a transcription factor which is active by binding to estrogen hormone enhancing DNA replication and mitosis in breast cells (Makki, 2015). This increases mutation risk causing cell cycle disruption, in addition to suppression of apoptosis and DNA repair processes increasing tumourigenesis. Furthermore, estrogen metabolism increases the formation of genotoxic

side products causing DNA damage and mutation (Deroo and Korach, 2006). ER antagonists or aromatase inhibitors are used to treat ER-positive BC patients. Aromatase is , responsible for androgens to estrogen conversion (Osborne, 1999, Normanno et al., 2005). PR is an intracellular receptor activated by binding to the steroid hormone progesterone. It is expressed in reproductive tissues, and is essential for females' reproductive cycle and pregnancy (Gadkar et al., 2002). About 65% of ER-positive BCs are also PR-positive, while 5% of BCs are PR-positive only (Horwitz and McGuire, 1978). Co-regulators of PR modulate its function either by enhancing or suppressing its transcription; hence, any mutation or aberrant expression of the co-regulators would affect normal PR function and disrupt normal mammary gland development leading to progression of BC (Gao and Nawaz, 2002). Finally, HER-2, also termed as ERBB-2, is a proto-oncogene and a member of the human epidermal growth factor receptor (HER/EGFR/ERBB) family. HER-2 membrane receptor is involved in transduction pathways enhancing cellular growth and differentiation (Bublil and Yarden, 2007). Increased HER-2 expression is observed in 30% of breast and reproductive tissue cancers and cancer pathogenesis (Quenel et al., 1995, Iqbal and Iqbal, 2014, Zhou and Hung, 2003). High expression of HER-2 associates with BC increased relapse and poorer prognosis (Ellsworth et al., 2008). So far, there is no worldwide-acknowledged definition for basal-like BC. Microarray-based profiling and immunohistochemical markers are being used to identify this subtype (Aas et al., 1996). The marker panels used to identify basal-like BC include lack of estrogen and progesterone hormone receptors and HER-2 what defines the triple-negative profile. In addition, high expression of genes is normally found in basal-like such as high weight cyto-keratins (Lehmann et al., 2011). Patients presenting with basal-like BC display typical features such as decreased response to therapy, specific recurrence sites and clinical outcome (Badve et al., 2011), in addition to high histological grade, and exceptionally high proliferation index. It accounts for up to 15% of all breast cancers, affect younger patients and often present as intervals (Badve et al., 2011).

These intrinsic subtypes have been also reported by several other studies with different genes included in the subtypes' signatures. Tissue microarray (TMA) technology also supported the validation of genes' signatures and revealed large panel of well-characterized commercially available biomarkers' groups (Abd El-Rehim et al., 2005). They constitute of two large subtypes with luminal phenotype, hormone receptors expression, HER2 over-expression, and absence of basal features. One subgroup was described by having high HER2 expression and lacking hormone receptors, but vary by MUC1 and E-cadherin expression; and another group was identified by strong basal epithelial marker expression, absence of hormone receptors expression and no luminal epithelial or

cytokeratin expression (Abd El-Rehim et al., 2005). These subtypes are similar to the intrinsic subtypes, what further confirms the heterogeneity of breast cancer, and validates the clinical relevance of the intrinsic subtypes (Abd El-Rehim et al., 2005). Furthermore, a signature containing 306 genes was determined which distinguishes the different breast cancer subtypes with differences observed on relapse-free and overall survival (Hu et al., 2006). Moreover, Parker et al reported PAM50 a 50-gene classifier that can be widely applied in clinical setting (Ades et al., 2014). PAM50 consists of hormone receptor and proliferation related genes, in addition to genes displaying myo-epithelial and basal characters (Gnant et al., 2014, Dowsett et al., 2013, Parker et al., 2009).

Though Sorlie's subtyping had been the standard for breast cancer classification, others also exist. For example, a signature of 706 cDNA probe elements which contains 3 luminal-like, 1 HER2-like and 2 basal-like subtypes was described (Sotiriou et al.; 2003). Moreover, Fan et al. described a signature of 70 genes that classify breast cancers into 4 groups only without identifying the normal-like subtype (Fan et al., 2006). Furthermore, Lehmann et al. divided TNBC into 6 subtypes, two basal-like (BL1 and BL2), one immunomodulatory (IM), one mesenchymal (M), one mesenchymal stem-like (MSL) and one luminal androgen receptor (LAR) (Lehmann et al. 2011). Briefly, the BL1 subtype is enriched with cell cycle and cell division components, inferring a potential response to anti-mitotic drugs, while the BL2 subtype displays unique genes involving growth factor signaling with basal and myo-epithelial characters; the IM subtype is enriched with genes involved in the cancer immune-profile; the M subtype displays a variety of unique genes involved in cell motility, ECM receptor interaction and cell differentiation. Moreover, the MSL subtype is similar to the M subtype and contains additional genes involved in growth factor signaling and low expression of claudin 3, 4, and 7. Finally, the LAR subtype is ER negative but displays luminal gene expression patterns (Lehmann et al. 2011).

1.1.2. Basal-like and Triple Negative Breast Cancer (TNBC)

According to gene expression arrays, most of basal-like BC samples have negative or reduced levels of ER, PR and HER-2 proteins and hence known as TNBC, and these two terms have often been used interchangeably (Rhee et al., 2008, Yadav et al., 2015, Anders and Carey, 2008). Similar to basal-like BC, TNBC comprises 10 to 15% of BC incidence and is highly hostile with worst prognosis (Stockmans et al., 2008, Rhee et al., 2008). They also associate with high grade tumour and nodal metastasis (Rhee et al., 2008). TNBCs are resistant to many typical BC drugs and have no specific recommended systemic regimen of treatment, hence the only current option is an aggressive cytotoxic chemotherapy (Foulkes et al., 2010). TNBC respond best to a combination therapy consisting of anthracycline and

taxane based chemotherapeutics, like doxorubicin, taxol, 5-fluorouracil (5-FU) and cyclophosphamide (Rouzier et al., 2005). Responses to such chemotherapeutic regimen are observed, but often relapse and drug resistance are observed (De Laurentiis et al., 2010, Arslan et al., 2009). Moreover, in the Cancer Genome Atlas project, patients with TNBC tumours displaying BL1, IM and MSL subtypes had an overall survival and disease-free survival double than that of patients with BL2, LAR, and M tumors, with IM patients having the best outcome (Mayer et al., 2014). In addition, a retrospective analysis of 130 TNBC cases treated with neoadjuvant doxorubicin, cyclophosphamide and paclitaxel, showed an overall pathologic complete response (pCR) rate of 28%; however, the BL1 subtype achieved the highest pCR rate at 52%, while the BL2, LAR, and MSL subtypes showed the lowest response of 0%, 10%, 23%, respectively (Masuda et al., 2013). Therefore, understanding the main mechanisms and factors underlying basal-like and TNBC development and its progression might help establish targets for more potent and selective therapies.

1.1.3. Breast Cancer Microenvironment

In response to stressful microenvironment such as chronic hypoxia, increased ROS and persistent inflammation, cancer cells develop. Simultaneously, these developing cells would further change the intracellular mechanisms and the surrounding micro-environment driving them to become increasingly more malignant, forming a vicious cycle (Bianchini et al., 2016). Consequently, this different microenvironment would induce further modifications in the cellular metabolism. Other changes, like hypoxia-induced angiogenesis, might lead to increased ROS level in neoplastic cells leading also to microenvironment changes, such as the composition of the cellular matrix and stromal cells. As a result, cells would further respond to these changes by modifying various metabolic mechanisms, hence, leading to more altered microenvironments. Therefore, the active interchange and co-adaptation between the tumour cells and the surrounding environment determine the interactions from the homeostatic to the more antagonistic and stressful status (Bianchini et al., 2016).

During the described alteration processes, various immune cells are also recruited from circulation, leading to further changes in the local stromal cell population and enabling immune response. However, diverse stromal cells and immune responses would be obtained in response to cancer initiation and development. Therefore, knowing the composition of the different immune and stromal cells within a tumour tissue, can reveal the main facilitators in cancer development, and may describe the primary cancer type. Transcriptomic data analyses can be used to estimate the relative subpopulation sizes of

different stromal cells and how the subpopulation sizes change as cancer advances. The tumour microenvironment might constitute of non-cancerous cells like fibroblasts, immune and epithelial cells (Weitzenfeld et al., 2016). Research studying the role of cellular microenvironment in tumourigenesis has increased considerably in the past years.

In addition to the presence of non-cancerous and immune cells in the tumour microenvironment, immune responses are essential during cancer developmental phases, namely initiation, progression and metastasis, and the release of specific proteins and released factors that could support cancer growth and metastasis (Weitzenfeld et al., 2016). However, immune responses play significant roles to cancer development. Specifically, immune surveillance and immune-editing are essential for detecting and inhibiting cancer development; however, chronic inflammation and the interaction between cancer and macrophages might be required for cancer development (Condeelis and Pollard 2006; Mantovani et al. 2006a).

In addition, several genes are important for the activity of tumor-associated macrophages (TAMs) and T-cells. For example, IL4 is an immune-cell gene known to stimulate differentiation of helper T-cells to type-2 helper T-cells (Th2) and activate macrophage M1 cells to become M2 cells (Sokol et al. 2008; Ho and Sly 2009; Martinez et al. 2013). Moreover, the combined signals of IL4 from T-cells and CSF1 from cancer cells stimulates TAMs to produce and release epidermal growth factor (EGF). CD4 (cluster of differentiation 4) is an immune cell-derived glycoprotein found on the surface of T helper cells and macrophages. CD8 and GZMA are both marker genes of cytotoxic T-cells, and KIR is a marker gene of natural killer cells. Moreover, epithelial cells induce the expression of myofibroblast markers, and increase the expression of chemokines, such as (C-X-C motif) ligand (CXCL) 12 or 14, which is important for cancer growth, invasion and metastasis (Natrajan et al., 2016, Makki, 2015). In addition, interleukin (IL)-6, IL-8, tumour necrosis factor (TNF), angiotensins, VEGF and CSF are overexpressed in BC (Kim et al., 2009, Goldberg and Schwertfeger, 2010, Weitzenfeld et al., 2016).

1.1.4. Genetics of cancer

Drug resistance is a major feature that limits the successful treatment of BC and occurs when tumours become insensitive to drugs with different mechanisms of action. It can occur by inhibiting interaction between the drug and its target, or preventing drug related signaling events that lead to cell death. Several genes, including p53, and proteins confer resistance in BC patients, and are relevant for clinical prognosis (Hanahan and Weinberg, 2011). The emergence of rapid deep sequencing technology has provided an

unprecedented opportunity to sequence large numbers of cancers for comparison with DNA sequences obtained from normal controls. In an interesting twist of fate, DNA sequencing is no longer the rate-limiting step in cancer genomics. Rather, it is the ability to analyze the copious amounts of data that are forthcoming from many laboratories and factory-like sequencing centers. From this perspective, the timing is good for bioinformaticians to enter cancer research with the possibility of adding substantively to our knowledge relating genomic changes to phenotypic changes in cancer patients.

Cancer genome sequencing provides considerable information on mutations and genomic changes existing in most cancers (Stratton et al. 2009; Pleasance et al. 2010; Garraway and Lander 2013; Alexandrov et al.m 2014; The Cancer Genome Atlas Research Network 2012, 2014; Alexandrov et al. 2013; Kandoth et al. 2013; Vogelstein et al. 2013). In a study of 3,281 tumors from 12 different types of cancer, 617,354 somatic mutations were identified including 398,750 missense, 145,488 silent and few other types such as nonsense, shift insertions or deletions (Kandoth et al. 2013). Interestingly, many mutations were identified in sequences that encode major pathways related genes such as transcription factors, cell cycle regulators, receptor tyrosine kinase, *MAPK, PI3K, TGFβ, WNT/β-catenin*, and *p53* gene which was the most frequently mutated (Kandoth et al. 2013). BC patient samples with high p53 levels show reduced survival rate, and almost 50% of BCs that express mutant *p53* form are associated with weak prognosis (Alsner et al., 2008, Aas et al., 1996, Wilson et al., 1997). In addition, *TP53* gene mutations or high p53 expression is reported in almost 85% of basal-like or TNBC incidents (Lacroix et al., 2006). Most *TP53* mutations occur in the DNA binding domain, which prevents p53 from inducing target genes transcription (Brady and Attardi, 2010). Upon stress, p53 regulates apoptosis through promoting mitochondrial outermembrane permeabilisation (MOMP) via interacting with the protective B-cell lymphoma 2- extra large (Bcl-xL), and B-cell lymphoma 2 (Bcl-2) proteins, releasing cytochrome c (Brady and Attardi, 2010, Mihara et al., 2003). Moreover, p53 is activated by cellular damage inducing the expression of genes important in cell cycle arrest and DNA repair to save cells, or genes associated with intrinsic apoptosis causing cell death (Brady and Attardi, 2010). Thus, mutant p53 can reduce tumour drug sensitivity in comparison to tumours with wild-type p53 (Longley et al., 2006). On the other hand, genome sequencing has also provided some observations that confirm cancer as a diverse and complex disease, and not only driven by mutations in the genome, and importantly the role of non-genomic changes that also contribute to tumorgenesis (Mack et al. 2014; Parker et al. 2014).

1.2. *Receptor-Interacting Serine-Threonine Kinase 2*

1.2.1. Receptor-interacting protein kinase (RIP) family

Receptor-interacting protein kinase (RIP) is a family of serine/threonine kinases. Figure 1.1 presents the 7 RIP protein members RIP1 to RIP7 (Zhang et al., 2010). RIPs have substantial role in inflammation and immunity (Zhang et al., 2010, Humphries et al., 2015). RIP1 is made up of a C-terminal death domain (DD) responsible for RIP1 recruitment to complexes inducing distinctive pathways. RIP2 is the only member having a CARD, caspase-activation and recruitment domain (Jacquet et al., 2008). RIP3 is made up of C-terminus-RIP-homotypic interaction motif (RHIM) (Son et al., 2008) also found in RIP1 and facilitates interaction between these two proteins. RIP4 and RIP5 are made of C-terminus ankyrin repeats, while RIP6 and RIP7 have leucine-rich repeats (LRR) important for pathogen recognition, stress or damage associated responses (Figure 1.1). RIP kinases and their C-terminal domains are important for relative RIP functions (Zhang et al., 2010). RIP1 facilitates various cellular mechanisms resulting in mitogen activated protein kinases (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) induction and cellular death. RIP2 is vital for activation of nucleotide-binding oligomerization domain (NOD) pathways triggering MAPKs and NF-κB. RIP3 is essential in necroptosis, and RIP4 takes part in NF-κB and MAPK induction (Figure 1.2).

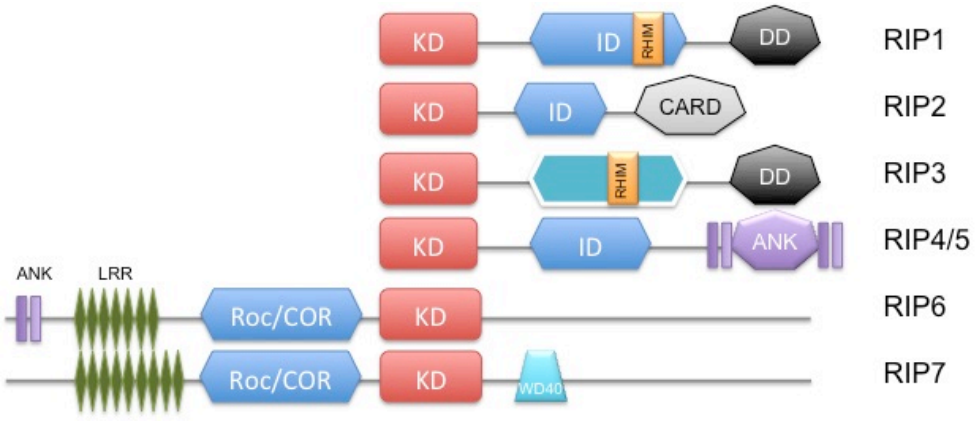


Figure 1.1: Domain structure of RIP proteins

All RIP proteins contain the kinase domain (KD). RIP1 and RIP3 contain C-terminal death and RHIM domains, RIP2 has a kinase domain and a CARD. RIP4, RIP5 and RIP6 have C-terminal ankyrin repeats (ANK). RIP6 and RIP7 have leucine-rich repeats (LRR) as well C-terminal Roc/COR domains. RIP7 contains a C-terminus WD40 motif.

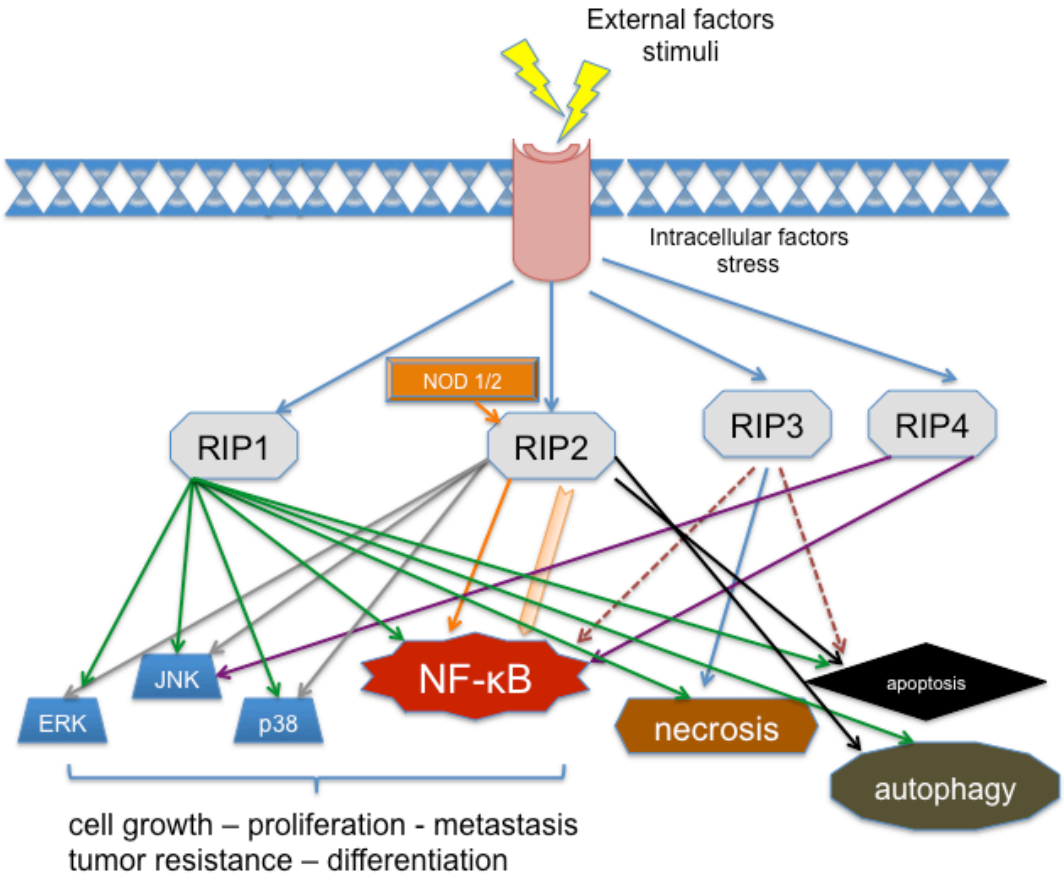


Figure 1.2 RIP family members' role in diverse cellular mechanisms.

All family members can induce NF-κB. RIP1 and RIP2 induce ERK, JNK, p38, and apoptosis. Additionally, RIP1 induces necroptosis and autophagy. RIP3 induces NF-κB and apoptosis, and RIP4 induces JNK.

1.2.2. RIP2

In 1998, different research groups identified RIP2 as a new protein involved in inflammatory responses and immunity and enhancer of NF-κB activity (McCarthy et al., 1998, Inohara et al., 1998, Thome et al., 1998). RIP2 is also known as CARD-like-apoptosis-regulatory protein kinase (CARDIAK) or RIP-like-interacting-caspase-like apoptosis-regulatory protein kinase (Thome et al., 1998). Like other RIP kinases, RIP2 activates various essential cellular mechanisms (Figure 1.2) (Zhang et al., 2010).

High RIP2 expression induces NF-κB, MAPK, and c-Jun N-terminal kinase (JNK) (Krieg et al., 2009, Navas et al., 1999, Thome et al., 1998). In addition, it has a major role in downstream signaling of cytosolic nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are intracellular sensors of pathogen-associated molecular patterns (PAMPs), and respond to bacterial peptidoglycan fragments (DAP) and muramyl dipeptide (MDP) (Girardin et al., 2003a, Girardin et al., 2003b, Inohara et al., 1998). NOD1 recognizes

meso-diaminopimelic acid (meso-DAP)-containing PGN fragments, while NOD2 senses MDP, which is found in the peptidoglycan (PGN) of nearly all Gram-positive and Gram-negative organisms. Stimulation of NOD receptors enhances the release of pro-inflammatory cytokines, for example, TNF, IL-1β and IL-6 are secreted (Brooks et al., 2011, Fritz and Gommerman, 2011). In addition, this facilitates NOD and RIP2 CARD-CARD interaction leading to RIP2 auto-phosphorylation and hence recruitment of ubiquitin (Ub) ligases such as inhibitor of apoptosis proteins (cIAP1, cIAP2, XIAP) and Pellino E3 Ubiquitin Protein Ligase Family Member 3 (Pellino3). As a result, K63-linked ubiquitin chains are added at Lysine 209, and linear ubiquitin chain assembly complex (LUBAC) to the RIP2 kinase domain (Bertrand et al., 2009, Damgaard et al., 2012). Ubiquitination induces proximity between RIP2 and TAK binding proteins (TAB2, TAB3), TGF-β-activated kinase (TAK1) as well as kappa-B (Konishi et al., 1997) inhibitor complexes comprising NEMO, IKK-alpha (α) and IKK-beta (β). Association of these complexes leads activates TAK1, which in turn induces MAP kinases activity and consequent induction of activator protein 1 (AP-1); in addition, IKK-β is activated and NF-κB is induced leading to release of pro-inflammatory cytokines (Figure 1.3) (Hasegawa et al., 2008). Moreover, the NOD/RIP2 pathway alone is sufficient to induce the production of the IFN response when stimulated with a potent form of MDP.

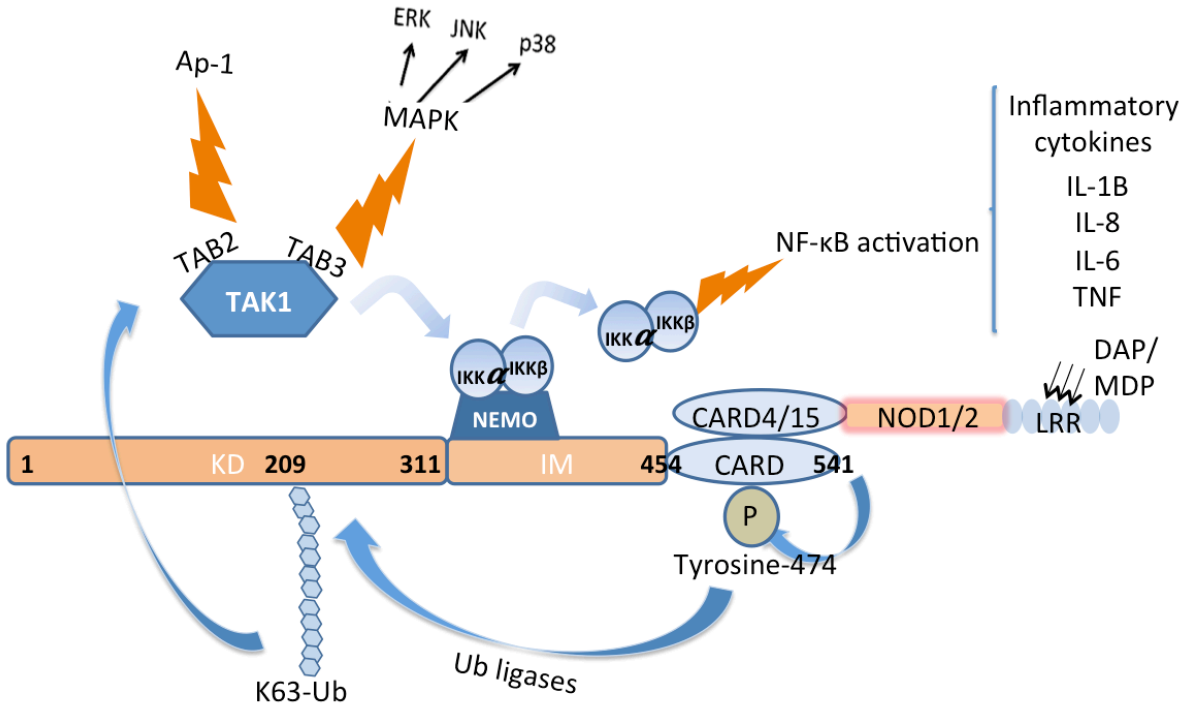


Figure 1.3 RIP2 activation and signaling

RIP2-CARD interaction with NOD-CARD domain leads to RIP2 auto-phosphorylation, which in turn causes Ub ligases recruitment and K-63 linked ubiquitination of RIP2, inducing proximity between RIP2 and TAB/TAK1 activating the MAPK pathway, and with IKK-β activating NF-κB leading to transcription of pro-inflammatory cytokines.

1.2.3. RIP2 activity

RIP2 was first recognized as a serine/threonine kinase (McCarthy et al., 1998, Thome et al., 1998), and was shown to auto-phosphorylate its Tyrosine-474 residue upon NOD signaling and CARD-CARD interaction leading to NF-κB activation (Tigno-Aranjuez et al., 2010). Moreover, RIP2 construct lacking CARD domain, has a Serine-176 auto-phosphorylation site that regulates RIP2 activity (Dorsch et al., 2006).

Several findings have reported that RIP2 protein expression and stability are dependent on RIP2 kinase activity, which is crucial to sustain RIP2 basal-like levels (Nembrini et al., 2009, Windheim et al., 2007). Mutation of an essential lysine residue (K47) in the adenosine triphosphate (ATP) binding site and two-conserved aspartic acid residues of RIP2 disables its kinase activity (McCarthy et al., 1998, Thome et al., 1998, Windheim et al., 2007) decreasing RIP2 expression levels (Hasegawa et al., 2008, Lu et al., 2005, Nembrini et al., 2009, Windheim et al., 2007). Inhibiting RIP2 kinase activity by SB203508 kinase inhibitor decreases protein stability (Windheim et al., 2007); in addition, the expression of kinase inactive RIP2 in murine bone marrow derived macrophages have results in less RIP2 levels in comparison to wild-type. However, messenger ribonucleic acid (mRNA) RIP2 transcript levels were unaffected (Lu et al., 2005, Nembrini et al., 2009). This further supports kinase function significance in RIP2 stability.

Additionally, CARD is another main domain essential in mediating RIP2 binding to activate signaling. Thome et al. (1998) identified potential CARD-containing proteins that might bind to RIP2, including cellular inhibitor of apoptosis proteins (c-IAP1 and c-IAP2) that form part of the tumour necrosis factor receptor (TNFR) signaling complex. Moreover, CARD facilitates solid interaction between RIP2 and caspase-1 independent of RIP2 kinase activity (Thome et al., 1998). Another study reported that dissociated Schwann cells highly express RIP2 which is associated with the nerve growth factor (NGF) receptor ($p75^{NTR}$). CARD domain binds to $p75^{NTR}$ and is essential to mediate NGF dependent NF-κB signaling enhancing survival in Schwann cells (Khursigara et al., 2001).

1.2.4. RIP2 downstream signaling

There is inconsistency in studies reporting the role of RIP2 kinase activity in downstream signaling. Initially, RIP2 kinase activity was shown not to be essential in activating NF-κB and JNK since these pathways are similarly activated by wild-type and kinase lacking RIP2 (Eickhoff et al., 2004, Thome et al., 1998). Moreover, other studies also reported that RIP2 kinase dead mutants expressed in HEK293 cells stimulated NF-κB and JNK activation more than the wild-type form (Eickhoff et al., 2004, Windheim et al., 2007). Besides, RIP2 over-expression highly provoked MCF-7 cell death independent of the kinase activity (McCarthy et al., 1998). Conversely, several findings indicate that RIP2 kinase activity is essential for NF-κB activation and inflammatory reactions and that kinase mutant RIP2 forms significantly lowered NF-κB activity (Ogura et al., 2001). Reduced NF-κB activation might be caused RIP2 instability which decreased upon the absence of its kinase activity, rather than being directly affected by RIP2 kinase activity (Hasegawa et al., 2008). Tigno-Aranjuez *et al*. (2010) also showed that HEK293 cells transfected with a kinase dead RIP2 mutant showed significant reduction in NOD2-dependent NF-κB activation and cytokine response (Tigno-Aranjuez et al., 2010).

Moreover, upon NOD stimulation, impaired inflammatory responses were observed in mice expressing RIP2-K47A mutant and inhibiting RIP2 kinase considerably decreased MDP induced NF-κB activation (Nembrini et al., 2009, Windheim et al., 2007). RIP2 kinase activity also inhibited apoptosis in B-lymphocyte cell line, and was shown to be involved in B-cell CLL/Lymphoma 10 (Bcl-10) phosphorylation which functions as upstream regulator in NF-κB signaling (Ruefli-Brasse et al., 2004).

1.3. NF-κB transcription factor

1.3.1. The NF-κB pathway

NF-κB is a nuclear factor that binds kappa light chain enhancer in B-cells (Sen and Baltimore, 1986). It has been considered one of the main inducible transcription factors essential in regulating and inducing immune reactions (Hayden and Ghosh, 2012).

NF-κB pathway is an ancient and well-conserved pathway in many organisms such as xenopus (Kao and Hopwood, 1991), zebra fish (Correa et al., 2004), and a homologue of the NF-κB activating protein (NKAP) has been identified in *Caenorhabditis elegans* (Chen et al., 2003). Mammalian NF-κB family constitutes of 5 members known as *Rel*A (p65), *Rel*B, c-*Rel*, NF-κB1 (p105/p50) and NF-κB2 (p100/p52) that can provoke or inhibit specific genes

expression through DNA binding (Perkins, 2007). Usually, NF-κB is an inactive cytosolic complex bound to kappa B inhibitors (IκB). Upon stimulation by several factors and stimuli such as bacterial products (LPS), viral proteins, inflammatory cytokines, cell stress or DNA damage, the IκB kinase phosphorylates the IκB complex degrading it and exposing the nuclear localization signal (NLS) of the NF-κB dimer leading to its activation. NF-κB usually refers to the *Rel*A/p50 hetero-dimer since it is the most commonly observed complex and is responsible for most defined NF-κB transactivation activity (Karin et al., 2002). *Rel*-B mainly binds p100 before being processed and translocated to the nucleus (Karin et al., 2002). However, association between *Rel*-B and c-*Rel* is rare and hence it does not bind to DNA as *Rel*-A/*Rel*-B heterodimers or *Rel*-B homo-dimers (Joyce et al., 2001, Karin et al., 2002). Other heterodimers exist but are less abundant and respond slowly and more research is required in order to reveal the role of different NF-κB complexes (Karin et al., 2002).

The NF-κB family regulates genes involved in several cellular processes, including immunity and inflammation, cell death, stress pathways, cellular interactions and division (Perkins, 2004). More than 200 factors that induce and regulate NF-κB are reported (Mohamed and McFadden, 2009). Constitutive activation or loss of expression of one or several κB proteins can drive multiple diseases such as cancer, diabetes, muscular and neurodegenerative diseases (Kumar et al., 2004). In the literature, dysfunctional NF-κB activity is described to be associated with cancer as will be discussed in Section 1.3.6.

1.3.2. The inhibitors of κB family (IκB)

The NF-κB inhibitor family, IκB, including IκB-alpha (α), IκB-beta (β), IκB-gamma (δ), IκB-epsilon (ε) and Bcl-3, interact with NF-κB DNA binding domain inhibiting its action (Perkins, 2007, Joyce et al., 2001). IκBδ splice variant, specific to lymphoid cells, encodes only the p105 C-terminus and is able to interact with p105/p50 and p100/p52 dimers only, while IκBε is specific to *Rel*-A and c-*Rel* complexes (Whiteside et al., 1997). Bcl-3 is a co-activator of p50 and p52 homodimers (Joyce et al., 2001). IκB proteins can act independent of NF-κB induction; however, they are expressed by NF-κB negative-feedback loop (Klement et al., 1996). Upon induction, IκBα, IκBβ and IκBδ get phosphorylated at the conserved serine residues causing K48-linked ubiquitination leading to proteasomal degradation. However, for IκBα, in response to the re-oxygenation of hypoxic cells, tyrosine Y42 phosphorylation leads to the dissociation of IκBα from NF-κB and thus its activation (Perkins et al., 1997).

IκB proteins are phosphorylated by the IκB kinase (Konishi et al., 1997) complex what releases NF-κB dimers. IKKs contain two catalytic subunits alpha (α) and beta (β), in

addition to NF-κB essential modulator (Tang et al., 2001). IKKα is essential in activating the non-canonical NF-κB pathway, while IKKβ activates the canonical pathway discussed in Section 1.3.3 (Perkins, 2007). IKKβ induces anti-apoptotic and proliferative mechanisms leading to cell survival and proliferation (Tang et al., 2001, Widera et al., 2006a, Widera et al., 2006b). IKKβ can also promote cell growth by phosphorylation and inhibition of the tumour suppressor FOXO3 thus inhibiting apoptosis leading to tumourigenesis and enhancing chemoresistance (Hu and Hung, 2005, Tezil et al., 2012). IKKβ can also induce proteolytic degradation of p105 activating the MAP kinase (MAPK) pathway and promoting the cell proliferation (Perkins, 2007).

1.3.3. Canonical NF-κB pathway

Upon stimulation, IκBα is degraded releasing *Rel*-A/p50 or c-*Rel*/p50 heterodimers, that are translocated to the nucleus regulating gene transcription (Perkins, 2007) (Figure 1.4). Pro-inflammatory factors stimulate the IKK complex phosphorylating IκBα causing its polyubiquitination and proteasomal degradation (Karin et al., 2002, Perkins, 2006, Perkins and Gilmore, 2006). Consequently, fast and temporary response to diverse stimuli occur which is is fundamental in lymphocytes survival and proliferation (Sun, 2011, Jost and Ruland, 2007).

Canonical NF-κB activation is primarily dependent on IKKβ (Senftleben et al., 2001). It provokes a negative feedback loop leading to transcription and synthesis of target genes that inhibit NF-κB like IκBα and A20 deubiquitinase by binding to NF-κB dimers and causing their translocation to the cytoplasm in an inactive dimer form (Perkins and Gilmore, 2006). Also, A20 reduces IKK activity through interacting with TNF receptor associated factor (TRAF) 6 and RIP1, inactivating IKK complexes and consequently hindering IκB degradation, inactivating NF-κB (Krikos et al., 1992, Shembade and Harhaj, 2012).

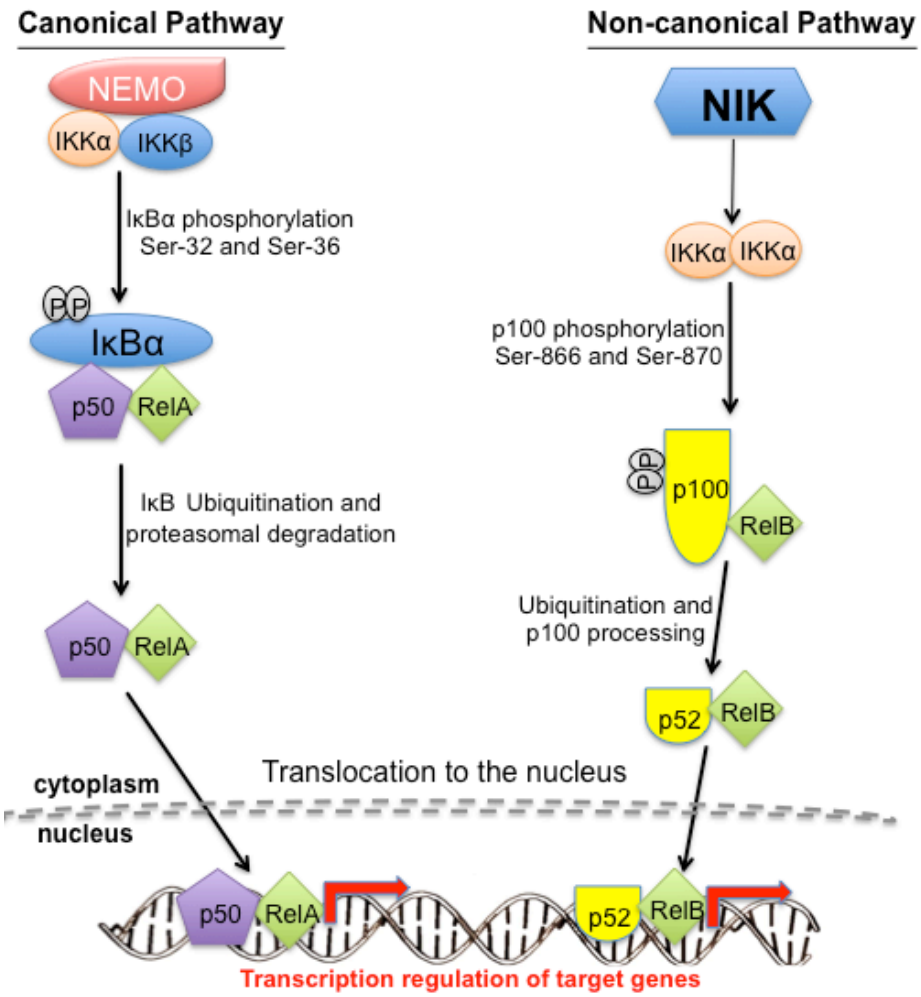


Figure 1.4: Canonical and non-canonical NF-κB activation pathways.

Upon stimulation of the canonical pathway, the IKK-NEMO complex phosphorylates IκBα on Serine-32 and Serine-36 leading to ubiquitination. Subsequent proteasomal degradation of IκBα releasing Rel-A-p50 dimers occurs and then translocates to the nucleus regulating target gene transcription. In the non-canonical pathway, NIK phosphorylates a IKKα complex upon specific stimulation which then phosphorylates p100 on Serine-866 and Serine-870. Then ubiquitination and processing of p100 into p52 occurs and a dimer with Rel-B and translocates to the nucleus regulating the transcription of target gene.

1.3.4. Non-canonical NF-κB pathway

The non-canonical or alternative pathway leads to processing of p100 to p52 and p52/*Rel*B heterodimers release (Figure 1.4). The IKK complexes recruited and the signaling mechanisms involved are distinct from those of the classical pathway (Perkins and Gilmore, 2006, Sun, 2011).

Several stimuli induce the non-canonical pathway, among which are the CD40 ligand (Coope et al., 2002), LPS (Mordmuller et al., 2003), viral proteins (Atkinson et al., 2003) and the transcription factor Stat-3 (Nadiminty et al., 2006). Upon stimulation, (NF-κB inducing kinase) NIK phosphorylates IKKα complexes on Serine-177 and Serine-188, and becomes

activated leading to p100 processing to p52 active form. p100 processing inhibits the IκB-like activity of p100, leading to the translocation of *Rel*-B/p52 complexes to the nucleus enhancing specific gene transcription and protein expression (Sun, 2011, Karin et al., 2002, Senftleben et al., 2001).

The TNFR superfamily members also stimulate the non-canonical pathway. They are death receptors that bind to cytokines inducing downstream signals and various responses such as cell survival, cell proliferation or apoptosis (Sun, 2011). NIK level crucially regulates the non-canonical pathway. TRAF3 maintains low NIK levels in most cells, hence de-novo synthesis of the protein is stimulated in order to increase its level (Sun, 2011). The non-canonical pathway stimulation is slow and leads to long-term and specific response based on the stimuli nature (Coope et al., 2002, Novack et al., 2003).

Similar to the canonical pathway, the non-canonical NF-κB pathway can be aberrantly activated by mutations or constantly active subunits leading to progression of various tumours. NF-κB2 is intensely associated with lymphomas, due to chromosomal translocation producing a constitutively active protein form (Karin et al., 2002).

1.3.5. Other NF-κB activation pathways

In addition to the NF-κB pathways described above, other mechanisms that are slower and with less efficiency have been reported (Rothwarf and Karin, 1999). Upon exposure to certain stimuli such as genotoxic stress, ultraviolet (UV) or hypoxia, these alternative pathways can be activated in an IKK dependent or independent manner and usually lead to activation of the canonical NF-κB complex (Perkins and Gilmore, 2006). In the IKK dependent mechanisms and upon cells exposure to genotoxic drugs, such as etoposide or ionizing radiation, only NEMO will be translocated into the nucleus where it gets sumoylated, i.e conjugated to a small ubiquitin-related protein-1. Then, mono-ubiquitination will take place at the same site in an ATM (ataxia telangiectasia mutated) dependent manner replacing the sumoylation, and gets directed back to the cytoplasm to activate IKK complexes (Perkins, 2007). Several mechanisms activate the alternative IKK-independent NF-κB, including UV stimulation or HER-2 oncogene expression. This leads to phosphorylation of the IκBα C-terminus, and hence its degradation and subsequent NF-κB induction (Perkins, 2007). Upon exposure to UV, CK2 phosphorylation of IκBα is dependent on the p38-MAP kinase and inhibition of this pathway will further induce UV-mediated cell death (Kato et al., 2003). HER-2 oncogene is overexpressed in 30% of BCs leading in PI3K-Akt dependent CK2 activation, phosphorylating IκBα what causes its degradation (Pianetti et al., 2001, Romieu-Mourez et al., 2002). This IKK-independent NF-κB pathway can be blocked by inhibition of HER-2 receptor or by activation of tumour suppressors (Pianetti et

al., 2001). IκBα phosphorylation can also take place on Tyr-42 by the c-Src kinase post hypoxia and H_2O_2 stimulation enhancing its degradation (Rothwarf and Karin, 1999). This c-Src dependent phosphorylation of IκBα was reported in bone marrow cells after TNFα stimulation, which activates the atypical NF-κB pathway rather than the non-canonical pathway (Abu-Amer et al., 1998). Moreover, delayed activation of NF-κB can occur in an IKK-independent manner upon drug treatment such as doxorubicin but the mechanism is still not known (Tergaonkar et al., 2003).

1.3.6. NF-κB target genes

Several target genes that are transcriptionally regulated by NF-κB activation have been described. These target genes have been categorized into different types including chemokines, cytokines, immune-receptors, acute phase proteins, stress response genes, cell surface receptors, regulators of apoptosis, growth factors, ligands and modulators, early gene responses, transcription factors and their regulators (Del Prete et al., 2011, Pacifico and Leonardi, 2006). Moreover, grouping the target genes based on function can give four main categories (Karin et al., 2002), including the cell proliferation genes, such as SKP2 or cyclin D1 (Schneider et al., 2006, Schumm et al., 2006), apoptosis regulatory genes such as Bcl-2 family (Chen et al., 1999), immune-regulatory genes such as TNFα and IL-6 (Libermann and Baltimore, 1990, Shakhov et al., 1990), and NF-κB regulatory genes such as *Rel-B*, IκB ∎ and A20 (Bren et al., 2001, Sun et al., 1993, Shembade and Harhaj, 2012). The interest of this project is in the apoptosis and immune-regulatory genes that are important for the development and proliferation of BC cells.

1.3.7. NF-κB in cancer

In addition to the role of NF-κB in infection and inflammation, it has also been reported as a pro-survival factor promoting tumourigenesis (Kawai and Akira, 2006). Irregular activation of NF-κB might be caused by stable activation of the IKK complex or defect in the negative feedback loop. It is also linked to cancer hallmarks promoting tumour growth and development (Hanahan and Weinberg, 2011, Perkins, 2012); however, the activation of NF-κB in cancer cells and the role or mutations of NF-κB subunits still requires to be further elucidated.

The link between NF-κB and cancer was first determined when v-*Rel* and c-*Rel* homologue found in the avian reticuloendothelial virus induced B-cells tumour in chicken (Gilmore, 1999). c-*Rel* seems to be the most oncogenic subunit, since its overexpression is enough to promote tumour formation, independent of any the other NF-κB subunits (Karin et al., 2002). It is overexpressed in lymphoma and almost half of B-cell non-Hodgkins' lymphomas, and associated with poor prognosis (Rayet and Gelinas, 1999). c-*Rel* mutations

are frequently observed in cancer and target IKK phosphorylation increasing c-*Rel*'s associated gene transformation (Perkins, 2012). A point mutation in *Rel*-A was described in multiple myeloma and was found to affect *Rel*-A/DNA-binding and transactivation activity, but the underlying mechanism is not known (Trecca et al., 1997). Other point mutations in *Rel*-A were identified in lung and ovarian cancer without any identified or known effect (Perkins, 2012). Similarly, *Rel*-B point mutations were found in ovarian cancer but their direct impact is still not known (Perkins, 2012). In addition, chromosomal translocation of the NF-κB2 gene locus was reported to cause C-terminal deletion of p100 hence constitutively processing p52 and inhibiting IκB-like function of p100 (Neri et al., 1991, Perkins, 2012, Xiao et al., 2001), and was found to be associated with the development of B and T cell lymphomas, chronic lymphocytic leukemia (CLL) and multiple myeloma (Trecca et al., 1997, Karin et al., 2002). Furthermore, in mice, C-terminal deletion of NF-κB2 gene results in abnormal p52 products increasing lymphocyte proliferation and gastrointestinal hyperplasia (Ishikawa et al., 1997). Moreover, NF-κB2 mutations were also reported in lung cancer but their significance is not yet understood although they are thought to induce p100 processing (Perkins, 2012).

NF-κB mutations in BC have not been widely reported but its increased activity is commonly described. *Rel*-A and p50 subunits are constitutively active in prostate cancers, and the p52 subunit is activated in almost 19% of prostate cancers although it is not clearly associated with any specific pathological feature (Seo et al., 2009). Moreover, NF-κB was found abnormally activated in BC (Sovak et al., 1997), leukemia and lymphoma (Karin et al., 2002). Continuous NF-κB activation leads to over-expression of target genes that might be involved in tumourigenesis, such as Cyclin-D1 protein which is over-expressed in up to 50% of human breast tumours, hence increasing its proliferation (Fu et al., 2004). *Cyclin-D1* is characterized as an NF-κB target gene, and in BC its regulation is mediated via the non-canonical pathway (Guttridge et al., 1999, Demicco et al., 2005). Furthermore, p52 and Bcl-3 are over-expressed in breast tumour tissues, and IKKα is also activated in mammary epithelium leading to p100 processing into p52 (Cogswell et al., 2000, Cao et al., 2001). Stimulation of NF-κB by several infectious agents induces the release of growth, survival and angiogenic factors; these factors are important regulators of cell cycle, anti-apoptotic and invasive protease genes' expression, what might enhance tumourigenesis (Karin, 2006).

1.4. Cell Death

Cell death is a basic developmental mechanism in multicellular organisms important for tissue maintenance (Lockshin and Zakeri, 2007). Several cell death modalities were defined based on the cell morphological, biochemical or immunological features (Galluzzi et al., 2007) including apoptosis, necrosis, autophagy, mitotic catastrophe, granzyme mediated, paraptosis and recently necroptosis; however, different forms of cell death have similar features and are difficult to be distinguished (Galluzzi et al., 2007, Galluzzi et al., 2012, Kroemer et al., 2009, Zeiss, 2003). The cell death process is determined by the kind, intensity or strength of the inducing stimulus, and its atypical regulation triggers several pathologies, especially cancer (Carson and Ribeiro, 1993, Favaloro et al., 2012). The cell death pathways can be defined into different categories according to morphological and biochemical characteristics (Hotchkiss et al., 2009; Kroemer et al., 2009; Klionsky et al., 2012).

Apoptosis and autophagic cell death occurs under normal physiological conditions and as part of growth and renewal of cells, and important for maintenance of cellular homeostasis. They are controlled processes, and initiated by a defined signaling cascade and end by cell engulfment by neighbouring phagocytes without initiating pro-inflammatory immune responses (Savill and Fadok, 2000). Apoptosis is described in details in Section 1.4.1. Cells undergoing autophagy are characterized by the presence of double-membrane organelles called autophagasomes containing partially digested organelles (Hotchkiss et al., 2009; Kroemer et al., 2009; Galluzzi et al., 2012). There are several forms of autophagy, macroautophagy, chaperone mediated autophagy (CMA), micro-autophagy, aggrephagy and chaperone-assisted selective autophagy. When a cell is under stress, as is the case when a cell is experiencing nutrient deprivation, autophagy is induced to meet the energy requirements of the cell and thus acts as a pro-survival mechanism. However, if this stress becomes prolonged or acute, autophagy becomes self-destructive and the cell undergoes autophagic cell death (Maiuri et al., 2007; Kroemer and Levine, 2008). The term autophagic cell death is debated in the field. Autophagic cell death can describe a cell which commits to death due to activation of the autophagic machinery and digestion of the cell contents, or the condition where autophagy is induced and is contributing to the cell death (Kroemer and Levine, 2008).

On the other hand, necrotic cell death mainly occurs upon severe injury, and results in cell lysis and leakage of cellular contents into the extracellular space causing an

inflammatory response (Lockshin and Zakeri, 2004). Necrosis features include swelling of cellular organelles, transparent cytoplasm, expansion of the nuclear membrane, irregular nuclear spots and swelled cell volume that disrupts the plasma cell membrane (Hotchkiss et al., 2009; Kroemer et al., 2009; Galluzzi et al., 2012). Necrosis was originally described as a passive cell death as a result of acute stress. It has been described as unregulated bursting of a cell triggering inflammation and an immunogenic response (Kaczmarek et al., 2013). necrosis and known as necroptosis. However, necroptosis challenges the understanding of necrosis since it is a controlled form of necrosis and requires the activation of a distinct signaling pathway (Vandenabeele et al., 2010). Other forms of cell death have also been described as being necrotic-like and apoptotic-like and are often a result of a disruption in cell death pathway (Leist and Jäättelä, 2001).

1.4.1. Apoptosis

Apoptosis is described as cell suicide mechanism that occurs in a sequential and programmed manner without affecting surrounding cells or tissue (Kerr et al., 1972). It was described as a vital genetically determined process triggering cell death at specific developmental stages, and a mean of abolishing defective or damaged cells driving cell suicide (Zaleske, 1985, Norbury and Hickson, 2001). Apoptotic cells are distinguished from healthy cells by a set of morphological features that can be detected using electron microscopy and have well-defined biochemical features and pathways that define controlled cell demolition (Kerr et al., 1972, Hengartner, 2000).

Apoptotic morphology is characterized by shrinking cells and condensing and fragmented genetic material with blebbing membrane as demonstrated in Figure 1.4. Membrane blebs appear like irregular cell membrane format and are due to phosphorylation of myosin regulatory light chain which affects its interaction with actin (Elmore, 2007). Other actin interacting proteins have been also implicated during apoptosis, such as fodrin, a cytoskeletal protein cleaved by aspartic proteases known as caspases and is followed by nuclear membrane disruption allowing diffusion of fragmented chromatin through the cytoplasm (Mills et al., 1998, Vanags et al., 1996). Finally, apoptotic bodies displaying membrane phosphatidylserine (PS) are formed (Descamps et al., 2001). PS induces phagocytosis limiting any immune response (Kurosaka and Kobayashi, 2003). In healthy cells, PS is held within the cell by aminophospholipid translocase and is inactivated upon apoptotic stimulus then flipped to the external part of the cell membrane (Bratton et al., 1997).

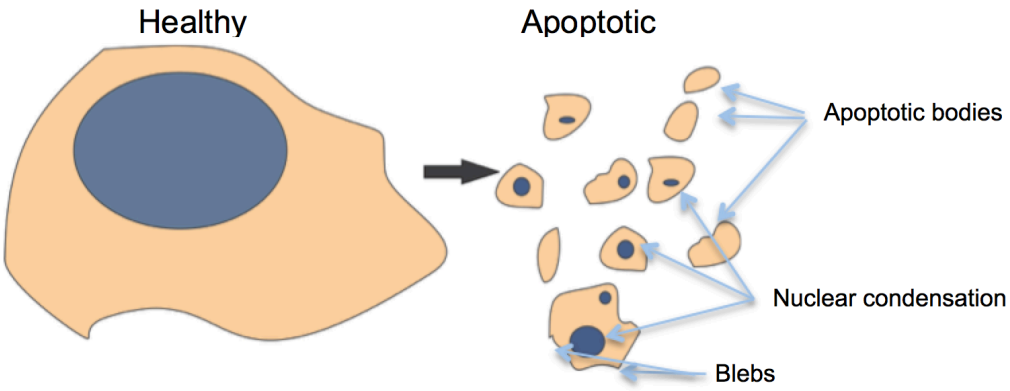


Figure 1.5: Apoptotic morphology

Apoptotic cells are characterised by shrinkage and condensation of genetic material. Breakdown of the cytoskeleton forms the membrane blebbing and apoptotic bodies.

1.4.2. Molecular basis of apoptosis

There are two main forms of apoptosis namely the intrinsic mitochondrial mediated or the extrinsic death receptor mediated apoptosis (Elmore, 2007). Two crucial protein groups are involved, the B-cell lymphoma-2 (Bcl-2) family essential for the intrinsic pathway, and cysteine-dependent aspartate-directed proteases (caspases) involved in intrinsic and extrinsic apoptosis (Cory and Adams, 2002, MacKenzie and Clark, 2012).

- ***The Bcl-2 family***

The Bcl-2 family is made up of four main Bcl-2 homology domains (BH1, 2, 3 and 4) with a transmembrane domain (Nakshatri et al., 1997, Chalfant et al., 2002). These proteins interact with one another, and are classified into groups based on function and/or domain as shown in Figure 1.6 (Lama and Sankararamakrishnan, 2008, Oltvai et al., 1993). The groups comprise the pro-survival Bcl-2 proteins that generally contain all 5 domains and include Bcl-2, Mcl-1, Bcl-xL, Bcl-w, Bcl-B and NRH/Nr-13, and the pro-apoptotic family members that generally lack one or more of the BH domains. The pro-apoptotic family is further sub-grouped into pro-apoptotic multi-domains such as BAX, BAK and BOK, and pro-apoptotic BH3 only proteins (BOP) like BAD, BIK, BIM, BMF, BNIP3, HRK, NOXA, PUMA, SPIKE and tBID (Leber et al., 2007). If the pro-apoptotic proteins dominate, BAX translocates to the exterior membrane of the mitochondria and homodimerises leading to membrane permeabilisation inducing apoptosis (Leber et al., 2007). Bcl-2 proteins roles can overlap and are regulated at multiple levels. According to the rheostat model, the balance between pro- and anti-apoptotic proteins determines cell fate; for example, higher levels of pro-apoptotic in comparison to anti-apoptotic proteins would direct the cell towards apoptotic cell

death or vice versa (Ploner et al., 2008). Another model, named the neutralization model, states that Bcl-2 pro-apoptotic proteins bind anti-apoptotic proteins and neutralize their effect, and the ratio between pro- and anti-apoptotic proteins affects the ability to neutralize and determines cell fate (Nickells, 2010).

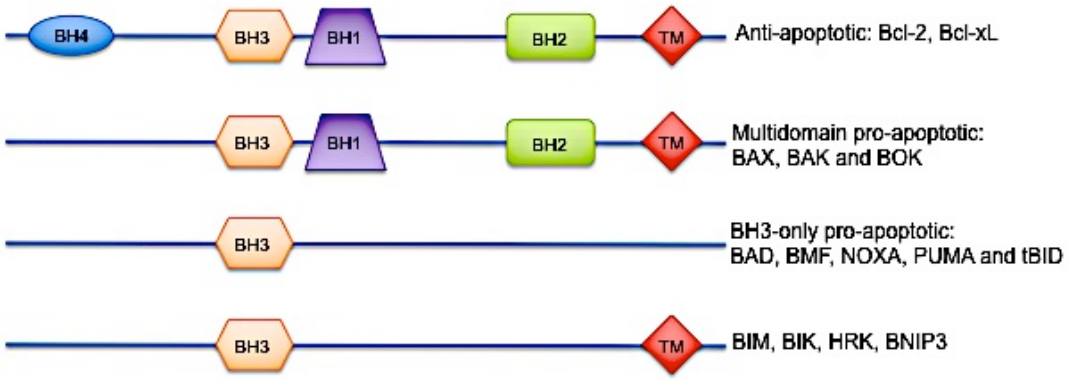


Figure 1.6: The Bcl-2 family

The Bcl-2 family is classified according to structure and function of proteins. They are composed of one or more BH domains with or without a transmembrane domain. Pro-survival or anti-apoptotic members are generally made up of all 5 domains and include Bcl-2 and Bcl-xL as the main proteins. Pro-apoptotic family is sub-grouped into multi-domain members (BAX, BAK and BOK) having BH1-3 and TM domains, the BH3-only members (BAD, BMF, NOXA, PUMA, tBID), and others have both BH3 and TM domains (BIM, BIK, HRK, BNIP3).

- ***Caspases***

Caspases are cysteine proteases with a conserved penta-peptide sequence (QACXG) and three catalytic residues, with a Cys-285 active site that specifically cleaves substrates following Asp residues (Fischer et al., 2003). Caspases are produced as inactive zymogens containing pro-domain fragment, p20 large fragment and p17 small fragment and activated upon cleavage of the zymogen precursor into individual fragments, the p20 and p17 and removal of the pro-domain (Li and Yuan, 2008). The pro-domain mediates their interaction with adaptor proteins and includes death effector domain (DED), caspase recruitment domain or pyrin domain (PYD) (Jacquet et al., 2008, Chang and Yang, 2000). Some caspases are inflammatory with major roles in cytokine maturation and T-cell proliferation such as caspase-1, -4, -5, -11 and -13; others are apoptotic that cause cell death upon activation and include the initiator caspases (caspases-2, -8, -9, -10, -12). Apoptotic caspases recruit upstream signaling events and often cleave downstream caspases (caspases-3, -6, -7) activating them resulting in series of biochemical and morphological changes leading to cell death (Earnshaw et al., 1999, Shi, 2004). Such changes are observed when inhibitor of caspase-activated-DNAse (ICAD) is cleaved by caspase-3 activating caspase-activated-DNAse (CAD) which degrades DNA (Sakahira et al., 1998, Tang and Kidd, 1998); moreover, poly-ADP-ribose-polymerase-1 (PARP-1) is cleaved

by caspase-3 and -7 during apoptosis and become deactivated and hence can no longer detect DNA damage (Kaufmann et al., 1993). Caspases are known to recognize a specific tetrapeptide sequences cleaving it. Inflammatory caspases, caspase-1, -4, -5, and -11 cleave with a preference for the sequence Trp-Glu-His-Asp (WEHD); caspases 2, 3 and 7 cleave after DEXD while caspases 6, 8, and 9 cleave (L/V) EXD where X is V, T or H (Fischer et al., 2003). Caspase-12 has polymorphisms in its catalytic function and active only in rodents (Saleh et al., 2004).

1.4.3. Intrinsic apoptosis

The intrinsic or mitochondrial apoptotic pathway is induced by intracellular stimuli that regulate main cellular events, such as transcription, translation and post-translation, in order to initiate apoptotic cell death by modification of mitochondrial permeability. For example, reduction in essential factors required for cell survival like glucose, hormones and growth factors, or exposure to toxins, viruses, radiation, stress, and increase in reactive oxygen species can provoke apoptosis (Elmore, 2007). Such stressors trigger Bcl-2 family expression increasing the levels of pro-apoptotic proteins in the cell. As a result, pro-apoptotic Bcl-2 protein BAX is translocated to the mitochondria and dimerises with BAK creating pores in the outer mitochondrial membrane (OMM) releasing proteins that are usually held within the intermembrane space such as apoptotic proteins, cytochrome-c, Smac/DIABLO, apoptosis-inducing factor (AIF), endonuclease G and Omi/htra2. These proteins either trigger caspases or caspase-independent cell death (Green and Kroemer, 2004). Then, the apoptosome, which is composed of cytochrome-c, apoptotic-peptidase activating-factor-1 (APAF-1) and caspase-9, is formed. (Saikumar et al., 2007). In healthy cell conditions, APAF-1 exists in an inactive conformation and bound to dATP; however, conformational change occurs upon cytochrome-c binding, due to nucleotide exchange, and exposing the AAA+ ATPase domain leading to oligomerization of several APAF-1 proteins forming a circular apoptosome complex to which caspase-9 is recruited (Bratton and Salvesen, 2010). Crystallization studies revealed the apoptosome as a wheel shaped platforms made up of 7 APAF-1 molecules and 7 cytochrome-c molecules (Bratton and Salvesen, 2010). Caspase-9 binds to APAF-1 in the complex via CARD-CARD interaction and becomes activated; then caspase-3 gets recruited to the apoptosome and activated via direct cleavage by caspase-9 (Cain, 2003). Activated caspase-3 cleaves diverse substrates leading to cellular damage and death (Saikumar et al., 2007). Additionally, apoptosis is induced by mitochondrial molecules that sequester inhibitor of apoptosis proteins (IAPs) (Shiozaki and Shi, 2004). Omi/htra2 is also believed to have protease activity leading to degradation of XIAP, cIAP1, cIAP2 and anti-apoptotic PED/ PEA-15 (Vande Walle et al.,

2008). AIF and endonuclease-G translocate into the nuclear compartment inducing chromosomal condensation and nucleic acid fragmentation (Lorenzo and Susin, 2007).

1.4.4. Extrinsic apoptosis

The extrinsic apoptosis or the death receptor pathway is induced by extracellular factors binding to corresponding receptors that belong to the TNF death receptor family (Figure 1.5.2) (Locksley et al., 2001). The TNFR family constitutes of around 20 proteins with the external cysteine domain and internal death domain (Magalhaes et al., 2011). The TNF family of receptors includes TNFR-1, TNF-Ligand, CD95/Fas and TNF-related apoptosis-inducing ligand (TRAIL) that couple to specific ligands and transmit their death-inducing signal through the death domain (DD) (Fulda and Debatin, 2004). Death receptors trimerise upon activation and ligand binding leading to recruitment of adaptor proteins and caspase-8 forming a death inducing signaling complex (DISC) which results in caspase-8 auto-activation cleaving caspase-3 and activating it and leading to cell death (McIlwain et al., 2013, Wajant et al., 2003). Caspase-8 inhibition occurs upon binding to c-FLIP which enhances its degradation, or by TRAF2 tags it with ubiquitin and determines a limit for extrinsic apoptosis (Li and Yuan, 2008, MacKenzie and Clark, 2012, McIlwain et al., 2013). In some cases, caspase-8 recruitment at the DISC is not sufficient for caspase-3 activation, therefore, switch to the intrinsic pathway occurs through cleaving the Bcl-2 family member, BID forming truncated BID, tBID (Cory and Adams, 2002). tBID then binds cardiolipin in the mitochondrial matrix enhancing BAX/BAK oligomer accumulation and pore formation (Kim et al., 2009).

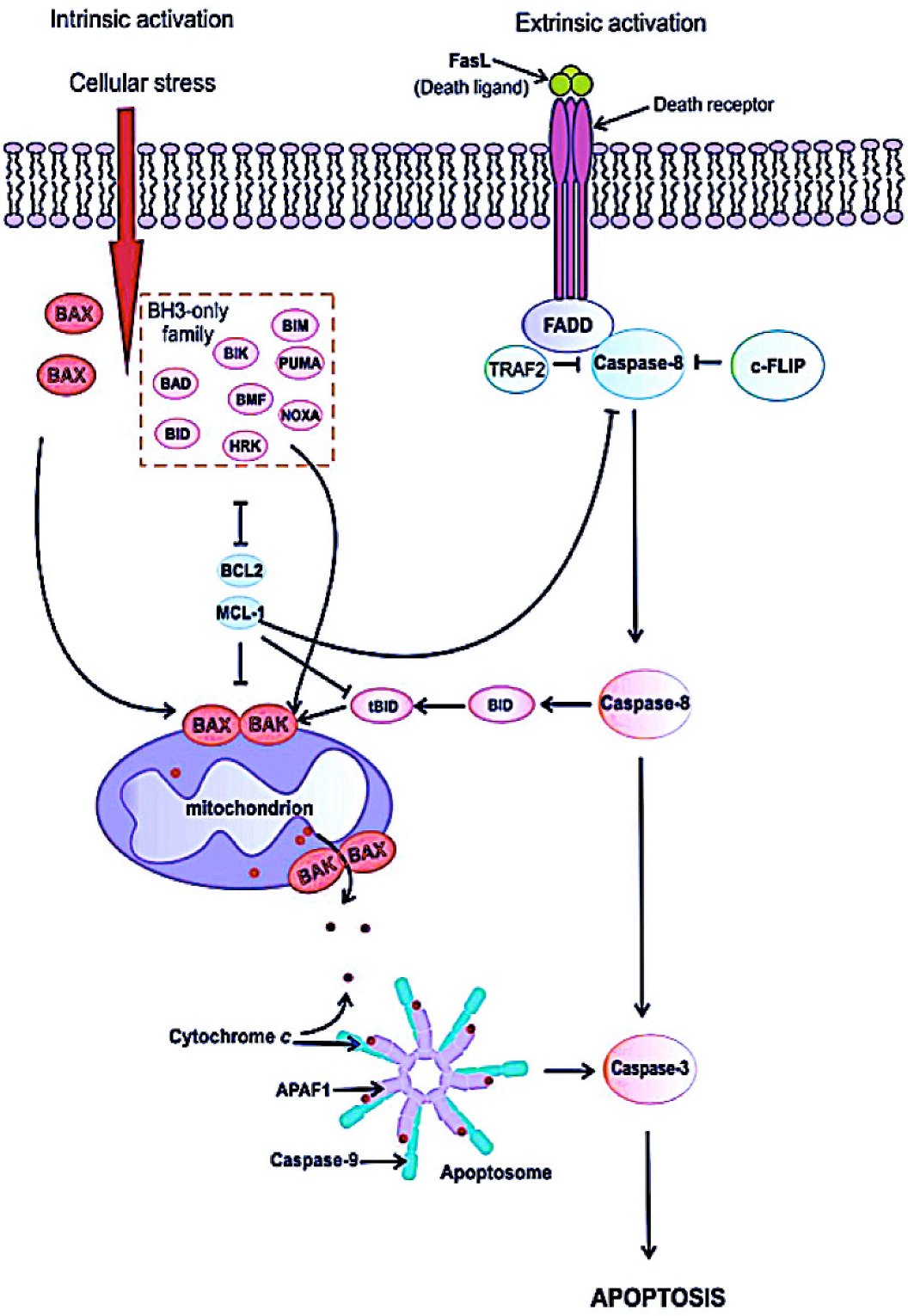


Figure 1.7: Intrinsic and extrinsic apoptosis pathways

The intrinsic apoptosis is initiated by stimuli activating Bcl-2 family members in response to stress signals and cause mitochondrial membrane permeabilisation. BAX translocates to the mitochondria, and heterodimerises with BAK forming pores in the OMM releasing cytochrome-c from the inter-mitochondrial space into the cytosol leading to apoptosome formation and activation of caspase-9 and downstream caspase-3. The extrinsic pathway is

activated upon binding of specific ligands to death receptors recruiting adaptor proteins and activating caspase-8 and caspase-3.

1.4.5. Immunogenic cell death

Immunogenic cell death (ICD) is a form of cancer cells' death triggered by cytostatic agents such as bortezomib, or by radiotherapy and photodynamic therapy (Garg et al., 2010). Different than programmed cell death which is mostly non-immunogenic, immunogenic cell death stimulates effectively an anti-tumour immune response by activating dendritic cells, followed by specific T-cell immune response activation (Spisek et al., 2007). Most agents that induce immunogenic cell death target the endoplasmic reticulum causing ER stress and production of reactive oxygen species (ROS), that play a main role in intracellular signalling pathways that control ICD (Krysko et al., 2012). ICD is characterized by secretion of damage-associated molecular patterns (DAMPs), such as calreticulin (CRT) that gets translocated after the induction of immunogenic apoptosis from the ER to the surface of dying cells, where it functions as an "eat me" signal for professional phagocytes. Other important surface exposed DAMPs are the heat-shock proteins (HSPs) that become translocated to the plasma membrane upon stress, where they will have an immune-stimulatory effect by interacting with antigen-presenting cell (APC) surface receptors like CD91 and CD40. Additionally, they facilitate cross presentation of antigens that are derived from tumour cells on MHC class I molecule, leading to the CD8+ T cell response. Amphoterin (HMGB1) and ATP are other important DAMPs. HMGB1 is considered a late apoptotic marker that is released to the extracellular enhancing the presentation of tumour antigens to dendritic cells. It also binds to several pattern recognition receptors expressed on APCs such as Toll-like receptor (TLR) 2 and 4. Recently, ATP was reported to be secreted during immunogenic cell death, and functions as an attracting signal for monocytes to the site of cell death (Garg et al., 2010).

1.5. Heat Shock Response

The heat shock response (HSR) is a cell protection effect in response to different stimuli (Ritossa, 1996, Lindquist, 1986). Several factors and cell stresses can trigger an increase and accumulation of misfolded proteins that may be detrimental causing cell damage and alter biological activities (Jolly and Morimoto, 2000). HSPs are group of chaperones stimulated by stress and contribute to protein folding, translocation and refolding (Schmitt et al., 2007, Ellis et al., 1992). They bind to improperly folded proteins and facilitate their refolding. Few have catalytic properties in the folding process and others can dis-aggregate loose protein aggregates (Georgopoulos and Welch, 1993). They are highly conserved biomolecules present in both eukaryotes and prokaryotes, in addition they were

first reported as various proteins powerfully induced by heat or other stressors (Lindquist and Craig, 1988). Heat shock factor-1 (HSF1) is responsible for stress induced transcription of HSPs and is considered the regulator of HSP proteins expression in vertebrates (Akerfelt et al., 2010). There are five categories of HSPs in mammals based on the molecular size including HSPH (HSP100), HSPC (HSP90), HSPA (HSP70), DNAJ (HSP40) and HSPB (Vos et al., 2008). HSP90, HSPB and HSPA impact cancer progression and associate with bad clinical presentation, and inhibiting HSP90 sensitizes cells to treatment and induces apoptosis. Moreover, HSP90 is involved in immunity and necroptosis. (Solarova et al., 2015). HSPB1 and HSPA1 are discussed further below.

1.5.1. Heat Shock Protein Beta-1 (HSPB1)

Heat shock protein beta-1 (HSPB1), also known as HSP27, is a member of the HSPB family of molecular chaperones whose expression is enhanced upon stress (Arrigo et al., 2007, Taylor and Benjamin, 2005). HSPB1 is a 27 kDa protein with a conserved α-crystalline domain composed of a highly structured β-pleated sheet allowing oligomer formation and stabilization (Kim et al., 1998). In addition, its N-terminal segment is composed of a WDPF domain and the carboxy terminal is a non-conserved and flexible domain important for solubility, chaperoning and oligomerization (Sunayama et al., 2005). HSPB1 form oligomers of about 1000 KDa that bind to the cytoskeleton and have a dynamic quaternary structure and mediate ATP independent chaperone activity (Haslbeck et al., 2005). HSPB1 oligomerization depends on its phosphorylation and exposure to stress and is a reversible process that plays a crucial role in providing protection against aggregation of proteins (Schmitt et al., 2007).

- ***HSPB1 role in apoptosis***

HSPB1 can function as an anti-apoptotic protein by inhibiting different stages of the extrinsic and intrinsic apoptotic machinery or mediating proteasomal degradation of target proteins (Concannon et al., 2003). HSPB1 is induced upon cellular stress inhibiting apoptosis, and hence, cells expressing high HSPB1 are most likely resist stress related cell death (Konishi et al., 1997). It can bind to various pro-apoptotic proteins inhibiting caspase and procaspase-9 activation (Garrido et al., Samali et al.). Moreover, HSPB1 expression blocks the release of reactive oxygen species and suppresses mitochondrial membrane potential. Additionally, it can bind to the apoptosome inhibiting caspases or negatively regulates cytochrome c-dependent induction of pro-caspase-3 (Samali et al., Pandey et al., 2000). Also, some studies showed that HSPB1 is able to negatively regulate BID re-distribution to the mitochondria preventing cytochrome c release and consequent activation of the intrinsic apoptotic pathway thus protecting the mitochondria from any damage (Paul et

al., 2002). Other anti-apoptotic mechanisms have also been reported such as inhibiting conformational BAX activation, oligomerization and translocation to the mitochondria, release of cytochrome c and apoptosis inducing factor (AIF) via a PI3-kinase dependent mechanism (Havasi et al., 2008).

HSPB1 post-translational modification is also important for eliciting its protective function. In response to stress, it gets phosphorylated at serine residues 15, 78 and/or 82 in humans by MAPK associated proteins, which are in turn phosphorylated by p38 kinase (Ito et al., 2005, Stokoe et al., 1992). As a result of Ser-78/Ser-82 phosphorylation of HSPB1, oligomers dissociate disturbing the interaction with the cytoskeleton, while Ser-15 phosphorylation has a small effect on oligomerization (Gusev et al., 2002).

As mentioned earlier, HSPB1 also targets misfolded proteins for proteasomal degradation (Georgopoulos and Welch, 1993). Upon stress, HSPB1 bind to Ub enhancing proteasomal degradation of unfolded or misfolded proteins (Parcellier et al., 2003). On the other hand, some reports showed that HSPB1 could also inhibit proteasomal degradation of proteins like eukaryotic initiation factor 2α (EIF2α), hence inducing resistance to androgen ablation and chemotherapy in prostate cancer (Andrieu et al., 2010).

1.5.2. Heat Shock Protein Alpha 1 (HSPA1)

HSPA1, also known as HSP72, is a large heat shock protein with an approximate molecular weight of 72 kDa. It belongs to HSP70 family and the main heat-inducible member (Multhoff, 1997). It is important in various cell mechanisms such as protein assembly, folding, translocation and complexes formation (Nelson et al., 1992). HSPA1 inhibits SMAC/Diablo release and XIAP degradation preventing apoptosis (Zhang et al., 2015). It also suppresses caspase cascades and stress-activated MAPK inhibiting apoptosis in response to different stresses (Park et al., 2002). Moreover, it interacts with Apaf-1 and apoptosis-inducing factor leading to inhibition of caspase-dependent and -independent apoptosis, respectively (Beere et al., 2000, Ravagnan et al., 2001, Saleh et al., 2000). It also inhibits the JNK signaling pathway and the p38 MAPK pathway (Park et al., 2002). HSPA1, being a heat shock protein, also acts as a molecular chaperone. It stabilizes existing proteins and mediates folding of newly translated proteins in the cytosol, which is essential to many molecular mechanisms (Mayer, 2005, Wang et al., 2013). Moreover, it maintains the function of mutant proteins and participates in DNA repair protecting against DNA damage (Mayer, 2005, Duan et al., 2014). HSPA1 expression is very low in normal cells but is significantly induced upon stress to protect cells against serious consequences (Ryu et al., 2013).

1.5.3. HSPB1 and HSPA1 in BC

The expression of HSPs is high in BC and associates with negative clinical presentation (Calderwood and Gong, 2012). HSPB1 and HSPA1 are important in survival and maintenance of cancer cells. It has been shown that expression of HSPB1 increases in BC, and high levels of HSPB1 enhance resistance to therapy and play an important role in the survival of cancerous cells in the body (Vidyasagar et al., 2012). However, the exact role of HSPB1 in breast tumours is still unclear; however, it plays a part in the prevention of programmed cell death and is associated with high-grade tumours (Straume et al., 2012). HSPA1 is associated with the mitotic spindle and significantly correlated to cell proliferation while HSPB1 is not. This suggests that HSPB1 is more involved with cell growth arrest and possibly cell differentiation, while HSPA1 is possibly involved in cell proliferation (Vargas-Roig et al., 1997). Due to the anti-apoptotic and cyto-protective role, several studies are ongoing to understand if the association of altered HSPB1 and HSPA1 expression is causal or correlative across tumours and investigating their role in oncogenesis and cell death (Jolly and Morimoto, 2000). Therefore, understanding HSPB1 and HSPA1 cellular signaling mechanisms and function with respect to cellular context and disease pathogenesis is important.

1.6. Endoplasmic Reticulum Stress

The Endoplasmic Reticulum (ER) is a cellular organelle responsible for protein synthesis and proper folding (English et al., 2009, Voeltz et al., 2002). The unique oxidative and calcium rich environment of the ER is essential for the assembly of chaperone and foldase proteins that are responsible for essential post-translational modifications and folding of proteins into their native most stable conformation (Braakman and Bulleid, 2011, Stevens and Argon, 1999). Abnormal changes in the cells adenosine triphosphate (ATP) supply, imbalance in the intracellular redox state, disruption of calcium homeostasis, accumulation of unfolded proteins, and inhibition of protein glycosylation can cause stress stimulating a series of signaling events known as the unfolded protein response (UPR) (Kaufman, 2002).

1.6.1. The unfolded protein response

There are two modes of the UPR known as the adaptive UPR and the apoptotic UPR (Jager et al., 2012). The adaptive UPR reduces the stress in the ER by reducing protein synthesis and increasing chaperones in order to enhance protein folding or target misfolded proteins to proteasomal degradation and thus alleviating the burden of protein accumulation in the ER. However, in cases of severe stress then the apoptotic UPR stimulates death

(Hetz, 2012, Rasheva and Domingos, 2009). Three distinct UPR pathways occur, the PERK pathway (protein kinase R-like ER kinase), the ATF6 pathway (activating transcription factor-6) and the IRE1α pathway (Inositol requiring enzyme-1-α). These transmembrane proteins are inactively binded to GRP78 in a normal cell; however, under stress GRP78's chaperoning activity is required, so it dissociates to target unfolded proteins; hence PERK, ATF6 and IRE1 become activated and induce downstream adaptive and apoptotic pathways (Szegezdi et al., 2006, Schroder and Kaufman, 2005).

1.6.2. PERK Signaling

PERK is a serine/threonine kinase and belongs to the eukaryotic initiation factor-2 (EIF2) kinase family (Donnelly et al., 2013). It is activated upon dissociation from GRP78 through homodimerization and subsequent cytoplasmic domain trans-autophosphorylation (Schroder and Kaufman, 2005). PERK then phosphorylates EIF2α blocking cap dependent protein translation relieving ER stress (Donnelly et al., 2013). The activated transcription factor 4 (ATF4) is one protein translated in a cap-independent manner; however, during enhanced EIF2α phosphorylation ATF4 translation is increased, due to the fact that the two upstream open reading frames (uORFs) of ATF4 are inhibited from blocking downstream ATF4 expression (Wek et al., 2006). ATF4 signaling induces the transcription of pro-survival genes needed for amino acid import and metabolism, or those that reduce endogenous peroxidase levels to maintain the ER oxidative environment (Harding et al., 2003). However, if the protein burden on the cell is severe, PERK signaling can switch to an apoptotic mode by preventing further EIF2α phosphorylation and restoring cap-dependent protein translation (van Huizen et al., 2003). Also, active PERK up-regulates ATF4 mediated CHOP expression (Szegezdi et al., 2006). In turn, CHOP up-regulates Bcl-2 pro-apoptotic proteins BIM and PUMA, and down-regulates the anti-apoptotic Bcl-2 proteins promoting apoptotic cell death (Cazanave et al., 2010, Ghosh et al., 2012, Yamaguchi and Wang, 2004, McCullough et al., 2001).

1.6.3. ATF6 Signaling

In normal cells, ATF6 is found as a monomer, dimer or oligomer and stress reduces it to a monomer and dissociates from GRP78 (Nadanaka et al., 2006). In the ATF6 monomer, the Golgi localization signals (GLS1 and GLS1) are exposed facilitating their transport to the Golgi apparatus (Shen et al., 2002). In the Golgi, ATF6 N-terminal fragment known as ATF6-N is cleaved and gets translocated to the nucleus, activating transcription of genes encoding chaperone proteins, folding enzymes and proteins associated with ER-associated degradation (ERAD) and X-box binding protein (XBP1) (Szegezdi et al., 2006).

1.6.4. IRE1 Signaling

In mammals, IRE1 consists an endoribonuclease domain and a serine/threonine kinase domain and exists as alpha and beta active isoforms (Chen and Brandizzi, 2013). Upon stress, IRE1 is dissociated from GRP78, dimerizes and autophosphorylates activating itself (Szegezdi et al., 2006). IRE1 endoribonuclease domain then cleaves XBP1 mRNA to spliced XBP1 (sXBP1) which promotes cell survival (Jager et al., 2012). The RNAse activity of IRE1 also splices a subset of mRNA through a process known as Regulated IRE1 Dependent Decay (RIDD) (Hollien 2009, Maurel 2013). If the stress on the ER cannot be prevented, IRE1 signaling would switch to apoptotic signaling recruiting TRAF2 which activates apoptosis signal-regulation kinase 1 (ASK1) inducing a series of phosphorylation events, ultimately leading to phosphorylation and activation of c-JUN terminal kinase (Urano et al., 2000).

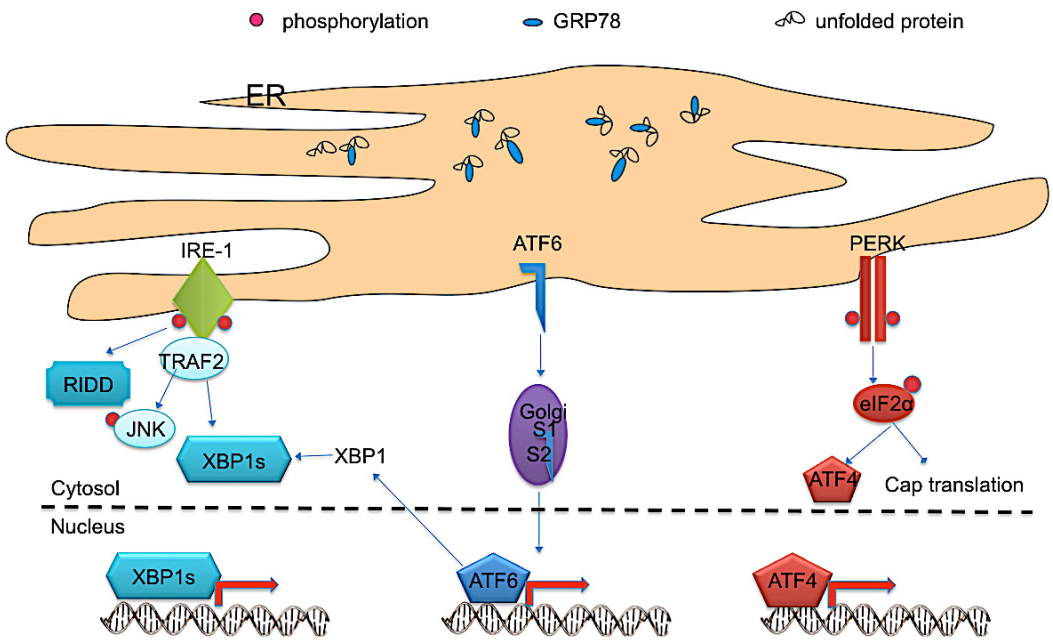


Figure 1.8 The unfolded protein response

Upon stress, IRE1, ATF6 and PERK dissociate from GRP78 and become active. In the PERK arm, EIF2α gets phosphorylated, blocking cap-dependent translation and enhancing cap-independent translation of ATF4 which, upregulates genes for amino acid synthesis, redox balance and CHOP. ATF6 gets translocated to the Golgi, and is cleaved into active N-terminalform which upregulates XBP1, ERAD and chaperone genes. IRE1 can cleave mRNAs by RIDD, or recruit TRAF2 leading to JNK phosphorylation. IRE1's RNAse domain can splice XBP1 to XBP1s, upregulating ERAD, chaperones and genes needed for phospholipid synthesis.

1.6.5. IRE1-XBP1 and Malignancies

Rapid tumour growth generates a distinctive microenvironment conditions including oxidative stress, nutrient deprivation and hypoxia leading to activation of the UPR in various

solid tumours (Vandewynckel et al., 2013). Ma et al., confirmed XBP1 role in oncogenesis (Ma 2004). In 2007 an analysis of a range of cancer subtypes identified IRE1 mutations as possible drivers of oncogenic processes in glioblastoma and hepatocellular carcinomas (Parsons et al., 2008, Guichard et al., 2012). The functional effects of these mutations still need to be identified but the role of IRE/XBP1 has been associated to several cancer types. Overexpression of XBP1 has been reported in TNBC presenting an aggressive phenotype and required for a complete hypoxic response through direct binding to hypoxia –inducible factor 1-alpha (HIF1α) (Chen et al., 2014). Moreover, XBP1 is required for B-cell differentiation and defense mechanisms, enhances survival and growth in prostate cancer and has been studied extensively in multiple myeloma (Reimold et al., 2001).

Aim

As discussed earlier, basal-like and TNBC are insensitive to treatment and rapidly develop resistance; therefore, there is no effective and targeted treatment regimen for basal-like and TNBC (De Laurentiis et al., 2010). Therefore, identifying and developing more selective and efficacious drugs for the treatment of this BC subtype is needed. Revealing key players and understanding the basic molecular mechanisms that drive development and resistance in basal-like and TNBC can highlight new targets for therapy and basis for drug development.

The main aims of the thesis are:

- Analyze BC patient databases for mRNA gene expression and its correlation with disease prognosis and survival, to identify key pathways and genes that might be responsible for basal-like BC tumourigenesis
- Analyze the expression and survival impact of RIP2 in BC patients, and determine its role and mechanism of action in MDA-MB-231 cell line model
- Analyze the expression and survival impact of HSPA1 and HSPB1 in BC patients, and understand their role in breast cancer cells survival, and determine whether there is any pathway crosstalk between UPR and HSR

Chapter 2: Materials and Methods

2.1. List of Suppliers

Alomone Labs, Jerusalem 91042, Israel

BD Biosciences-Pharmingen, San Diego, CA 92121, USA

Biomatik, Wilmington, Delaware, 19809, USA

Biosciences, Dun Laoghaire, Co Dublin, Ireland

Bio-Techne, Abingdon, OX14 3NB, United Kingdom

Calbiochem, Nottingham, NG9 2JR

Cell Signaling Technology (CST), Inc., Beverley, MA 01915, USA

Enzo Life Sciences, Exeter, EX2 4DG, United Kingdom

Fisher Scientific Ireland, Dublin 15, Ireland

Genecopoeia, Rockville, MD, USA

Integrated DNA technologies, Leuven, B-3001, Belgium

Invitrogen, Crofton Rd., Dun Laoghaire, Dublin, Ireland

Medray Imaging systems, Dublin 22, Ireland

Merck Millipore, Billerica, MA 01821, USA

Mybio Ltd., Kilkenny, Ireland

New England Biolabs (NEB), Ipswich, MA01938-2723, UK

Pierce, Subsidiary of Thermo Scientific Ltd, Rockford, IL 61105 USA

Promega, Mulhuddart, Dublin 15, Ireland

R&D systems, Abingdon, OX14 3NB, UK

Santa Cruz Biotechnologies, Inc., Santa Cruz, CA 95060, USA

Sigma-Aldrich Ireland, Dublin, Ireland

All chemicals were obtained from Sigma-Aldrich unless otherwise stated

2.2. Expression array analysis and statistics

Online databases were downloaded from NCBI Gene Expression Omnibus (Geo) database and analyzed using SPSS software. These datasets were chosen based on the cancer related clinical information outlined including tumour subtype, tumour stage and grade, progression free survival (PFS), metastatic free survival and/or overall survival. The breast cancer expression datasets were separately normalized then pooled for analysis. Briefly, probes were mapped using Entrez identifiers and Human Genome Organization gene symbols and then averaged for gene-level analysis. Missing values were calculated using nearest neighbor averaging. Data analysis was conducted using tools in R/Bioconductor. Datasets were standardized by scaling and finally checked using principal component analysis (PCA) in SPSS. Triple-negative samples were predicted from the annotated ER-negative samples using the subtyping tool, TNBCtype (http://cbc.mc.vanderbilt.edu/tnbc/). The median expression of identified proteins was used to divide each dataset into two cohorts in order to study the expression in samples of different BC clinical subtypes. Descriptive statistics were done to identify sample size and databases information. P-values within boxplots were determined using the Kruskal-Wallis test. Survival curves were evaluated by Kaplan-Meier estimators with log-rank P-values reported. Univariate and multivariate Cox regression with Pearson correlation coefficient was performed. P-values were reported for univariate Cox proportional hazards model; overall log-rank P-values are reported for the multivariate model using Cox proportional hazards regression model.

2.3. Cell Culture

2.3.1. Cell lines

MCF-7, T47D, SKBR3 and MDA-MB-231 were obtained from European Collection of Cell Cultures (ECACC) a Culture Collection of Public Health England. The non-transformed breast cell line MCF10A, and the TNBC cells lines MDA-MB-453, MDA-MB-468, BT-549, and HCC1806 were a kind gift from Dr. Sharon Glynn (Apoptosis Research Center, National University of Ireland, Galway) and obtained from the American Tissue Culture Collection (ATCC).

2.3.2. Culture conditions

MCF7 and MDA-MB-231, cancer cell lines were maintained in DMEM medium supplemented with 10% FBS, 1% L-Glutamine and 1% penicillin-streptomycin (Pen/Strep). T47D, SKBR3, MDA-MB-453, MDA-MB-468 and BT-549 BC cell lines were maintained in

RPMI medium supplemented with 10% FBS and 1% L-glutamine. MCF10A were maintained in F-12 Ham's medium supplemented with 5% horse serum, 20 ng/ml epidermal growth factor (EGF)-long, 100 ng/ml cholera toxin, 0.01 mg/ml insulin and 500 ng/ml hydrocortisone. Cell lines were incubated in a humidified incubator (5% CO_2, 37 °C) and routinely passaged every 2-3 days. For experiments, cells were seeded at specific cell density outlined in Table 2.1.

Table 2.1: Seeding density of BC cell lines optimized for transfection and treatment experiments

Cell line	Breast cancer subtype	Seeding density (number of cells per cm^2)
BT-549	TNBC	$3.5x10^4$
HCC1806	TNBC	$3x10^4$
MCF-7	luminal A, epithelial human breast adenocarcinoma (ER+)	$3.2x10^4$
MDA-MB-231	TNBC obtained from EACC	$3x10^4$
MDA-MB-231	TNBC obtained from ATCC	$5x10^4$
MDA-MB-453	TNBC	$5x10^4$
MDA-MB-468	TNBC	$5x10^4$
SKBR3	Luminal, human breast adenocarcinoma (overexpresses HER2)	$4x10^4$
T47D	Luminal A, infiltrating ductal carcinoma (ER+)	$3.5x10^4$

2.3.3. Freeze-down of cell lines

Freeze-down of cell lines was performed by suspending trypsinized cells in medium. Cells were maintained at a cell density of 2 x 10^6 cells/ml. Supplemented medium containing 20% DMSO was added to this cell suspension drop-wise giving a final cell density of 1 x 10^6 cells/ml and a final DMSO concentration of 10%. 1 ml aliquots of this cell suspension were added to cryogenic vials. Cells were gradually frozen to reduce cell death, cryogenic vials were loaded into a polystyrene holder and placed into -70 °C freezer. After 48 h storage in a -70 °C freezer, cryogenic vials were transferred into liquid nitrogen for long-term storage.

2.4. Transfection

2.4.1. Transient transfection of BC cells

At 24 h post seeding, cells were transfected with the desired plasmid using Turbofect transfection reagent (Fermentas). The transfection complex consists of 1:2 ratio of DNA:lipid in serum free media. The transfection mixture was gently pipetted to ensure mixing and was incubated at room temperature (RT) for 20 min. In the meantime, the wells were washed twice with DMEM containing 10% FBS. Alternatively, if the cells were cultured in antibiotic free media, then the media need not be changed prior to transfection; however, the media should

be replaced 4 h post-transfection. After incubation the DNA:lipid complex was added slowly drop-wise on to the cells and the plate was swirled gently and incubated at 37 ºC. At 4 h post transfection, media on the cells were changed to complete media and cells were treated as required. The typical transfection efficiency obtained was 90-95%. Based on the described optimization, volumes of DNA, Turbofect and media used were calculated depending on experiment and cell culture growth area and culture volume. For example, in a T25 cell culture flask, MDA-MB-231 cells were seeded at density of $3x10^4/cm^2$ in 4 ml of complete media. At 24 h post seeding, culture media were replaced with P/S free media. The P/S free media contain all required nutrients and FBS. The DNA:lipid transfection complex was then prepared by adding 5µl of Turbofect reagent to 2.5 µg plasmid DNA in 600 µl of fresh DMEM media. The complex was mixed well and incubated for 20 min, and then added drop wise to cells. The media is then replaced with complete media after 4 h and cells were left in culture for 24 h, at which required treatment or experimental procedure was done.

2.4.2. siRNA transfection

Cells were seeded in T25 flask till 60-70% confluency in pen/strep free media. 20 µl of media were mixed with 8 µl DharmaFECT (purchased form Dharmacon) and 50 ng siRNA (cat # L-005269-00-0005) were added to 20 µl serum-free media and incubated at RT for 5 min. The siRNA was then mixed with DharmaFECT/media and allowed to incubate at RT for 20 min. Media were replaced on cells and 4 ml of pen/strep free media were added. The siRNA mixes were then added drop wise and mixed by gentle swirling of flask. Cells were incubated for 4 h at 37 °C after which siRNA containing media were removed and fresh media were added. Cells were then harvested for Western Blot and DEVDase assay.

2.5. Plasmid constructs and cloning

2.5.1. Plasmid preparation

The DNA inserts encoding the N-terminal HA-tagged wild-type RIP2 (wt-RIP2) and CARD-only RIP2 eukaryotic expression constructs were generated by standard PCR techniques and subcloned into the mammalian expression vector pcDNA3.1. Cloning was performed in order to obtain VSV-tagged RIP2 constructs in pcDNA3.1 vector. Primers were designed to amplify the whole RIP2 gene. VSV-tag sequence was added to the forward primer at amino terminal of RIP2 protein. Primers used for wt-RIP2 were RIP2-EcoRI-F: 5'AAAGAATTCAAAATGGCATACACTGATATCGAAATGAACCGCCTGGGTAAGAAAATGAA CGGGGAGGCCAAAAA-3' and RIP2-XHOI-R: 5'AAAAAAATGTTACATGCTTTTATTTTGAAGTAAATTAAACTCGAGAAAAAA-3' and for CARD only were CARD onlyRIP2-F CCGGAATTCAAAATGATGGCTTACCCATACGACGTTCCAGATTACGCTCAGCCTGGTATA

GCCCAGCAGTGG, CARD onlyRIP2-R CCCAAGCTTTCACTTACATGCTTTTATTTTGAAGTAA. Mutation of lysine 209 to arginine for RIP2 (RIP2-K209R) was accomplished by the use of mutagenic primers (forward primer GGGCCAGTATCCGGCACGATATATAT and reverse primer ATATATATCGTGCCGGATACTGGCCC). The presence of the introduced mutation and fidelity of PCR replication was confirmed by sequence analysis done by LGC Genomics. shRNA-RIP2 (shRIP2) and shRNA scrambled control (sc) were purchased from Genecopoeia. VSV-tagged CARD-only RIP2 was provided by Prof. Jürg Tschopp from the University of Lausanne, Switzerland. PCR was optimized at 92 °C for 2 min, initial denaturation step at 94 °C for 30 sec, 30 cycles of 62 °C for 1 min and 72 °C for 1 min, and final elongation step at 72 °C for 10 min. Amplicons were gel extracted and ligated with pcDNA3.1 plasmid of which maxipreps were prepared.

Plasmids that inhibit the NF-κB pathway were a kind gift from Dr. Aideen Ryan, REMEDI, National University of Ireland Galway. The plasmids are the empty vector (EV) control pRC-β-actin and the IkBαS32/36A-pRC-β-actin plasmid, which is also known as the super-repressor (SR). The SR is a mutated human IκBβ gene where the phosphorylation sites (Serine 32 and 36) have been mutated into Alanine so the constitutively expressed human IκBα cannot be phosphorylated, ubiquitinated and degraded by the proteasome so endogenous NF-κB activation is greatly decreased.

2.5.2. Transformation of plasmids into *E. coli* cells

For the generation of all plasmids, DH5α *E. coli* cells were used. Plasmid DNA (~10 ng) was combined with 25 µl of DH5α competent *E. coli* cells and incubated on ice for 30 min to aid in the permeabilization of cells allowing them to take up the foreign DNA. The mixture was then heat shocked at 42 ºC for 40 sec. Cells were placed on ice for 2 min and combined with 475 µl of Luria-Bertani (LB) Broth. Cells were allowed to recover and then incubated at 37 º C for 1 h. The cells were spread on LB agar plate containing 50 µg/ml Ampicillin and grown overnight at 37 ºC. Next day, bacteria from single colonies were further inoculated into 5 ml of LB broth containing antibiotic. This starter culture was incubated overnight at 37 ºC with shaking. The resulting culture was centrifuged at 5000 g for 30 min to pellet the bacterial cells containing the plasmid of interest. Plasmid extraction was carried out using Qiagen maxi-prep kit as per manufacturer's guidelines (Qiagen, Cat. No. 12163)

2.5.3. Digestion of vector and insert

The full-length PCR products obtained using the method described in Section 2.5.1 were then subjected to restriction enzyme digestion. The forward primer contained the restriction site *EcoRI* and the reverse primer contained the restriction site *XhoI*. These

restriction sites were incorporated into the full-length RIP2 PCR products to permit insertion of the full length PCR product into the destination vector, pcDNA3.1+ (Invitrogen). For the restriction enzyme digestion 100 ng of the full-length PCR product (insert) or the pcDNA3.1+ (vector) was placed in an Eppendorf tube along with 1 U of *EcoRI*, 1 U of *XhoI*, 1 x BSA and 1 x NEB Buffer 3. The reaction was then incubated at 37 ^{o}C for 4 h. After the incubation 5 µl of the reaction products were visualized by gel electrophoresis with 1% agarose in 1 x TAE at 70 V.

2.5.4. Ligation of vector and insert

Following digestion of the vector and the insert with complementary restriction enzymes they were incubated together to ligate the insert into the vector. 50 ng of the restriction enzyme digested vector was placed into an Eppendorf tube along with 100 ng of insert. To this 1 U of T4 DNA ligase was added with 1 x ligase buffer. The reaction was incubated for 6 h at 16 ^{o}C.

2.6. Cell culture treatments

Table 2.2 summarizes the details of drug preparation, the stock concentration of the drugs and storage conditions of the drugs.

Table 2.2: Drugs used in cell culture treatments

Drug	Solvent	Stock	Storage
Bortezomib	DMSO	20 mM	-20 ºC
Doxurubicin	DMSO	50 µg/ml	-80 ºC
Etoposide	DMSO	100 mM	-20 ºC
MKC-8866	DMSO	25 mM	-80 ºC
N-acetyl sphingosine	DMSO	10 mM	-20 ºC
Taxol	DMSO	250 µg/ml	-80 ºC
PP2	DMSO	25 mM	-20 ºC
SKG797800	DMSO	1 mM	-20 ºC
SN50	DMSO	10 mM	-20 ºC
Thapsigargin	DMSO	4 mM	-80 ºC
Tunicamycin	DMSO	2 mg/ml	-80 ºC

2.7. Western Blotting

2.7.1. Protein sample preparation

Cells were seeded in T25 flaks, as per optimum seeding density outlined in Table 1. At the required time point post treatment, cells were scraped into the culture media and centrifuged at 2500 rpm for 5 min. Media were then removed and cells washed in 1 ml of cold

PBS and centrifuged again for 5 min. The supernatant was discarded and the cell pellet was washed twice in 1 ml of ice-cold PBS. The cells were centrifuged at maximum speed for 1 min. The supernatant was discarded and the cell pellet was lysed in 50 µl of whole cell lysis buffer (WCLB). WCLB is prepared by adding 1 M N-2-hydroxyethyl-piperazine-N-2-ethanesulphonic acid (HEPES) pH 7.5, 350 mM NaCl, 1 mM $MgCl_2$, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM ethyleneglycoltetraacetic acid (EGTA) and 1% Nonidet-P40 (NP-40)). Reducing agent, 1, 4-dithio-DL-threitol (DTT) (0.5 mM), and protease inhibitors phenylmethylsulphonyl fluoride (PMSF) (0.1%), and 1% aprotinin were added to the lysis buffer at the time of lysing. The lysis buffer also contained phosphatase inhibitor sodium fluoride (NaF) (5 mM) and sodium orthovanadate (Na_3VO_4) (1 mM). For detection of phosphorylated proteins, addition of DTT was avoided as this could inhibit the activity of phosphatase inhibitors. The cell pellet lysed in WCLB was allowed to swell on ice for 30 min and centrifuged at maximum speed for 5 min at 4 ºC. The supernatant containing the cytosolic proteins was transferred to a new 1.5 ml tube.

2.7.2. Protein quantification of samples

The protein concentration of samples was determined using Bradford method with bovine serum albumin (BSA) as the standard. Samples were stored at -20 ºC until further analysis.

2.7.3. Protein concentration from conditioned media

In order to detect the release of proteins in to media, conditioned media from cultured cells were collected and centrifuged at high speed to remove any cell debris. Supernatants were then transferred to Amicon® Ultra 15 mL Centrifugal Filters from Millipore and spun at 4000 xg in a swinging bucket rotor at 25°C for 15 min.

2.7.4. SDS-PAGE gel electrophoresis

Gels were cast the day prior to running the gel. The percentage of SDS-PAGE gel was prepared based on protein size. Details of gel composition are outlined in Table 2.3. 30 ng of proteins were mixed with with 5X loading buffer and heated at 95 ºC for 5 min and then loaded into gel lanes. The SDS-PAGE gels were then run in a tank with appropriate amounts of running buffer at 80 V until the sample passes through the stacking gel and then 100 V until the dye front reached the end of the gel. The gel was then prepared for transfer to nitrocellulose paper. The proteins were transferred onto the nitrocellulose paper for 90 min at constant voltage of 110 V in transfer buffer (25 mM Tris pH 8.3, 2 M glycine and 20% methanol)

Table 2.3: Composition of the western blot gel

Components	Running Gel Percentage			Stacking gel
	8%	12%	15%	
H_2O	1.9 ml	1.7 ml	1.2 ml	1.36 ml
30% acrylamide	1.8 ml	2 ml	2.5 ml	0.34 ml
1.5 M Tris-HCl pH 8.8	1.3 ml	1.3 ml	1.3 ml	-
1.0 M Tris-HCl pH 6.8	-	-	-	0.26 ml
10% SDS	0.05 ml	0.05 ml	0.02 ml	0.02 ml
1 mg/ml Ammonium Per Sulphate	0.05 ml	0.05 ml	0.05 ml	0.02 ml
Tetramethylethylenediamine	0.002 ml	0.002 ml	0.002 ml	0.002 ml
Total	**5 ml**	**5 ml**	**5 ml**	**2 ml**

Membranes were blocked in 5% non-fat milk in PBS containing 0.1% tween (PBS-T). The primary antibodies were incubated as described in Table 2.4. After primary incubation the membranes were washed three times for 10 min each in PBS-T. Membranes were then incubated in appropriate horseradish peroxidase conjugated secondary antibody in 5% non-fat milk PBS-T for 2 h at room temperature with agitation. Secondary antibodies were all obtained from Jackson labs and diluted in 5% milk-TBST for 2 h. Antibodies were visualized using Western Chemiluminescent HRP substrate (Advansta or Pierce).

Table 2.4: Antibodies used for probing proteins with relative conditions

Protein	Company (catalogue number)	Primary Antibody	Secondary Antibody
Actin	Sigma (A2066)	1/5000 in 5% milk -T	1/5000 anti-rabbit
Bcl-2	Santa Cruz (sc-7382)	1/1000 in 5% milk-T	1/5000 anti-mouse
Bcl-xl	Santa Cruz (sc-8392)	1/1000 in 5% milk-T	1/5000 anti-mouse
BIM	Advansta Enzo (ADIAAAP-330-E)	1/1000 in 5% milk-T	1/10000 anti-rabbit
Caspase-3	Cell Signaling (9662)	1/1000 in 5% milk-T	1/5000 anti-rabbit
cIAP1/2	Santa Cruz (sc-12410)	1/1000 in 5% milk-T	1/5000 anti-goat
HSPB1	Advansta Enzo (ADISPA-803-F)	1/5000 in 5% milk-T	1/10000 anti-rabbit
p-IκB	Advansta Enzo (CST 9246)	1/1000 in 5% BSA-T	1/10000 anti-mouse
p-RIP2	Cell signaling (14397)	1/1000 in 5% BSA-T	1/5000 anti-rabbit
PARP	Cell signaling (9532)	1/1000 in 5% milk-T	1/5000 anti-rabbit
RIP2	Santa-Cruz (sc-22763)	1/1000 in 5% milk-T	1/10000 anti-rabbit

*RT: room temperature; ON: overnight

2.7.5. Stripping the membrane

In order to re-probe the membranes using different antibodies, the membranes were washed twice with 10 ml of 1X PBST for 10 min at RT. The membranes were then incubated for 30 min at RT in 10 ml of the stripping buffer (2 M Tris HCl pH 6.8, 10% SDS, and 0.5 % Tween, 100 mM fresh β-mercaptoethanol) and then washed twice with 10 ml of 1X PBST for 10 min at RT. The membranes were then blocked with blocking buffer, incubated with desired primary antibody and developed as described in the previous section.

2.8. Gene amplification

2.8.1. RNA preparation

Total RNA was prepared form cell culture plates by adding 1 ml of TRI Reagent (Invitrogen) onto the cell monolayer for 2 min. All cells were scraped, transferred to 1.5 ml Eppendorf tubes, vortexed for 1 min and left at RT for 5 min. To separate RNA, 200 µl chloroform was added, mixed well and incubated for 2 min at RT. Samples were centrifuged at 12,000 g for 15 min. The upper clear phase was carefully pipetted into freshly autoclaved and labeled 1.5 ml tubes. In order to precipitate the RNA, 250 µl of isopropanol was then added to each sample and incubated at -20 ºC overnight. Samples were then centrifuged at 12,000 g for 10 min and the RNA pellet was washed in 75% ethanol. All of the ethanol was carefully removed and the pellet was air dried to allow all traces of ethanol to evaporate. The pellet was re-suspended in 15 µl of RNAse free water (Sigma) and heated at 65 ºC for 15 min to completely dissolve the RNA. RNA concentration was determined by measuring absorbance at 260 nm (A260 nm) using a spectrometer (Nanodrop). RNA samples were then stored at -80 ºC for further use.

2.8.2. Reverse transcription RT-PCR

To synthesize complementary DNA (cDNA), 2 µg of RNA was subjected to DNAse treatment with 1 µl DNAse and 1 µl buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl2) added per reaction and incubated for 15 min at RT. The DNAse was then inactivated using 1 µl of EDTA. The RNA was then reverse transcribed into cDNA using Superscript III first strand RT-PCR system and random hexamers (Invitrogen). To each reaction 1 µl of random hexamer was added, mixed well, and heated at 65 °C for 2 min, and then 25 °C for 2 min. First strand mastermix consisting of molecular grade water, first strand buffer, DTT, dNTPs and superscript III enzyme was added to each sample and mixed well. The tubes were then incubated at 65 ºC for 8 min in a 'Biometra T3' thermocycler. 1 µl of 0.5 µg/µl oligoDT 12-18 (the cDNA primer) was then added to the tubes and incubated at 65 ºC for 2 min to reduce secondary structures. This was followed by incubation at 42 ºC for 2 min, then 1X strand master mix was prepared containing 4 µl 5X First strand buffer, 2 µl of 0.1 M DTT, 1 µl of 10 mM deoxynucleotide triphosphate (dNTPs). 2.6 µl of diethylpyrocarbonate treated water was added to the mix and 0.4 µl of Superscript III was added just before the samples were incubated at 42 ºC for 50 min. The samples were then incubated at 75 ºC for 10 min to inactivate superscript reverse transcriptase and stored at 4 ºC.

2.8.3. Polymerase chain reaction (PCR)

cDNA (2 µl) were amplified using 10 mM forward and reverse primers corresponding to the gene of interest. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified in

parallel to confirm whether equal quality and quantity of DNA were used. The PCR mix was prepared on ice in a total reaction volume of 25 µl, containing 2 µl cDNA, 3 µl of primers each, 12.5 µl of 2 X Go Taq PCR master mix (Promega) and the volume was made up to 25 µl with DEPC water. The negative control used contained master mix and primers with water instead of cDNA. The PCR reagents were placed in the thermocycler and cDNA was amplified under the optimized conditions for corresponding genes. The PCR products were spun down and stored short-term at 4 °C or long-term (3-4 weeks) at -20 °C. The PCR conditions and primer sequence for each gene of interest are tabulated in Table 2.5 and Table 2.6.

Table 2.5: Optimized PCR conditions for amplification of respective genes

Steps	RIP2	GAPDH
Hot start	92 °C/ 2 min	95 °C / 3 min
Denaturation	94 °C / 30 sec	94 °C / 30 sec
Annealing	62 °C /1 min	58 °C / 1 min
Extension	72 °C / 1 min	72 °C / 1 min
Final extension	72 °C / 10 min	72 °C / 10 min
Size	1650 bp	452 bp

Table 2.6: Primer sequences for gene of interest

Gene	Primer sequence (5'…. 3')	
RIP2	Forward	GATATCGAAATGAACCGCCT
	Reverse	CATGCTTTTATTTTGAAGTAA
GAPDH	Forward	ACCACAGTCCATGCCATC
	Reverse	TCCACCACCCTGTTGCTG

2.8.4. Patient samples and real time PCR

BC patient samples were obtained from Dr. Roisin Dwyer, REMEDI Regenerative Medicine Institute at University College Hospital Galway (UCHG). Ethical Review and consent was obtained from UCHG Institutional Review Board for the use of patient samples in biomedical research. Approval from Dr. Dwyer was obtained in order to detect RIP2 expression in these samples. Poly (A)+RNA extraction from patient samples was performed according to the protocol for the total mammalian RNA extraction kit (Sigma-Aldrich). Reverse transcription for cDNA synthesis was carried out with 2 µg total RNA and oligo(dT) (Invitrogen) using 20 U Superscript II Reverse Transcriptase (Invitrogen). cDNAs for genes of interest were amplified during 33 cycles with the SYBR-green endogenous control PPIA and MRPL19 primers and SYBR-green RIP2 primer probes for real time PCR were obtained from IDT Technologies.

2.9. Flow Cytometry

2.9.1. Sample Preparation

Cells were cultured at optimized density outlined in Table 1. Culture medium of growing cells was transferred to 1.5 ml Eppendorf tube. Cells were then washed with Hank's Balanced salt solution and trypsinized. The corresponding supernatant was then added to the well and the cells collected in Eppendorf tubes. Cells were then processed according to protocol.

2.9.2. Tetramethylrhodamine, Ethyl Ester, Perchlorate (TMRE) staining

TMRE is tetramethylrhodamine-ethyl-ester-perchlorate used to measure mitochondrial transmembrane potential (ΔΨM) which occurs upon apoptosis. TMRE is a cell permeable, positively charged, red-orange-dye that readily accumulates in active mitochondria due to their relative negative charge while depolarized or inactive mitochondria have decreased ΔΨM and fail to sequester TMRE. Carbonyl-cyanide-m-chlorophenyl-hydrozone (CCCP) is an ionophore uncoupler of oxidative phosphorylation and destroying ΔΨM, and used as positive control by treating cells with 60 µM CCCP for 3 h prior to harvesting. Cells were seeded at optimum density in 24-well plate in 500 µl media. After experimental procedure and required treatment, cells were harvested, treated with 100 nM TMRE and incubated at room temperature for 30 min in the dark and analyzed by flow cytometry using BD FACSCanto on channel PerCP and PE. The analysis was carried out using Cyflogic software. At least 3 repeats of each experiment were done.

2.9.3. Cell cycle analysis

PI was used to allow quantification of cells in SubG1, G1, S and G2M phases of cell cycle. Cells were seeded at optimum density in T25 flasks in 4 ml media. Cells were then trypsinized and fixed in 70% ice-cold ethanol and then washed in PBS. Cells were then suspended in 150 µl ice cold PBS and then 350 µl ice cold ethanol was then added dropwise while cells were continuously vortexed. Cells were kept on ice for minimum of 1 h and then either processed or stored at -20 °C for further use. Cells were centrifuged for 5 min at 2000 rpm and washed in PBS. An RNAse-A containing Propidium Iodide (PI) mix was then prepared by adding 5 µl of RNAse-A (10 mg/ml stock) to 95 µl PI (20 µg/ml). Cell pellet was re-suspended in 100 µl of RNAse/PI and incubated on ice in the dark for 30 min. Finally, 200 µl PBS was then added and measured by flow-cytometry using BD FACSCanto on channel PE. The analysis was then carried out using Cyflogic software.

2.10. Other assays

2.10.1. Cell viability assay

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazonium bromide) is used to detect cell viability. Cells were seeded in a 96-well plate at desired density. After the desired experimental procedure, transfection or treatment with required drugs, 10 µl MTT at 5 mg/ml was added to the wells and incubated at 37 °C for 3 h. The reaction was then stopped by the addition of 100 µl of stop solution (20% SDS, 50% dimethylformamide in dH_2O) to the wells. The plate was left rocking in dark for 3 h at RT to re-dissolve the resulting formazan precipitate. The change in cellular reductase activity, indicative of the metabolic activity in live cells, was measured using Wallac Victor x3 plate leader at 550 nM and viability was measured relative to control.

2.10.2. Luciferase assay

Luciferase assay was used to detect firefly luciferase reporter gene expression in cells using One-Glo Luciferase assay kit (Promega, Southampton, UK). Cells were seeded in 96-well plate in 100 µl growth media and treated as per experimental design. Upon harvest, 60 µl of media were removed and 30 µl of One-Glo Luciferase substrate were added to plate and mixed gently. Luciferase activity was measured using Wallac Victor plate reader. To analyze the results the firefly luciferase activity was expressed as a ratio of the luminescence for each sample relative to the control.

2.10.3. Caspase 3/7 activity assay

Caspase-3/-7 like activity was measured using DEVDase assay measuring enzyme activity of caspases upon cleaving DEVD sequence during cell death by apoptosis. Cells were cultured in T25 flask in 4 ml media, and at experimental time point, they were scraped and centrifuged at 300 × g at 4 ^{o}C for 5 min. Pellets were washed in ice-cold PBS and centrifuged again at 20,000 × g for 10 s. The pellets were re-suspended in PBS and flash-frozen in duplicates in liquid nitrogen. 50 µM of DEVDase-substrate conjugated to amino-4-methyl-coumarin (DEVD-AMC) in reaction buffer composed of 100 mM N-2-hydroxyethyl-piperazine-N-2-ethanesulphonic acid (HEPES) of pH 7.5, 10% sucrose, 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 5 mM DTT, 0.01% Nonidet-P-40, was added into lysates and the release of free AMC was monitored at 37 ^{o}C at 60 s intervals over a 30 min period using a Wallac Victor plate reader (excitation 355 nm, emission 460 nm). Fluorescence units were converted to nmol of AMC released per minute per mg of enzyme using a standard curve generated with free AMC and subsequently related to protein concentration.

2.10.4. Colony formation assay

Cells were seeded at a density of 1000 cells per well in a 6-well plate and incubated overnight at 37 °C, 5% CO_2. Cells were subsequently treated with control or drug for 24 h after which the drug was washed away and fresh medium was added. Cells were allowed to grow for one week replacing fresh medium every three days. After one week colonies were washed using ice cold PBS and fixed in methanol for 5-10 min. Following fixation, crystal violet dye was added and incubated with the cells for 5-10 min. Crystal violet was washed away using cold PBS and colonies were left to dry overnight. Colonies were visualized by light microscopy and the number of colonies per well was determined by scoring colonies with greater than fifty cells. Plates were then be scanned for images.

2.10.5. Scratch assay

Cells were seeded and cultured till being confluent in a 6-well plate. Without changing the medium, the monolayer was scratched gently and slowly with a sterile 1 ml pipette tip across the well, three vertical and three horizontal scratches were made. While scratching across the surface of the well, the long-axial of the tip should always be perpendicular to the bottom of the well. After scratching, wells were gently washed twice with medium to remove the detached cell and media were replenished. Images were taken at time of scratch (T0), 24 h, 48 h and 72 h post scratch and the scratch width was measured at indicated time points. Thirty measurements were taken all over the well and the average was calculated as scratch width. Percentage wound healing was the average scratch width at indicated time point (24 h, 48 h or 72 h) as a percentage of the average scratch width at T0.

2.10.6. DAPI staining

Cells were seeded in a 24-well plate in 0.5 ml media and treated according to experimental plan. At harvest, media were removed and the cells were trypsinized and collected. 100 µl of cells were cyto-centrifuged on to the slides at 200 rpm soft spin for 5 min. The slides were allowed to air dry and 200 µl of 3.7% paraformaldehyde were added to the cells and left for 20 min at RT. Slides were then washed three times in 1X PBS and the slides were allowed to dry, then 3 µl of DAPI in VECTASHIELD (Vector Laboratories Ltd) were placed on the slide which was overlaid with a cover slip and the edges sealed with varnish. The DAPI stained nuclei were then visualized using the Olympus Fluorescence Microscope at 360 nm excitation and 460 nm emission.

2.11. Assays to detect secreted factors

2.11.1. Reduced serum conditions

In order to determine effect of external factors in TNBC, reduced serum condition was optimized for MDA-MB-231 cells. Cells were seeded in 6-well plate with 2 ml media and transfected 24 h post seeding with RIP2 plasmid constructs using Turbofect. At 4 h post transfection media were changed and cells were allowed to recover in 10% FBS media for 24 h. Then media were changed and 2 ml of 4% serum-containing media were added to cells for 24 h, 48 h and 72 h. Culture media were collected, centrifuged at high speed for 10 min and stored at -20 °C for later use. To ascertain if the effect was dependent protein factors, supernatant from transfected cells was heated to 55 °C for 30 min and this supernatant was used as control.

2.11.2. Supernatant swap experiments

To examine if our observations were due to extracellular factors we performed various supernatant swap experiments. Conditioned media were collected from pcDNA3.1 and wild-type RIP2 transfected MDA-MB-231 cells, centrifuged at high speed to remove and any cell debris and then added to cell culture as per experiment.

2.11.3. Cytokine array

Human XL cytokine Array kit was purchased from R&D systems (ARY022) and performed as per manufacturers' recommendations. Membranes were blocked in buffer 6 for 1 h at RT after which membrane was incubated in 2 ml of culture media overnight at 4 °C with gentle shaking. Membranes were then washed three times with wash buffer for 10 min each. Detection antibody cocktail was then added to membrane for 1 h at RT and then washed. Membrane was then incubated with 2 ml of Streptavidin-HRP for 30 min at RT. After washing again, 1 ml of chemiReagent Mix was added to membranes and the array was developed using Agfa films. Array intensity was quantified using Image J software.

2.11.4. ELISA

All ELISAs were purchased from R&D Systems to detect IL-6, IL-8 and Gro-α presence in extracellular media. 100 µl of diluted capture antibody were used to coat wells overnight at RT. Wells were washed three times in wash buffer, and dried by placing plate against clean paper towel. Reagent diluents were used to block wells for 1 h at RT. 100 µl sample or standard suspended in reagent diluents were added to each well, covered and incubated at RT for 2 h and washed again. 100 µl substrate solution were then added to each well and incubated for 20 min at RT in dark. Then 50 µl of stop reagent were then added gently tapping the plate to ensure mixing. Optical density of each well was then determined

using Wallac Victor x3 microtitre plate reader set to 540-570 nm. Values were determined based on standard curve.

2.12. Quantitative and Statistical analysis

2.12.1. Protein densitometry

Image J software was used to calculate the protein density for proteins detected by Western blot. First the developed films were scanned in black and white and then cropped down to size. The image J software then measured the protein density by placing small boxes around each protein band. This produced a graph of peaks and the software was then able to quantitate the relative density. Values from the Image J were recorded. Protein expression was measured as a ratio of protein value measured by image J to the value of actin of the same sample of the same experiment. Fold increase was calculated as ratio of protein of interest relative to the control value of that experiment as described in Table 2.7. Final fold increase was calculated as an average of 3 separate experiments.

Table 2.7: Calculations of protein expression fold change

protein expression in sample = a actin expression of same sample = b → protein expression = a/b = **X**	protein expression control sample (eg. untreated) = A actin expression of control sample = B → control protein expression = A/B = **Y**
➔ protein fold change = **X/Y**	

2.12.2. Statistical Analysis

Statistical analysis was carried out using SPSS software. Values are expressed as mean ± standard error mean of 3 independent experiments. The choice of statistical test used across this thesis in indicated in each chapter. Differences were considered statistically significant at $p < 0.05$.

Chapter 3

Expression Analysis Of Pro-Survival Pathways mRNA markers In Basal-like Breast Cancer Subtype

3.1. Introduction

Basal-like breast cancers are among the most aggressive and deadly BC subtypes, with high rates of tumour recurrence and poor overall survival (Lehmann et al., 2011, Foulkes et al., 2010). They are still poorly characterized at the molecular level and lack definitive prognostic markers, and, therefore, it is hard to specify a treatment plan especially that basal-like breast cancers do not respond to treatment regimens (Lehmann et al., 2011, Yadav et al., 2015). Therefore, identifying molecular hallmarks specific to basal-like breast cancer subtype would help understand the complex characteristics of this subtype and determine potential targets for therapeutic strategies.

In general, cancer results from defects in main regulatory pathways controlling normal cell proliferation and homeostasis. Any atypical expression of essential pathways related genes might disturb homeostasis leading to uncontrolled cell division and increased proliferation rate, and are usually accompanied with high expression of Ki-67. Ki-67, also known as (*MKI-67*), is a protein strictly associated with cell proliferation and described as a prognostic parameter in BC patients (Scholzen and Gerdes, 2000, Inwald et al., 2013).

The "Hallmarks of Cancer" highlights several reasons that would lead to cancer development including gene mutations, proliferation, and apoptosis evasion (Hanahan and Weinberg, 2000, Hanahan and Weinberg, 2011). Mutations in genes encoding main regulatory components of signaling pathways might lead to active growth or reduced cell death. Most common cancer mutations include retinoblastoma (*Rb)*, *TP53*, phosphate/tensin homologue deleted on chromosome 10 *(PTEN)* genes and several others (Hanahan and Weinberg, 2000). *TP53*, the guardian of the genome, encodes p53 tumour suppressor protein that regulates transcription, cell cycle and apoptosis (Brady and Attardi, 2010). In BC, *TP53* mutations are mainly point mutations leading to loss of wild-type p53 function and over-expression of mutant p53 in tumour cells (Lacroix et al., 2006). Basal-like BCs often show accumulation of mutant p53 which has been reported as a clinico-pathological characteristic of TNBC or basal-like subtype (Rhee et al., 2008, Yadav et al., 2015, Anders and Carey, 2008). Moreover, in cancer, aberrant regulation of some genes, up-regulation or down regulation, may lead to evasion of apoptosis or escape of growth inhibitory signals and cell-cell interaction (Hanahan and Weinberg, 2000).

Several pathways have been identified to be up-regulated and involved in cancer proliferation and anti-apoptotic activation, including the phosphatidylinositol-3'-kinase (PI3K)-Akt signaling pathway (Vanhaesebroeck et al., 2016), mTOR signaling pathway JAK/STAT pathway (Populo et al., 2012), mitogen activated protein kinase pathway (Dhillon et al., 2007),

HSR (Sherman and Multhoff, 2007), unfolded protein response (UPR) (Tameire et al., 2015) and the NF-κB pathway (Rayet and Gelinas, 1999). The relevance and function of associated proteins and the main regulators of these pathways were identified and are further described below.

The phosphatidylinositol 3'-kinase(PI3K)-Akt signaling pathway is a pro-survival pathway described to be involved in cancer formation (Vanhaesebroeck et al., 2016). It is activated by several stimuli and regulates essential cellular processes such as transcription, translation, proliferation, growth and survival. Growth factors binding to their receptor tyrosine kinase or G-protein-coupled receptors (GPCR) stimulates PI3K isoforms, the PI3K catalytic subunit alpha, beta, gamma and delta, and regulated by PI3K regulatory subunits alpha, beta and gamma, catalyzing the production of phosphatidylinositol-3,4,5-triphosphate (PIP3) at the cell membrane. PIP3 acts as a second messenger activating AKT, hence controlling main cellular functions through phosphorylation of substrates involved in apoptosis, protein synthesis, metabolism and cell cycle (Engelman et al., 2006, Duronio, 2008, Hers et al., 2011).

mTOR is a conserved serine/threonine kinase and a downstream effector of the PI3K/AKT pathway. It constitutes the mTORC complexes, the rapamycin sensitive mTORC1 and the rapamycin insensitive mTORC2 (Populo et al., 2012). mTORC1 is activated by diverse stimuli enhancing cell growth, proliferation and survival; mTORC2 activates protein kinase C alpha (PRKCA) and AKT and regulates the actin cytoskeleton (Populo et al., 2012). There are multiple main elements of the mTOR pathway such as the ribosomal protein S6 kinase (RPS6KB1), a serine-threonine kinase which promotes cell growth and proliferation, the eukaryotic translation initiation factor 4E binding protein (EIF4EBP1) which interacts with eIF4E inhibiting complex assembly repressing translation, and eukaryotic translation initiation factor 4E (eIF4E) which acts as a proto-oncogene. Deregulation of these genes has been reported in many types of cancers, where changes in major components of the mTOR pathway were reported to have significant effects on tumour progression (Guertin and Sabatini, 2007, Populo et al., 2012).

The Janus kinase/signal (JAK/STAT) pathway is another pathway involved in cancer defined as a set of pleiotropic cascade signals essential for cellular development and homeostasis (Kiu and Nicholson, 2012). The JAK/STAT pathway is the principal signaling mechanism for a wide array of cytokines and growth factors. Upon cytokines binding to their cognate receptor, STATs (1 to 6) become activated by JAK family of tyrosine kinase members (Jak 1, 2 and 3); thus, they dimerize and translocate to the nucleus modulating target genes' expression. In addition, JAKs mediate the recruitment of other molecules such as the MAP

kinases and PI3 kinase resulting in the activation of additional transcription factors (Morrison, 2012, Kiu and Nicholson, 2012). STATs are also negatively regulated by protein inhibitors of activated STAT (PIAS), affecting several mechanisms in the nucleus (Shuai, 2006). Research has elucidated the role of JAK/STAT in various cellular processes such as proliferation, apoptosis and migration. It is important to note that frequent aberrant regulation of the JAK/STAT pathway has been found in diverse types of cancer (Anders and Carey, 2008).

The mitogen-activated protein kinase (MAPK) cascade is a highly conserved module involved in various cellular functions, including cell proliferation, differentiation and migration. There are at least four distinctly regulated groups of MAPKs, extracellular signal-related kinases (ERK)-1/2, Jun amino-terminal kinases (JNK1/2/3), p38 proteins (p38alpha/beta/gamma/delta) and ERK5. These genes are activated by specific mitogen-activated protein kinase kinases (MAPKK) such as MEK1/2 for ERK1/2, MKK3/6 for the p38, MKK4/7 or JNKK1/2 for the JNKs, and MEK5 for ERK5 (Chen et al., 2001). Abnormalities in MAPK signaling play a critical role in the development and progression of cancer. The ERK pathway is known to be responsible for cell proliferation, and is the best studied MAPK pathway. Evidence states that aberrant regulation of this pathway is linked to tumour phenotype and deregulated in approximately one-third of all human cancers (Dhillon et al., 2007). ERK is activated upon phosphorylation by MEK which is itself activated upon phosphorylation by Raf (Raf-1, B-Raf and A-Raf) (Dhillon et al., 2007).

In addition to the pathways mentioned above, the HSR, UPR and NF-κB pathways, discussed in detail in Chapter 1, are also known to play critical roles in cancer. It is generally known that the majority of tumours experience stressful conditions activating cellular stress-response pathways, including the HSR and the UPR. Recent studies have shown that the HSR and the UPR play important roles in tumourigenesis; however, the induction of HSR does not occur if the stress is too severe. HSPs, including HSP90, HSPA1 and HSPB1 are atypically expressed at high levels in many solid tumours and haematological malignancies (Calderwood and Gong, 2012). Activation of any of the UPR arms has been reported in several cancers, and many human tumour samples show an increased expression of ER chaperones and UPR target genes (Vandewynckel et al., 2013). These pathways participate in carcinogenesis and cell death resistance by inhibiting effector molecules of apoptosis (Jager et al., 2012, Graner et al., 2007). Therefore, targeting HSR and UPR using specific inhibitors is currently under investigation as a potential anti-cancer approach.

Finally, the NF-κB pathway is a evolutionary well-conserved pathway and one of the important inducible transcription factors essential in the regulation of immunity specific genes and hence the development of immunity (Hayden and Ghosh, 2012). In normal conditions,

NF-κB activation is a reaction to infection and inflammation; however, it can act as a pro-survival factor and promote tumourigenesis in cells along with other factors such as AP-1, and pronto-oncogenes like c-Fos and c-Jun (Kawai and Akira, 2006). Therefore, irregular activation of NF-κB is linked to cancer hallmarks promoting tumour growth and development (Perkins, 2012, Hanahan and Weinberg, 2011). Continuous NF-κB activation in tumour cells results from stable activation of the IKK complex or defect in the negative feedback inhibition mechanism (Perkins, 2007).

All of these pathways described have a pro-survival effect and can adversely regulate apoptosis enhancing cancer progression. Apoptosis, described in detail in Chapter 1, is a cell death process regulated by pro-survival pathways such as the PI3K pathway, HSR, UPR and NF-κB (Elmore, 2007, Duronio, 2008, Zhang et al., 2015, Baldwin, 2012, Galluzzi et al., 2012). Cancer cells have adapted anti-apoptotic mechanisms to resist cell death, through activation of survival pathways and over-expression of key proteins that inhibit pro-apoptotic proteins or the activity of the apoptotic effectors (Hanahan and Weinberg, 2011).

The focus of this chapter is to elucidate the factors that characterize basal-like BCs, enhance their anti-apoptotic and pro-survival function and may be involved in progression and worse prognosis. This is done by integrative analyses of patient gene expression databases.

The specific aims of this chapter include:

- Compare the role of pro-survival pathways among different BC subtypes using gene expression analysis.
- Identify genes that are overexpressed in basal-like BC subtype and their association with adverse-event (AE) free survival and various histological markers including tumour size, tumour grade, lymph node (LN) involvement and clinical outcome.
- Identify genes that are associated with *TP53* and Ki67 proliferation marker expression in BC

3.2. Results

3.2.1. Patient databases

Patient mRNA protein expression databases were downloaded from NCBI Gene Expression Omnibus (GEO) and outlined in Table 3.1. mRNA expression level of genes associated with proliferation and anti-apoptotic pathways including PI3K-AKT signaling pathway, mTOR signaling pathway, JAK/STAT pathway, MAPK pathway, HSR, UPR and the NF-κB pathway were analyzed. Genes included in the analysis of this chapter and their functions are outlined in Table 3.2. Data collected from databases include patients' number, BC subtype, *TP53* gene status and histological markers including tumour size, tumour stage and lymph node (LN) involvement. Clinical survival outcomes outlined as adverse-event (AE) free survival includes overall survival, metastasis free survival and relapse free survival. In total 16 databases were analyzed with total of 2518 patients.

Table 3.1: Datasets downloaded from online GEO database.

DATASET	Cohort	Contributor	Array type	n
GSE19615	DF/HCC	Li	HG-U133_Plus_2	115
GSE12276	EMC	Bos	HG-U133_Plus_2	204
GSE6532-GPL570	GUYT	Loi	HG-U133_Plus_2	87
GSE9195	GUYT2	Loi	HG-U133_Plus_2	77
GSE12093	IO, NCI, TUM, CCF (1992-2000)	Zhang	HG-U133A	136
GSE11121	Mainz (1988-1998)	Schmidt	HG-U133A	200
GSE1378	MGH (1987-2000)	Ma	Arcturus 22k	60
GSE1379	MGH (1987-2000)	Ma	Arcturus 22k	60
GSE9893	Montpellier, Bordeaux, Turin (1989-2001)	Chanrion	MLRG Human 21K V12.0	155
GSE2034	Rotterdam (1980-1995)	Wang	HG-U133A	286
GSE1456-GPL96	Stockholm (1994-1996)	Pawitan	HG-U133A	159
GSE7378	UCSF	Zhou	U133AAofAv2	54
E-TABM-158	UCSF, CPMC (1989-1997)	Chin	HG-U133A	117
GSE3494-GPL96	Uppsala (1987-1989)	Miller	HG-U133A	236
GSE4922-GPL96	Uppsala (1987-1989)	Ivshina	HG-U133A	249
GSE2990	Uppsala, Oxford	Sotiriou	HG-U133A	125
GSE7390	Uppsala, Oxford, Stockholm, IGR, GUYT, CRH (1980-1998)	Desmedt	HG-U133A	198

Table 3.2: Pathways and genes included in the analysis.

Pathway	Gene	Function
PI3K/AKT	*PIK3CA* *PIK3CB* *PIK3CD* *PIK3CG*	PI3K isoforms alpha beta and gamma and delta. Oncogenes, activate signaling cascades involved in cell growth, survival, proliferation, motility and morphology
	PIK3R1 *PIK3R2* *PIK3R3*	Isoforms of PI3K regulatory protein. It phosphorylates the inositol ring of phosphatidylinositol at the 3' position.
	AKT *(1, 2, 3)*	Serine/threonine-specific protein kinase that plays a key role in multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation, transcription and cell migration. AKT isoforms are overexpressed in a variety of human tumors
mTOR	*mTOR*	Serine/threonine kinase that is present in two distinct complexes. It promotes cellular growth by phosphorylating substrates that potentiate anabolic processes such as mRNA translation and lipid synthesis.
	RPTOR	Regulatory-associated protein of mTOR
	RICTOR	Subunit of mTOR2, phosphorylates AKT
	RPS6KB 1	It is also known as S6K1. It binds to eIF3 and detaches following phosphorylation by mTOR/Raptor. Free S6K1 is then able to phosphorylate a number of its targets, including eIF4B promoting protein synthesis and cell proliferation
	EIF4EBP 1	Repressor of translation initiation that regulates EIF4E activity by preventing its assembly into the eIF4F complex
	eIF4E	Eukaryotic translation initiation factor involved in directing ribosomes to the cap structure of mRNAs. Its activity is implicated in mitosis, embryogenesis and apoptosis. It is overexpressed in several human cancers
	PRKCA	It is a member of protein kinase C (PKC) family of serine-threonine specific protein kinases. Increased activation of PRKCA is associated with the growth and invasion of cancers
JAK/STAT	*STAT* *(1, 2, 3)*	Signal transducer and activator of transcription family are intracellular transcription factors that mediate many aspects of cellular immunity, proliferation, apoptosis and differentiation
	JAK *(1, 2, 3)*	Janus kinases, activate STATs and implicated in angiogenesis increase and tumor survival
	PIAS *(1, 2, 3)*	Protein inhibitor of activated STAT
NF-κB	*NFKB1* *NFKB2*	Protein complex that controls transcription of DNA, cytokine production and cell survival. Activation of NFkB is linked to cancer development
	RelA *RelB*	Subunits of the NFkB complex, v-rel avian reticuloendotheliosis viral oncogene homolog A or B
	IKBK *(B, G, E)*	NFkB kinase inhibitors

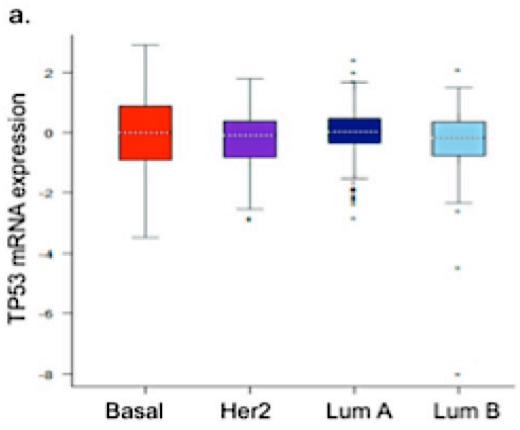
Table 3.2.1b continued…

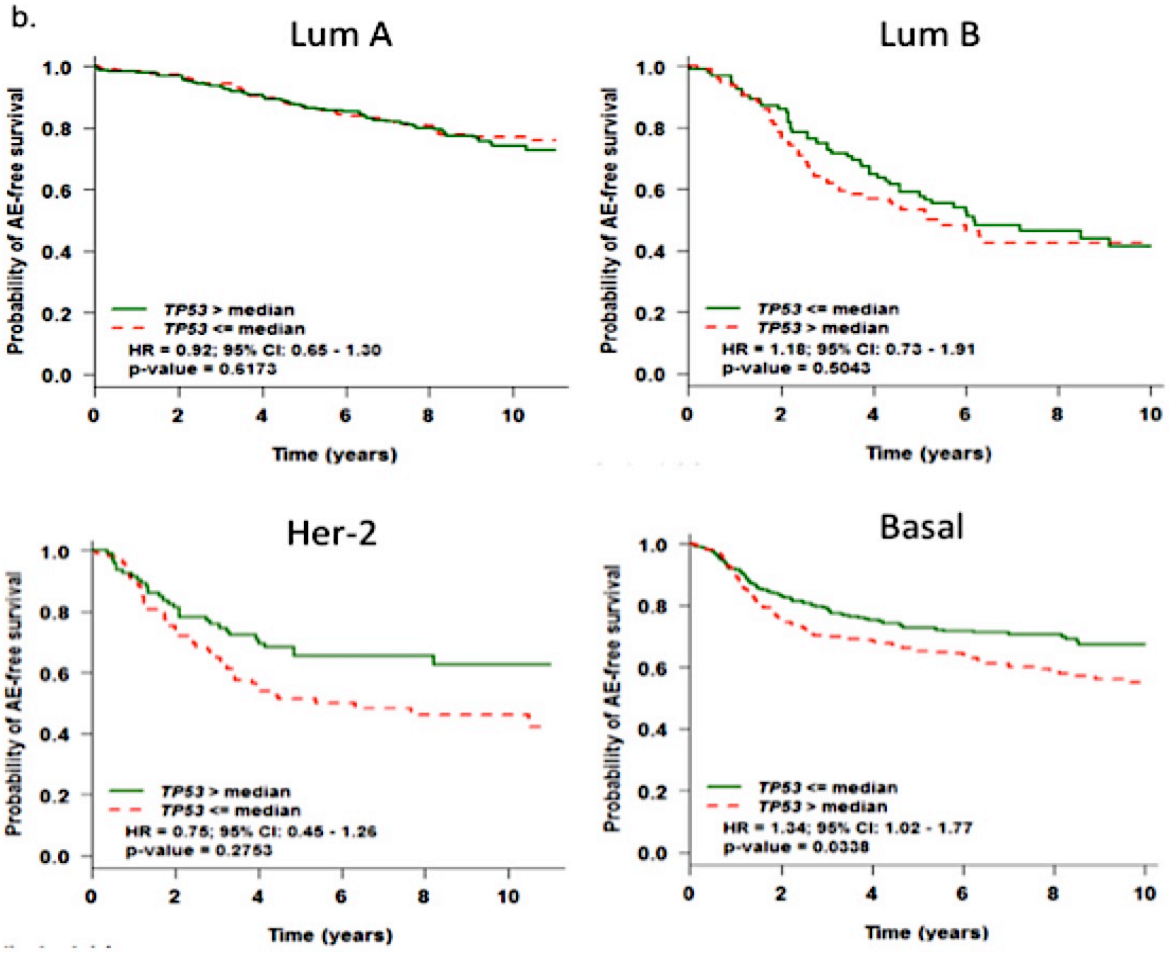
HSR	*HSF (1, 2, 5)*	Isoforms of HSF transcription factor that regulate the expression of the heat shock proteins
	HSP90 (AA1, AB1, B1)	HSP90 isoforms responsible for proteins folding, stabilization, and aids in protein degradation. It also stabilizes a number of proteins required for tumor growth
	HSPA (1A, 1B, 2)	Hsp70 isoforms that are an important for protein folding, and help to protect cells from stress. Implicated in several cancers
	HSPB (1, 2, 3)	HSP27 isoforms, responsible for are chaperone activity, thermotolerance, inhibition of apoptosis, regulation of cell development, and cell differentiation
UPR	*IRE (1 and 2)*	Isoforms of IRE protein and possesses intrinsic kinase activity and an endoribonuclease activity and it is important in altering gene expression as a response to endoplasmic reticulum-based stress signals
	XBP1	Transcription factor that regulates the expression of genes important to the proper functioning of the immune system and in the cellular stress response.
	EIF2AK3	Eukaryotic translation initiation factor 2 alpha kinase 3, also known as PERK, pro-apoptotic
	eIF2a	Eukaryotic translation initiation factor implicated in cancer
	ATF4	Activating transcription factor 4, overexpressed in several cancers
	CHOP	Pro-apoptotic, down regulates the anti-apoptotic mitochondrial proteins causing mitochondrial damage, cytochrome c release and caspase-3 activation.
	ATF6	Stress-regulated transmembrane transcription factor that activates the transcription of ER molecules.

3.2.2. *TP53* and basal-like BC

- ***TP53 mRNA expression in basal-like BC is associated with poor prognosis***

Patient databases were analyzed for *TP53* mRNA expression. No significant difference in *TP53* expression between the subtypes was observed (Figure 3.1a) with no significant effect on AE-free survival in HER-2, Lum A and Lum B BC subtypes; however, in basal-like BC subtype, higher *TP53* levels had a worse prognosis ($p = 0.03$) as indicated by probability of AE-free survival (Figure 3.1b). Moreover, high *TP53* mRNA expression was associated with higher percentage of patients with increased tumour grade and LN metastasis in basal-like BCs (Figure 3.1c).


a.
TP53 mRNA expression
Basal
Her2
Lum A
Lum B


b.
Lum A
Probability of AE-free survival
TP53 > median
TP53 <= median
HR = 0.92; 95% CI: 0.65 - 1.30
p-value = 0.6173
Time (years)
Lum B
Probability of AE-free survival
TP53 <= median
TP53 > median
HR = 1.18; 95% CI: 0.73 - 1.91
p-value = 0.5043
Time (years)
Her-2
Probability of AE-free survival
TP53 > median
TP53 <= median
HR = 0.75; 95% CI: 0.45 - 1.26
p-value = 0.2753
Time (years)
Basal
Probability of AE-free survival
TP53 <= median
TP53 > median
HR = 1.34; 95% CI: 1.02 - 1.77
p-value = 0.0338
Time (years)

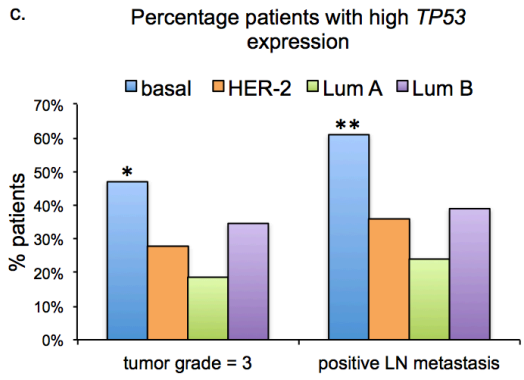
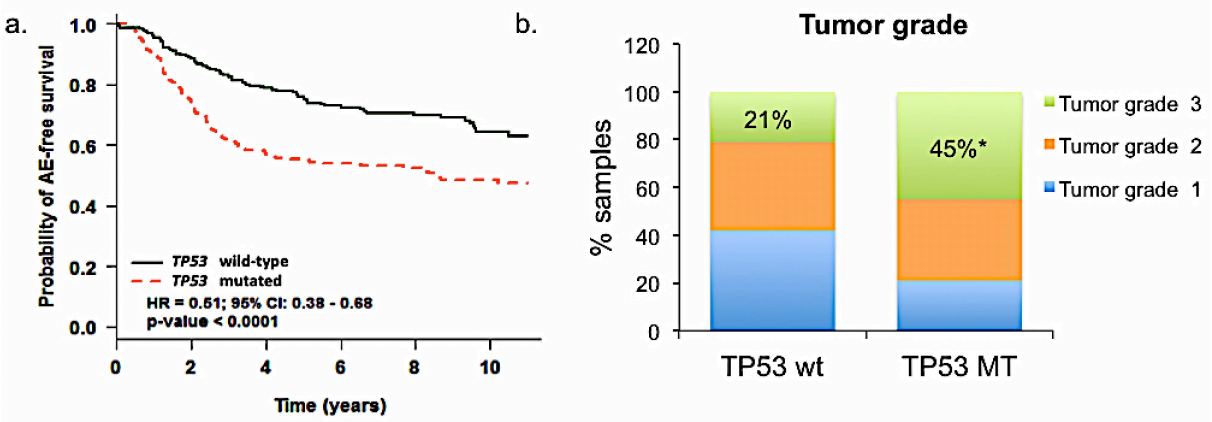

c.
Percentage patients with high TP53 expression
basal
HER-2
Lum A
Lum B
% patients
*
**
tumor grade = 3
positive LN metastasis

Figure 3.1: *TP53* mRNA expression is associated with worse prognosis in basal-like BC.

*a. Patient databases analyzed for TP53 mRNA expression in different BC subtypes (basal-like, HER-2 positive, Luminal A (Lum A) and Luminal B (Lum B)); b. Kaplan-Meier survival analysis based on TP53 expression. Probability of adverse event (AE) free survival in different BC subtypes was stratified based on TP53 mRNA expression relative to mean (higher than mean (> mean) or lower or equal to mean (< mean)); c. Tumour grade and lymph mode metastasis association with BC subtypes and high TP53 mRNA expression. Statistical significance was calculated using Kruskal-Wallis analysis, * indicates p < 0.05 and ** indicates p < 0.01.*

- ***TP53 mutation in BC is associated with poor prognosis and higher tumour grade***

Previous studies have reported p53 expression as a prognostic marker of metastatic BC (Yang et al., 2013, Dookeran et al., 2010), however, this high expression is due to accumulation of mutant form of p53 in the nucleus, since wild-type p53 has a short life time and is degraded fast (Alsner et al., 2008, Yamashita et al., 2006). Patient data was further analyzed based on *TP53* mutation status. Only three databases E-TABM-158, GSE3494-GPL96 and GSE4922-GPL96 identified *TP53* mutations in 159 patients (26.4%). These databases were only included in this analysis. Mutated *TP53* was associated with worse AE-free survival p-value < 0.001 (Figure 3.2a). Moreover, mutated *TP53* was associated with higher tumour grade where 45% of samples of mutated *TP53* are high-grade tumours and poorly differentiated (grade 3), while only 21% of wild-type *TP53* are grade 3 tumours (p < 0.01) (Figure 3.2b). Moreover, patients with mutated *TP53* have significantly higher tumour size and more LN metastasis (Figure 3.2c).



c.

	average tumor size (cm)	positive LN metastasis
TP53 wt	2.36	27%
TP53 MT	3.91	41%
	p = 0.03	p = 0.57

Figure 3.2: *TP53* mutation is associated with worse prognosis and higher tumour grade.

*a. Kaplan-Meier adverse event (AE) free survival analysis based on TP53 status (wild-type or mutated); b. Tumour grade distribution among BC patients based on TP53 status (MT: mutated, wt: wild-type); c. Average tumour size and LN involvement in BC patients based on TP53 status. p-value was calculated using One-way Anova, * indicates p < 0.05.*

3.2.3. *MKI-67* mRNA expression is associated with basal-like BC subtype

Ki-67 is a marker of proliferation and is used as a histo-pathological marker for cancer and a predictor of poor prognosis in TNBC (Li et al., 2015b, Li et al., 2015a). In this analysis, MKI-67 mRNA expression was higher in basal-like BC patient samples (Figure 3.3a); 71% of patients with basal-like phenotype express high levels of *MKI-67* mRNA (Table 3.3) and associated with poor prognosis (Figure 3.3b). However, no association with *MKI-67* expression was observed in other BC subtypes.

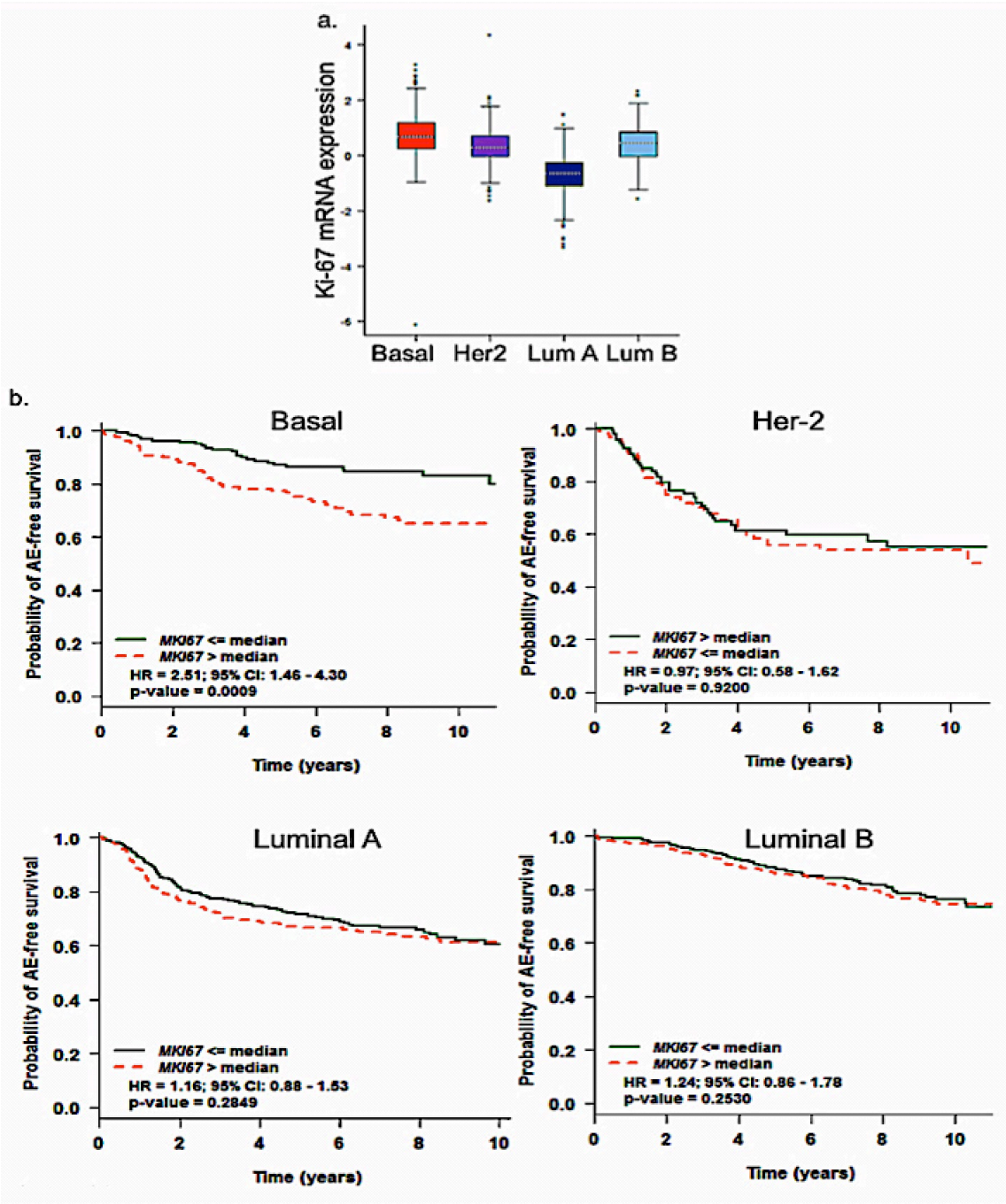


Figure 3.3: *MKI-67* mRNA expression in BC subtypes.

Subtypes: (basal-like, HER-2 positive (HER-2), Luminal A (Lum A) and Luminal B (Lum B)); a. Box plot expression of MKI-67 mRNA in different BC subtypes; b. Kaplan-Meier survival analysis based on MKI-67 expression level in BC subtypes. Probability of adverse event (AE) free survival in different BC subtypes was stratified based on mRNA expression level relative to mean (higher than mean (> mean) or lower or equal to mean (≤ mean)).

Table 3.3: Percentage patients expressing high *MKI-67* mRNA

MKI-67 gene expression	Percentage Patient Samples of Molecular Subtype			
	Basal-like	HER-2	Luminal A	Luminal B
High	71%**	44%	4%	25%

3.2.4. PI3K/AKT signaling pathway in basal-like BC

- ***PI3K/AKT signaling pathway gene expression in BC is not associated with subtype***

In order to determine whether the PI3K/AKT pathway has any impact on BC basal-like subtype, patient databases were analyzed for mRNA expression of PI3K isoforms alpha (*PIK3CA*), beta (*PIK3CB*), gamma (*PIK3CG*), and delta (*PIK3CD*), PI3K regulatory subunits alpha (*PIK3R1*), beta (*PIK3R2*), and gamma (*PIK3R3*), and *AKT1*, *AKT2* and *AKT3*. No significant difference in the expression of the four PI3K isoforms or the regulatory PI3K forms was observed among the different BC subtypes (Figure 3.4a and b). Similarly, as shown in Figure 3.4c, AKT levels were comparable among the different BC subtypes except for *AKT1* which was significantly higher in HER-2 BC subtype with ($p < 0.05$).

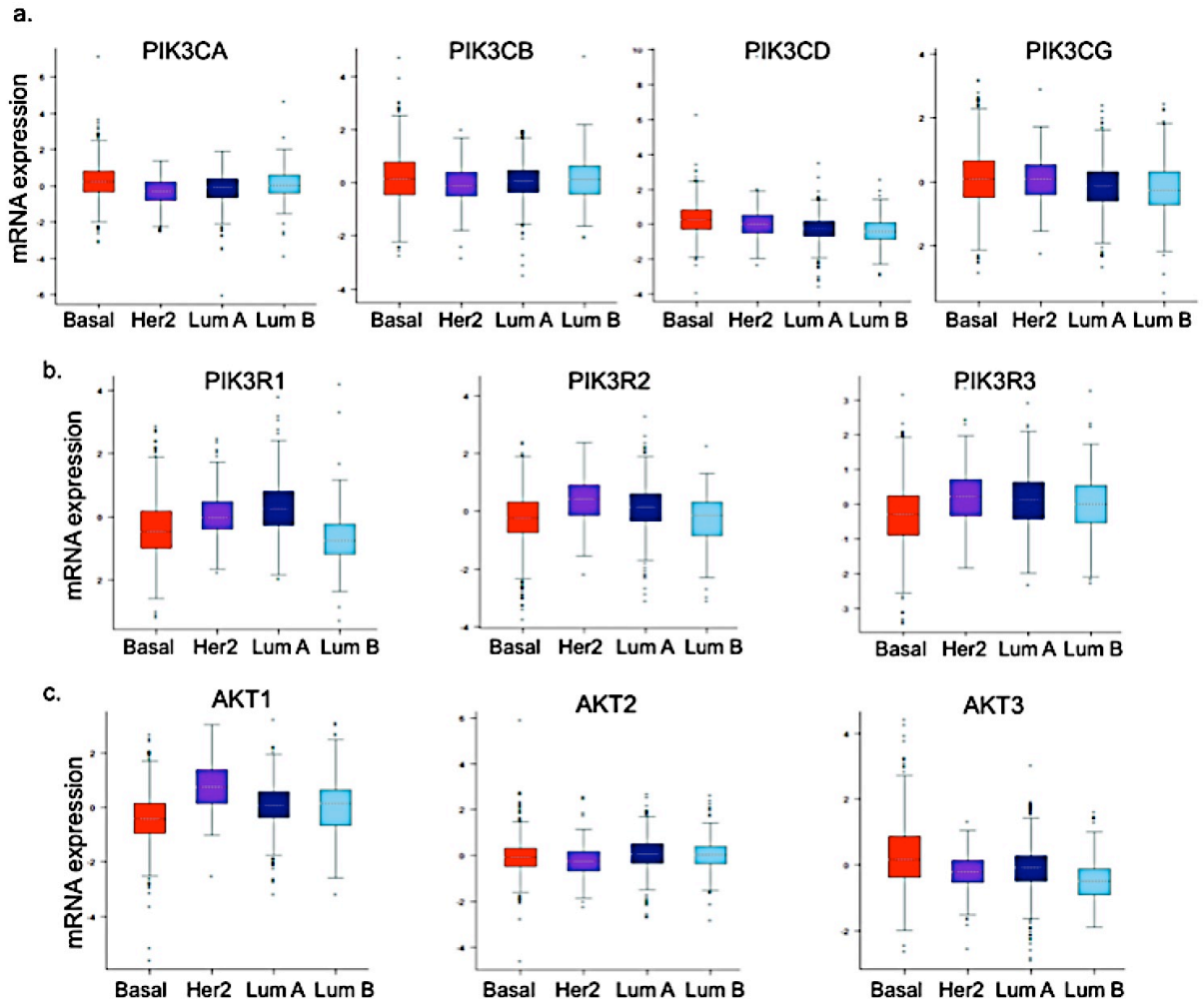


Figure 3.4: PI3K/AKT associated mRNA expression in BC subtypes.

Subtypes: (basal-like, HER-2 positive (HER-2), Luminal A (Lum A) and Luminal B (Lum B)); a. Box plot expression of PI3K isoforms along different BC subtypes; b. Box plot expression of PI3K regulatory subunits along different BC subtypes; c. Box plot expression of AKT1, AKT2 and AKT3 along different BC subtypes.

- ***PIK3CA and PIK3CD mRNA levels are high in basal-like BC but do not affect patient outcome***

Patient samples were further analyzed for high mRNA expression levels of PI3K/AKT related genes, where the mean expression was considered the cut-off point; 46% of basal-like and 37% of luminal B BC subtypes express significantly high *PIK3CA* mRNA levels, 48% of basal-like BC express significantly high *PIK3CD* mRNA levels, 49% of luminal A subtype express significantly *PI3KR1* mRNA high levels, 59% and 68% of HER-2 subtypes express significantly *PIK2R3* and *AKT1* mRNA high levels, and 46% of basal-like subtype express significantly more *AKT3* mRNA high level (Table 3.4). Furthermore, observing the effect of higher protein expression on AE-free survival, Kaplan-Meier survival plots shows only significant impact on decreased AE-free survival in luminal B BC subtype with mRNA *PIK3R2* expression level higher than mean, and in HER-2 subtype with *PIK3R3* mRNA expression level higher than mean (Figure 3.5). Moreover, in HER-2 subtype decreased AE-free survival was observed with *PIK3CG* mRNA levels less than mean (Figure 3.5). No significant effect on AE-free survival was observed in other BC subtypes.

Table 3.4: Percentage patients expressing high mRNA of AKT related genes

High mRNA gene expression	Percentage Patient Samples of Molecular Subtype			
	Basal-like	HER-2	Luminal A	Luminal B
PIK3CA	46%*	17%	29%	37%*
PIK3CG	40%	39%	26%	25%
PIK3CD	48%*	37%	20%	19%
PIK3CB	42%	29%	36%	41%
PIK3R1	20%	28%	45%*	9%
PIK3R2	21%	43%	37%	32%
PIK3R3	25%	59%*	39%	26%
AKT1	18%	68%*	36%	36%
AKT2	29%	20%	39%	37%
AKT3	46%*	15%	25%	11%

*High levels of mRNA were considered as higher than mean. * indicates p-values less than 0.05 as calculated by One-way Anova.*

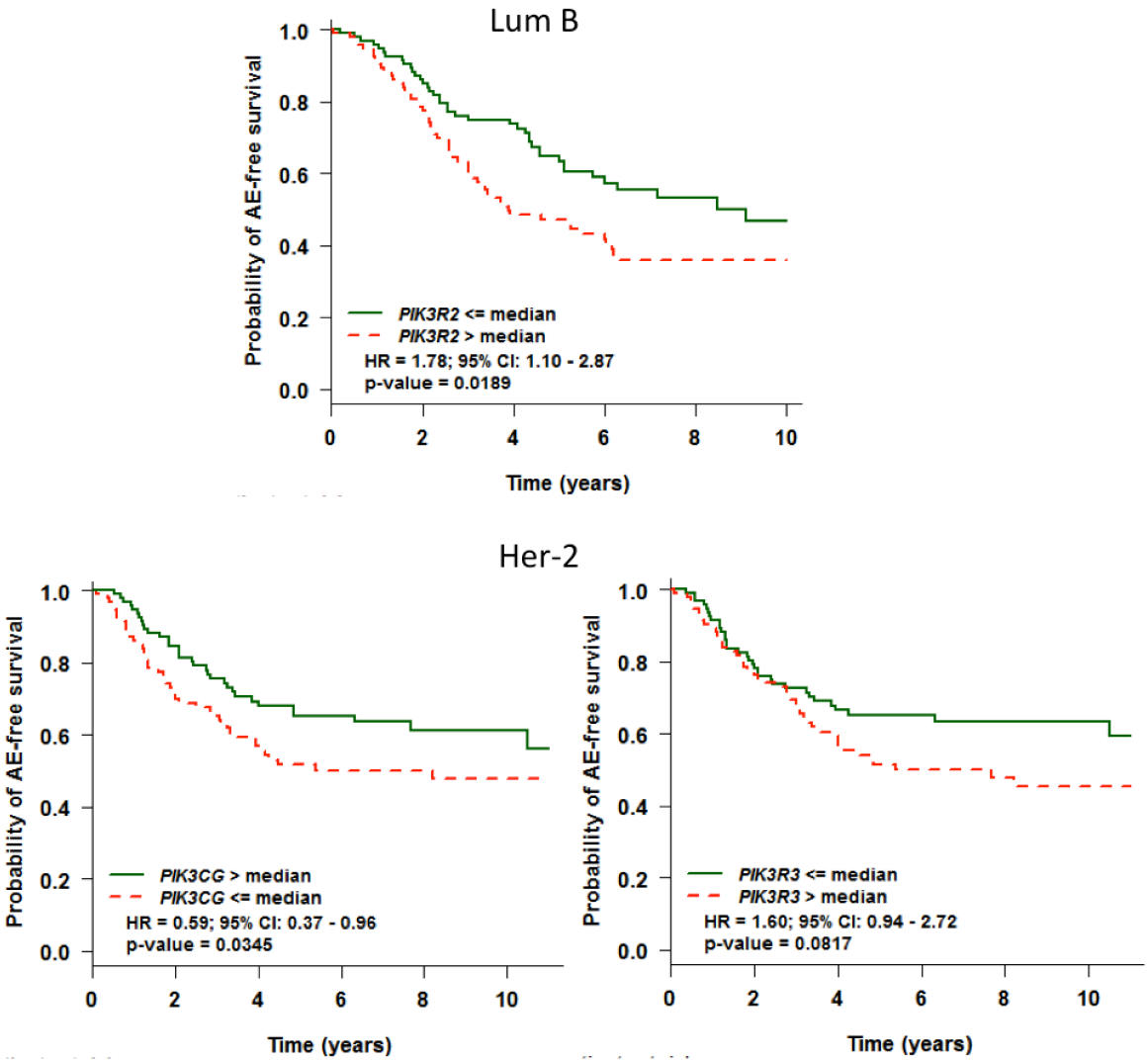


Figure 3.5: Impact of PI3K/AKT associated mRNA expression levels on BC subtypes.

Kaplan-Meier survival analysis based on PIK3R2 expression level in luminal B subtype, and PIK3R3 and PIK3CG in HER-2 subtype. Probability of adverse event (AE) free survival in different BC subtypes was stratified based on mRNA expression level relative to mean (higher than mean (> mean) or lower or equal to mean (≤ mean).

3.2.5. *mTOR* signaling pathway in basal-like BC

- ***mTOR signaling pathway genes expression is not associated with basal-like subtype***

Aberrant mTOR signaling is involved in the development of several cancers (Populo et al., 2012). BC databases were analyzed for mRNA expression of mTOR pathway genes and their impact on different BC subtypes. Starting with genes expressing proteins composing the mTOR complexes, *mTOR*, *RPTOR*, associated with mTOR complex 1, and *RICTOR*, associated with mTOR complex 2, expression of these genes is not different among the different BC patient subtypes (Figure 3.6a). Similarly, the mRNA expression of downstream

effectors of mTOR pathway the *RPS6KB1*, *eIF4E* and *PRKCA* is relatively equivalent in the different subtypes; however, the eIF4E inhibitory protein *EIF4BP1* had significantly lower expression in luminal A BC subtype (Figure 3.6b).

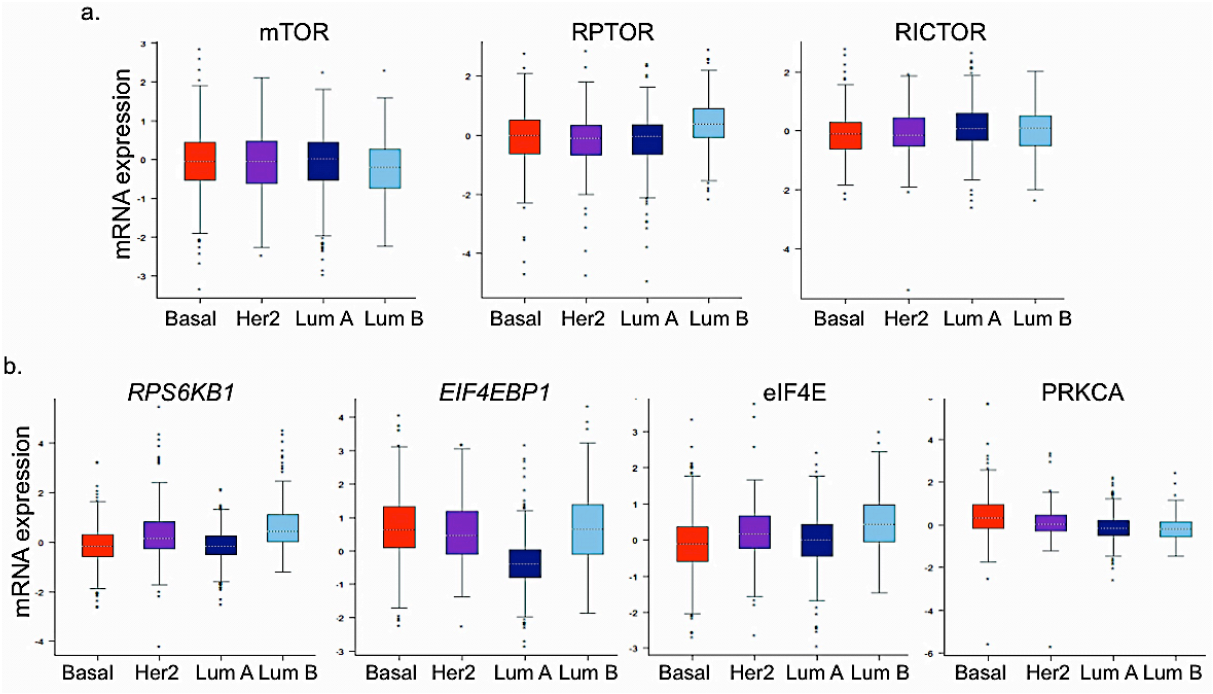


Figure 3.6: mTOR pathway associated mRNA expression levels in BC subtypes.

Subtypes: (basal-like, HER-2 positive (HER-2), Luminal A (Lum A) and Luminal B (Lum B). a. Box plot mRNA expression of mTOR, RPTOR and RICTOR in different BC subtypes; b. Box plot expression of RPS6KB1, EIF4EBP1, eIF4E and PRKCA in different BC subtypes.

- ***mTOR associated mRNA is not linked to basal-like subtype prognosis***

To further determine the impact of mTOR pathway in development and prognosis of basal-like BC, subtypes were compared based on percentage of samples expressing mRNA higher than mean, 53% of patients with basal-like subtype showed significantly higher *PRKCA* mRNA levels in comparison to all other subtypes and 46% percent of patient samples with HER-2 subtype express higher levels of transcription factor *RPS6KB1* as outlined in Table 3.2.8. Significantly, less patient samples with luminal A subtypes express high levels of the eIF4E inhibitor (*EIF4EBP1*) only 11% in comparison to more than 50% of patients with other 3 subtypes (Table 3.5). As for luminal B subtypes, significantly more patients express high levels of the *RPTOR* (54%), *RPS6KB1* (58%) and *eIF4E* (59%) (Table 3.5). No significant difference in percentage of patients expressing high levels of other mRNA was identified between the different subtypes.

Table 3.5: Percentage patients expressing high mRNA of AKT related genes

High mRNA gene expression	Percentage Patient Samples of Molecular Subtype			
	Basal-like	HER-2	Luminal A	Luminal B
mTOR	31%	34%	35%	24%
RPTOR	34%	27%	26%	54%*
RICTOR	26%	29%	38%	35%
RPS6KB1	27%	46%*	24%	59%*
EIF4EBP1	61%	53%	11%*	58%
eIF4E	27%	38%	30%	58%*
PRKCA	53%*	34%	22%	19%

*High levels of mRNA were considered as higher than mean. * indicates p-values less than 0.05 as calculated by One-way Anova.*

Observing the impact of gene expression on AE-free survival, only low levels of *RPS6KB1* had significantly poorer outcome in patients with HER-2 subtype ($p = 0.04$) and high levels of *RPS6KB1* had a significantly poorer outcome in patients with luminal A subtype ($p = 0.06$) (Figure 3.7a). Observing the impact of mTOR associated genes expression on tumour size, grade and LN involvement, patient samples with mRNA expression higher than mean were analyzed and presented in Figure 3.2.7b. No significant impact of any of high levels of mTOR associated genes expression was observed on tumour size in any subtype. More patients with grade 3 tumours are associated with high levels of *RICTOR* expression in HER-2 and luminal B BC subtypes ($p < 0.05$), with high levels of *RPTOR* in luminal A and luminal B subtypes and with high expression of *eIF4E* in luminal B subtype ($p < 0.01$). However, significantly less patients with grade 3 tumours are observed with high *EIF4EBP1* expression levels in basal-like subtype (Figure 3.7b). Finally, only high levels of *eIF4E* mRNA have significant effect on LN involvement in HER-2 BC patients (Figure 3.7b).

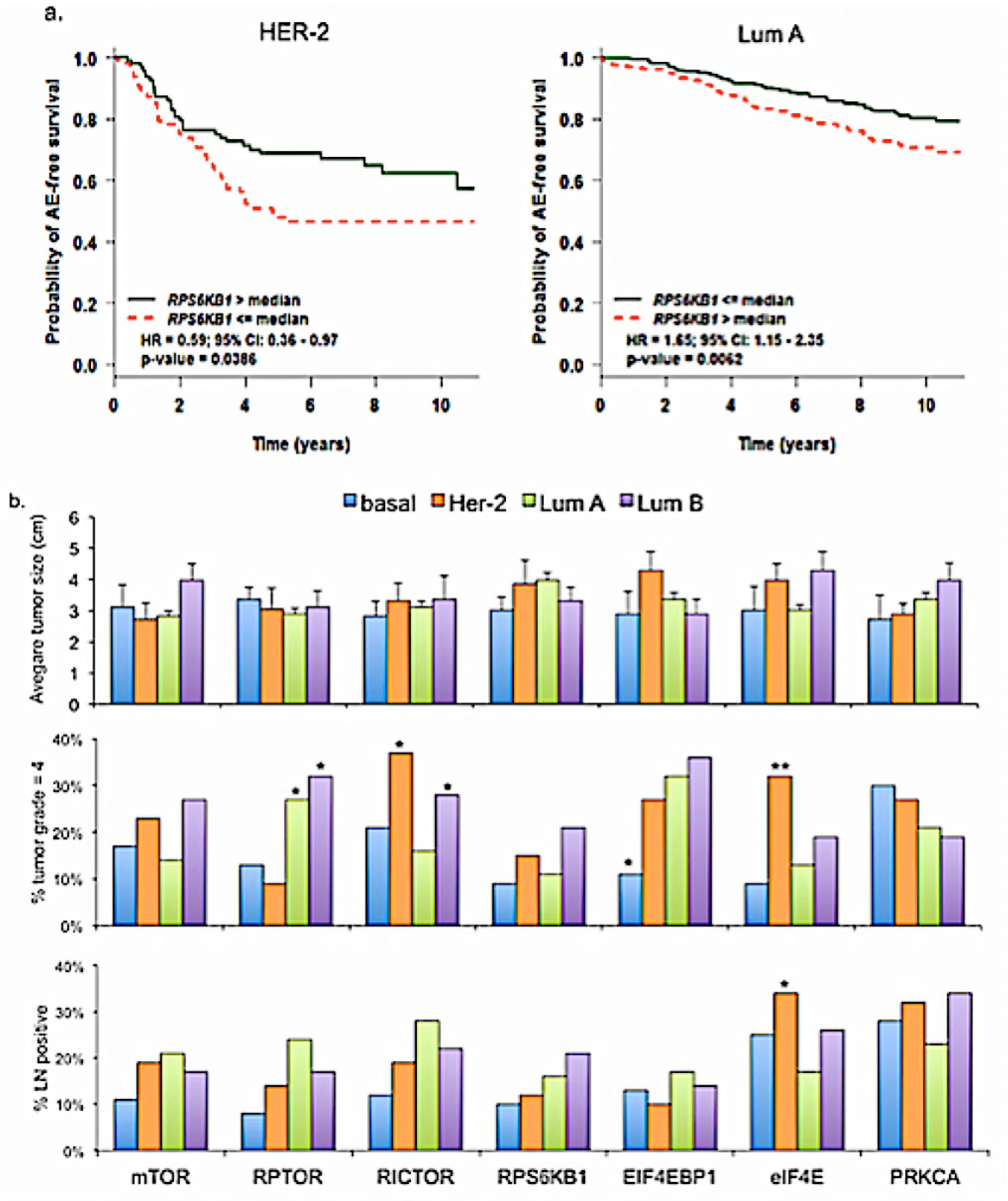


Figure 3.7: mTOR pathway associated mRNA expression levels and BC subtypes.

*a. Kaplan-Meier survival analysis based on RPS6KB1 mRNA expression level in HER-2 and Lum B subtypes. Probability of adverse event (AE) free survival in different BC subtypes was stratified based on mRNA expression level relative to mean (higher than mean (> mean) or lower or equal to mean (<mean)); b. Impact of mTOR pathway associated mRNA high levels on average (Av) tumour size ((cm), tumour grade 3 and LN involvement in different BC subtypes. * indicates p-values less than 0.05 and ** indicates p-values less than 0.05 as calculated by One-way Anova determining significant difference in expression among the subtypes.*

3.2.6. JAK/STAT signaling pathway in basal-like BC

- ***JAK/STAT pathway mRNA expression in BC is not associated with subtype***

Over-activation of the JAK-STAT pathway can cause cancer by bypassing apoptosis and cell cycle checkpoints (Springuel et al., 2015). BC databases were analyzed for JAK/STAT pathway genes mRNA expression and their impact on different BC subtypes. mRNA expression level of the different STAT isoforms (1, 2 and 3), luminal A BC subtype express significantly lower level of *STAT1* mRNA (Figure 3.8a) while no significant difference in mRNA expression of JAK isoforms (1, 2 and 3) or STAT inhibitor *PIAS* (1,2 and 3) was observed among the different subtypes (Figures 3.8 b and c).

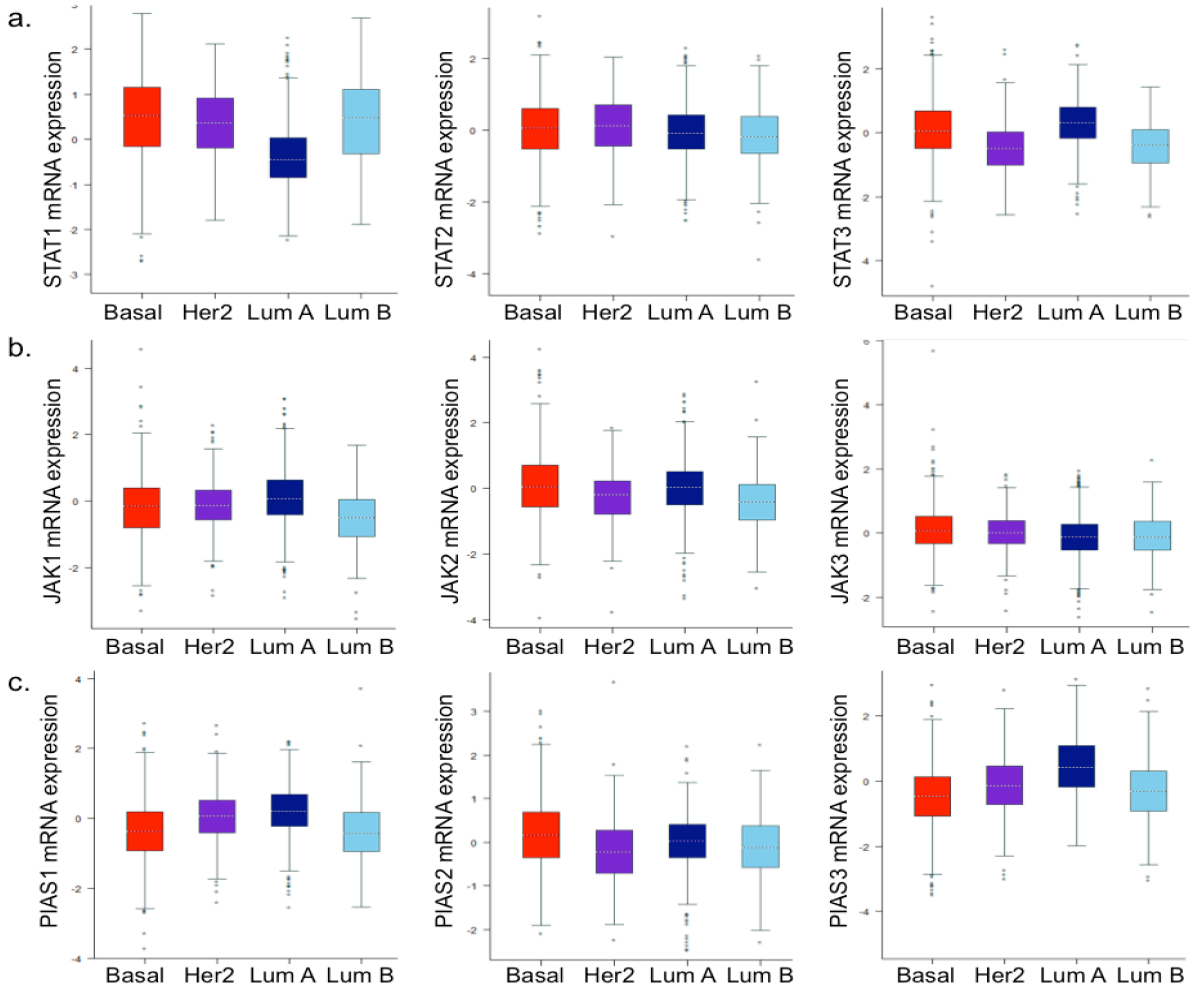


Figure 3.8: JAK/STAT pathway associated mRNA expression levels in BC subtypes.

Subtypes: (basal-like, HER-2 positive (HER-2), Luminal A (Lum A) and Luminal B (Lum B)); a. Box plot expression of STAT1, STAT2 and STAT3 along different BC subtypes; b. Box plot expression of JAK1, JAK2 and JAK3 along different BC subtypes; c. Box plot expression of PIAS1, PIAS2 and PIAS3 along different BC subtypes .

- ***JAK/STAT pathway gene expression is not associated with basal-like BC subtype***

Patient samples compared based on high mRNA expression showed that 40% of patients with basal-like subtype express significantly high *STAT3* mRNA and 41% of patients with basal-like subtype express significantly high JAK2 levels in comparison to all other subtypes. Also, significantly more patients with luminal A subtype express higher mRNA levels of *STAT3* (50%) and *PIAS3* (52%) and only 15% of patients with luminal A subtype express high levels of *STAT1* (Table 3.6).

Table 3.6: Percentage patients expressing high mRNA of JAK/STAT pathway related genes

High mRNA gene expression	Percentage Patient Samples of Molecular Subtype			
	Basal-like	HER-2	Luminal A	Luminal B
STAT1	55%	48%	15%*	53%
STAT2	36%	42%	29%	26%
STAT3	40%*	16%	50%*	17%
JAK1	27%	24%	38%	15%
JAK2	41%*	24%	35%	19%
JAK3	38%	35%	26%	29%
PIAS1	21%	35%	41%	19%
PIAS2	46%	26%	35%	29%
PIAS3	17%	27%	52%*	23%

*High levels of mRNA were considered as higher than mean. * indicates p-values less than 0.05 as calculated by One-way Anova.*

Observing the impact of gene expression on AE-free survival, high levels of *STAT1* and *JAK2* in basal-like BC, high level of *JAK2* in HER-2 subtype, high level of *STAT3* in luminal A subtype and high levels of JAK1, 2 and 3 in luminal B subtype have better prognosis as observed with higher probability of AE-free survival (Figure 3.9 a and b, and 3.10). Observing the impact of JAK/STAT associated genes expression on tumour size, grade and LN involvement, patient samples with mRNA expression more than mean were analyzed and presented in Figure 3.10. High expression of STAT3 was associated with significantly larger tumour size in HER-2 patients. Increased number of patients with grade 3 tumours was associated with high STAT2 mRNA expression in luminal A and B subtypes, with high STAT3, JAK2 and PIAS3 mRNA expression in HER-2 subtype. Moreover, significantly fewer patients with tumour grade 3 were observed with high PIAS2 mRNA expression in basal-like BC. No significant impact of high JAK/STAT associated genes expression was observed among different subtypes on LN involvement (Figure 3.11).

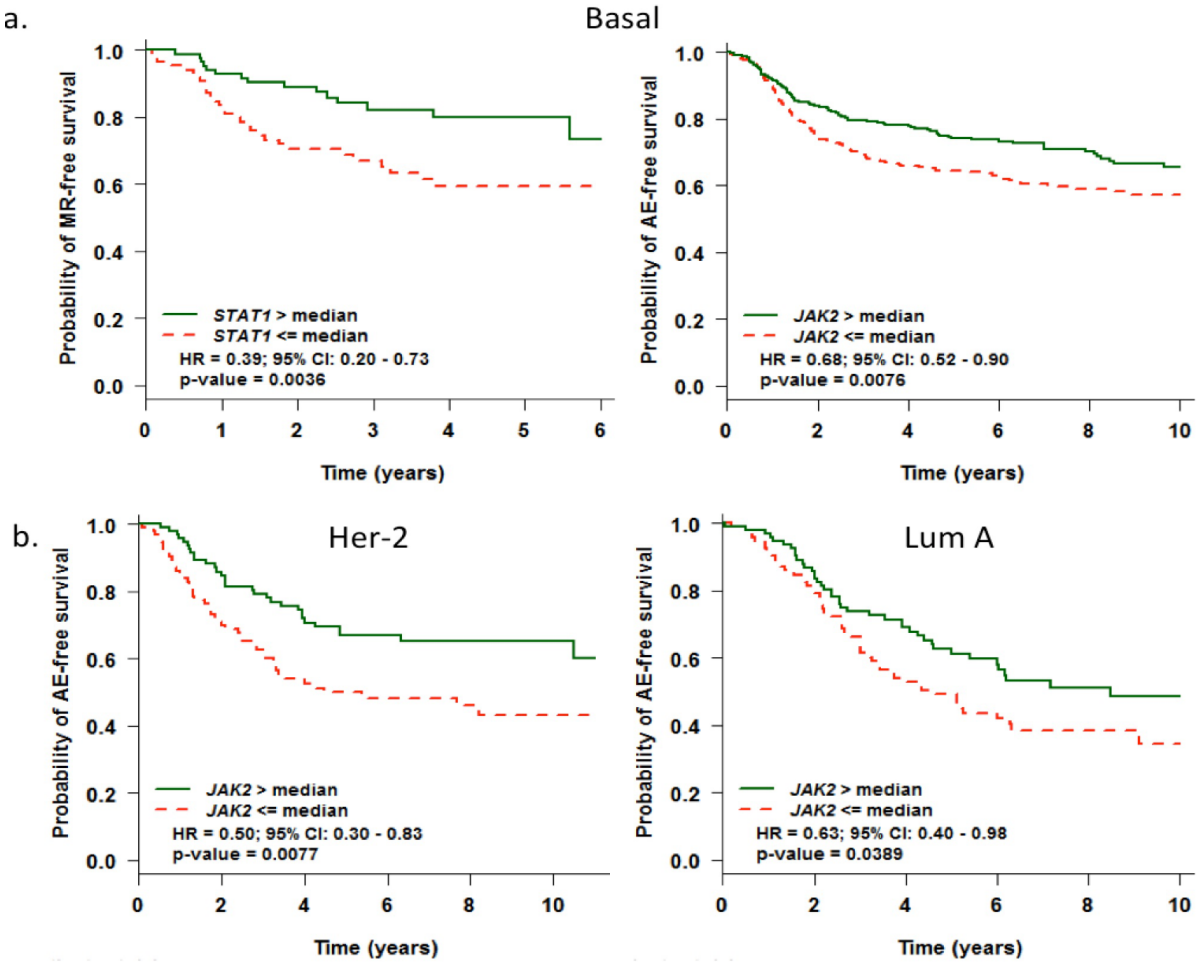


Figure 3.9: Impact of JAK/STAT pathway associated mRNA expression levels on BC subtypes prognosis.

a. Kaplan-Meier survival analysis of basal-like subtype based on mRNA expression level of STAT1 and JAK2. Probability of adverse event (AE) free survival in different BC subtypes was stratified based on mRNA expression level relative to mean (higher than mean (> mean) or lower or equal to mean (≤ mean)); b. Kaplan-Meier survival analysis of based on JAK2 mRNA expression level in HER-2 subtype and of STAT3 in luminal A subtype. Probability of adverse event (AE) free survival in different BC subtypes was stratified based on mRNA expression level relative to mean (higher than mean (> mean) or lower or equal to mean (≤ mean)).

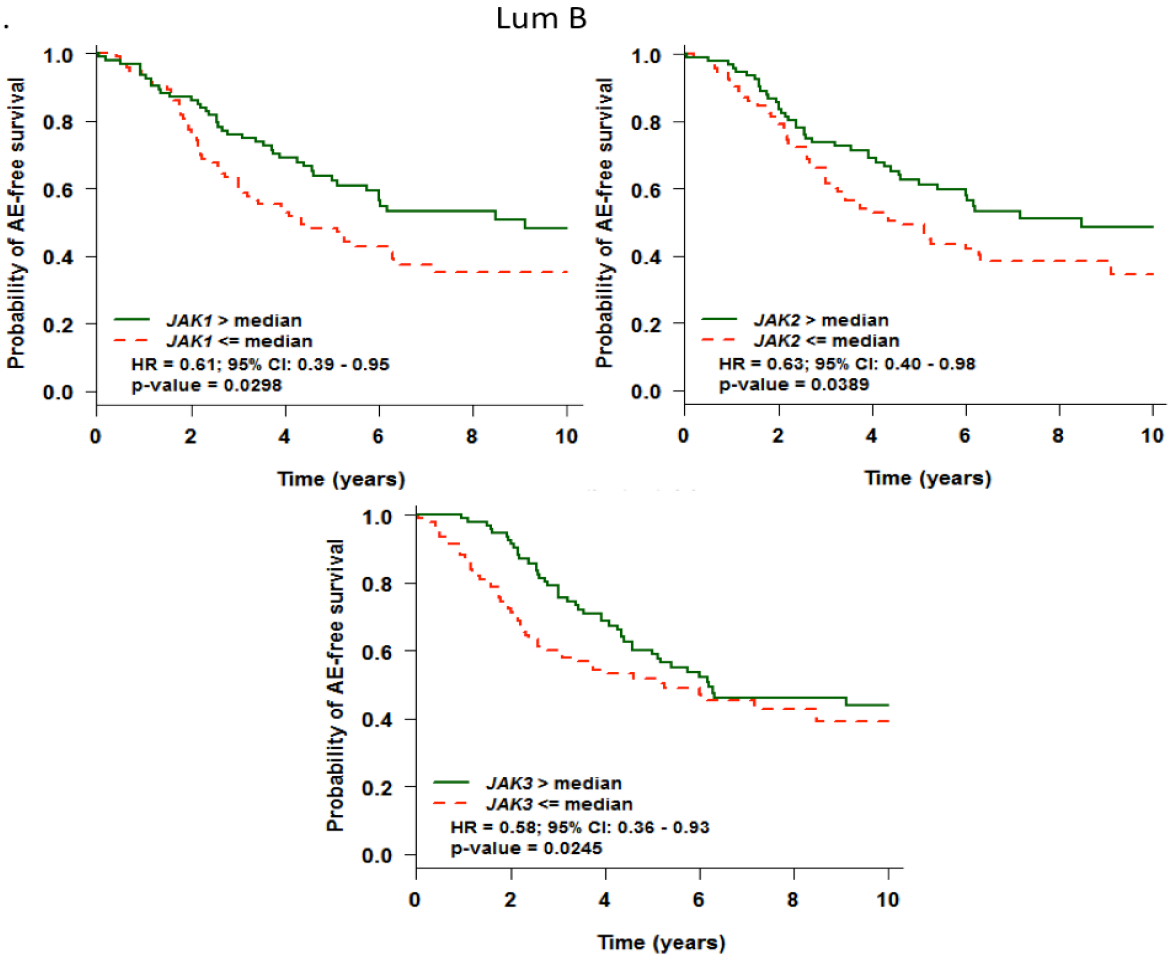


Figure 3.10: Impact of JAK/STAT pathway associated mRNA expression levels on Luminal B BC subtypes prognosis.

*Kaplan-Meier survival analysis of basal-like subtype based on mRNA expression level of JAK1, JAK2 and JAK3 in luminal B subtype. Probability of adverse event (AE) free survival in different BC subtypes was stratified based on mRNA expression level relative to mean (higher than mean (> mean) or lower or equal to mean (≤ mean)); d. Impact of JAK/STAT pathway associated mRNA high levels on average (Av) tumour size (Guichard et al., 2012), tumour grade 3 and LN involvement in different BC subtypes. * indicates p-values less than 0.05 as calculated by One-way Anova determining significant difference in expression among the subtypes.*

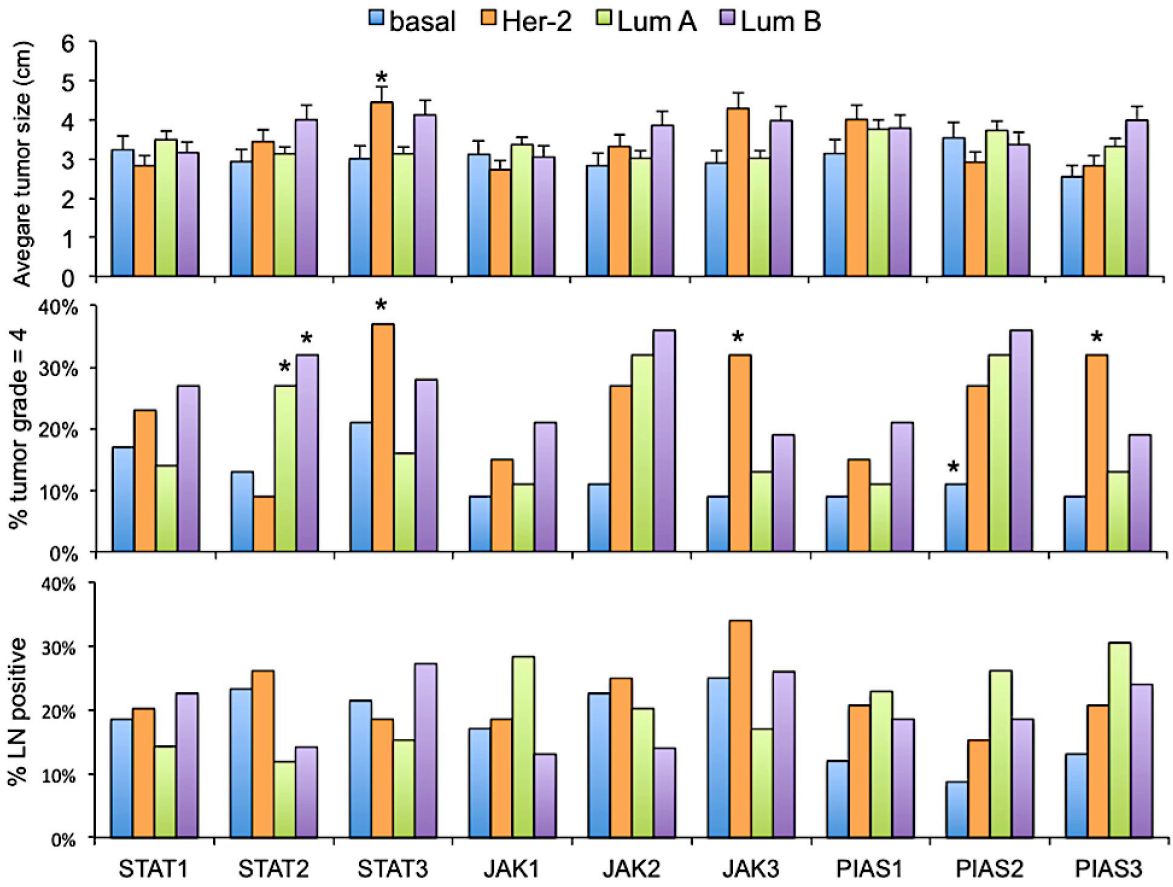


Figure 3.11: JAK/STAT pathway associated mRNA expression levels and BC subtypes prognosis.

*Impact of JAK/STAT pathway associated mRNA high levels on average (Av) tumour size (Guichard et al., 2012), tumour grade 3 and LN involvement in different BC subtypes. * indicates p-values less than 0.05 as calculated by One-way Anova determining significant difference in expression among the subtypes.*

3.2.7. NF-κB gene expression in basal-like BC

- ***NF-κB gene expression in different BC subtypes***

Increased activity of NF-κB has been reported in human BC cell lines and in BC tissue (Rayet and Gelinas, 1999). NF-κB pathway genes expression has been analyzed according to subtype in human BC patient databases. *NF-κB1*, *NF-κB2*, *Rel-A* and *Rel-B* subunits and the NF-κB pathway inhibitors *IKBKB*, *IKBKG* and *IKBKE* mRNA expression levels were analyzed and their relative impact on BC prognosis were determined. Basal-like mRNA expression levels of *NF-κB1* and *NF-κB2* were not different between the BC subtypes (Figure 3.12a). Similarly, *Rel-A* mRNA expression was relatively similar, however, *Rel-B* subunit mRNA expression was higher in basal-like breast cancer subtype (Figure 3.12b). As for the NF-κB pathway inhibitors, lower level of *IKBKB* mRNA expression was observed in basal-like and HER-2 BC subtype in comparison to luminal subtypes, while no significant difference in mRNA expression of *IKBKG* and *IKBKE* were observed (Figure 3.12c).

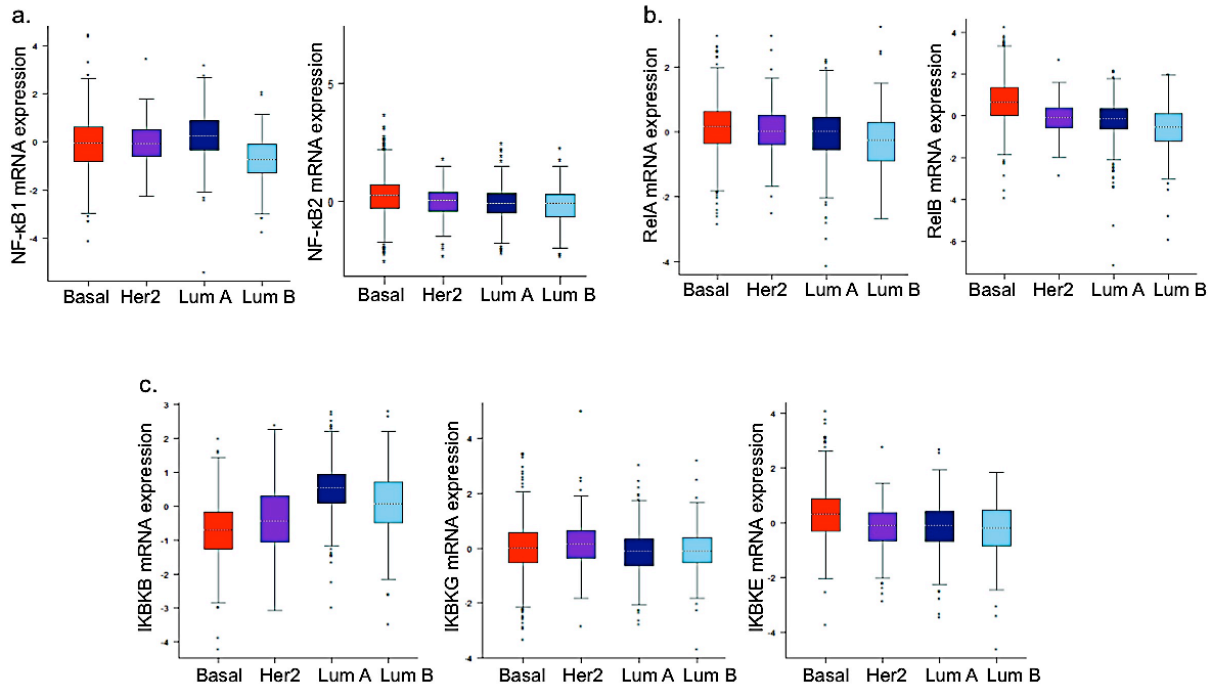


Figure 3.12: NF-κB pathway associated mRNA expression levels in BC subtypes.

Subtypes: (basal-like, HER-2 positive (HER-2), Luminal A (Lum A) and Luminal B (Lum B)); a. Box plot expression of NF-κB1 and NF-κB2 along different BC subtypes; b. Box plot expression of Rel-A and Rel-B along different BC subtypes; c. Box plot expression of IKB inhibitors (IKBKB, IKBKG and IKBKE) along different BC subtypes.

- ***NF-κB pathway genes' expression is associated with poor prognosis in basal-like subtype BC***

Patient samples were compared based on high mRNA expression relative to mean, only 11% of patients with luminal B subtype express significantly high *NF-κB1* mRNA, 46% of patients with basal-like subtype express significantly high *NF-κB2* mRNA levels in comparison to all other subtypes, and 62% of patients with basal-like subtype express high *Rel-B* levels which is significantly more than patients of HER-2 and luminal subtypes (Table 3.7). In addition, only 9% of basal-like subtype patients express high *IKBKB* mRNA, and 49% of basal-like subtype patients express high levels of *IKBKE* mRNA (Table 3.7).

Table 3.7: Percentage patients expressing high mRNA of NF-κB pathway related genes

High mRNA gene expression	% Patient Samples Molecular Subtype			
	Basal-like	HER-2	Luminal A	Luminal B
NFKB1	34%	31%	45%	11%*
NFKB2	46%*	30%	27%	25%
Rel-A	42%	34%	34%	26%
Rel-B	62%*	26%	24%	17%
IKBKB	9%*	23%	63%	38%
IKBKG	36%	39%	26%	29%
IKBKE	49%*	26%	30%	31%

High levels of mRNA were considered as higher than mean. * indicates p-values less than 0.05 as calculated by One-way Anova.

Observing the impact of gene expression on AE-free survival, high *NF-κB1* mRNA expression is associated with better prognosis as determined by higher probability of AE-free survival in HER-2 BC subtypes (p = 0.04) (Figure 3.13). In basal-like BC, high level of *NF-κB1*, *NF-κB2* and *Rel-B* are associated with poor prognosis in basal-like subtype, and high level of *IKBKGE* mRNA was associated with better prognosis in basal-like subtype BC (Figure 3.13b). Observing the impact of NF-κB associated genes expression on tumour size, grade and LN involvement, patient samples with mRNA expression higher than mean were analyzed and presented in Figure 3.13c. Significantly larger tumour size was observed in basal-like subtypes with high *NF-κB1*, *NF-κB2* and *Rel-B* mRNA expression, and more patients with tumour grade 3 were associated with high *NF-κB1*, *NK-κB2*, *Rel-A* and *Rel-B* levels in basal-like subtype (Figure 3.14). Moreover, in HER-2 subtype, significantly high percentage of patients with grade 3 tumours was observed with high levels of *Rel-A* and *IKBKE* mRNA (Figure 3.14), and more positive LN involvement was observed only in basal-like BC subtypes with high *NF-κB1, NF-κB2, Rel-A, Rel-B* and *IKBKG* mRNA expression (Figure 3.14). This data indicates an important role of the NF-κB pathway related genes expression in development of basal-like BC subtype.

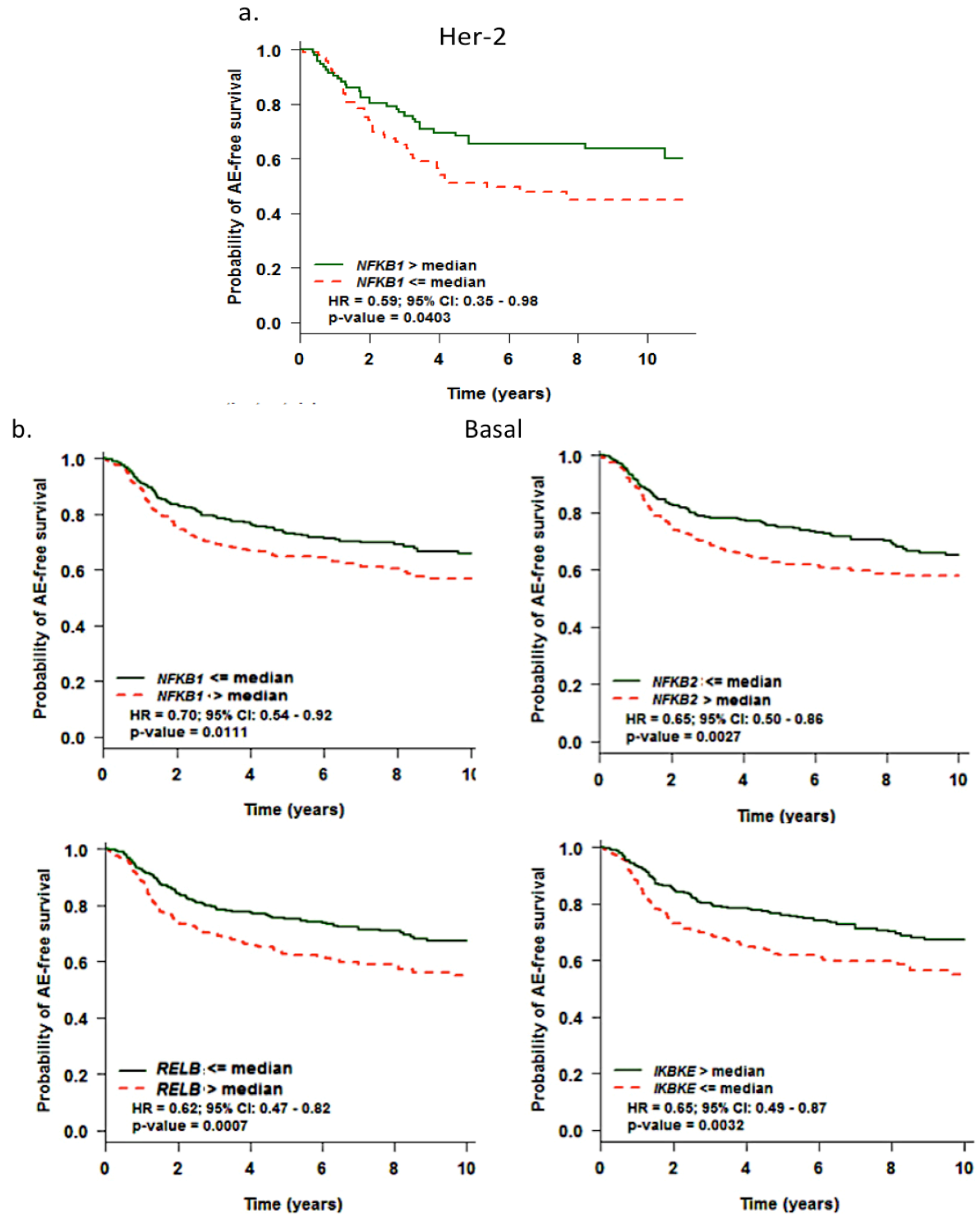


Figure 3.13: Impact of NF-κB pathway associated mRNA expression levels on basal-like BC survival.

a. Kaplan-Meier survival analysis of NF-κB1 expression mRNA in HER-2 subtype. Probability of adverse event (AE) free survival in different BC subtypes was stratified based on mRNA expression level relative to mean (higher than mean (> mean) or lower or equal to mean (≤ mean)); b. Kaplan-Meier survival analysis of based on NF-κB1, NF-κB2, Rel-A and IKBKE mRNA expression level in basal-like subtype. Probability of adverse event (AE) free survival in different BC subtypes was stratified based on mRNA expression level relative to mean (higher than mean (> mean) or lower or equal to mean (≤ mean)).

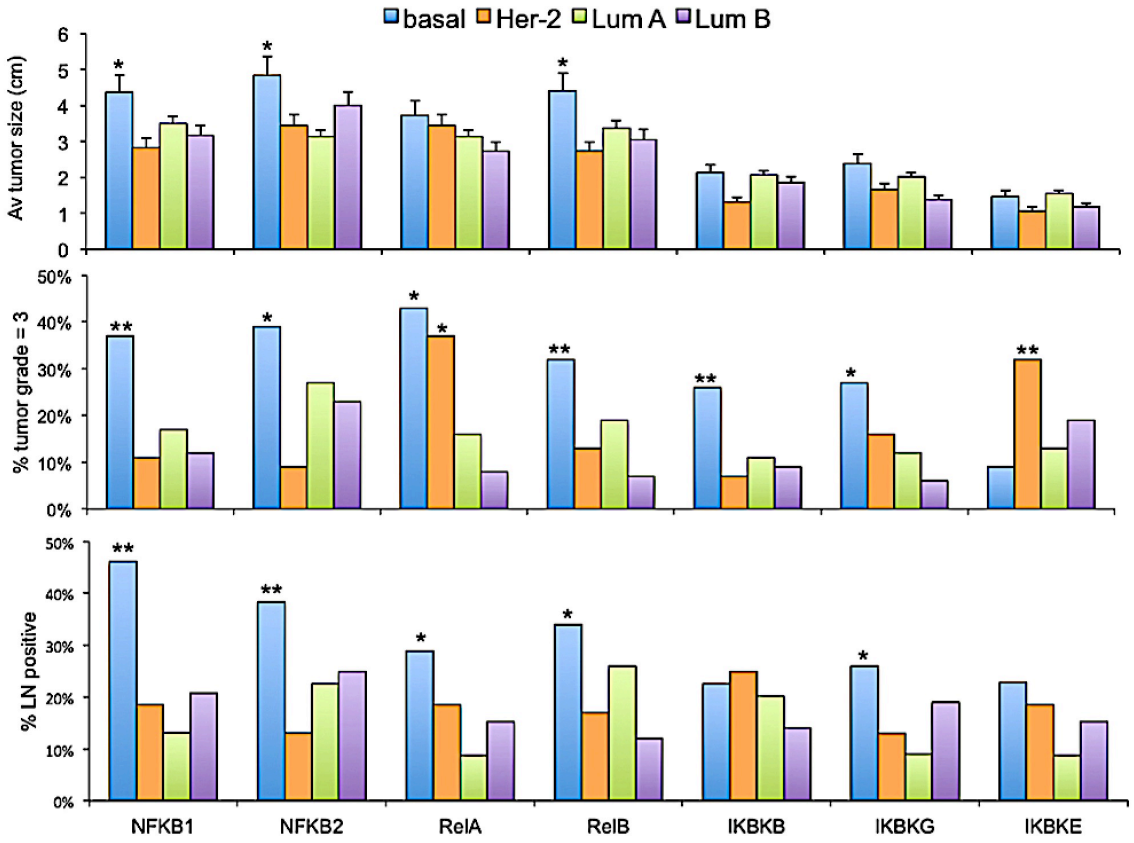


Figure 3.14: NF-κB pathway associated mRNA expression levels and basal-like BC prognosis.

*Impact of NF-κB pathway associated mRNA high levels on average (Av) tumour size (Guichard et al., 2012), tumour grade 3 and LN involvement in different BC subtypes. * indicates p-values less than 0.05 and ** indicates p-values less than 0.01 as calculated by One-way Anova determining significant difference among the BC subtypes.*

3.2.8. HSR and basal-like BC

- ***mRNA expression level in BC subtypes***

HSPs are known to inhibit apoptosis by direct physical interaction with apoptotic molecules and are also overexpressed in several tumour cells (Ciocca and Calderwood, 2005). HSP pathway associated genes including heat shock factor (HSF) isoforms 1, 2 and 5, *HSP90* isoforms (AA1, AB1 and B1), HSP70 or HSPA isoforms (*HSPA1A, HSPA1B* and *HSPA2*) and HSP27 or HSPB isoforms (*HSPB1, HSPB2* and *HSPB3*) mRNA expression have been analyzed according to subtype in human BC patient databases. *HSF1* mRNA expression is higher in basal-like and luminal B subtypes, while no difference in *HSF2* or *HSF3* mRNA levels were observed (Figure 3.15a). *HSP90AA1*, *HSP90AB1* and *HSP90B1* mRNA levels were relatively comparable in all subtypes with slightly less in luminal A subtype (Figure 3.15b). *HSPA1A* mRNA expression was higher in basal-like and luminal B subtype and no differences were observed in *HSPA1B* or *HSPA2* (Figure 3.15c). Finally, *HSPB1* mRNA expression level was lowest in basal-like subtype and highest in luminal B BC (Figure 3.15d).

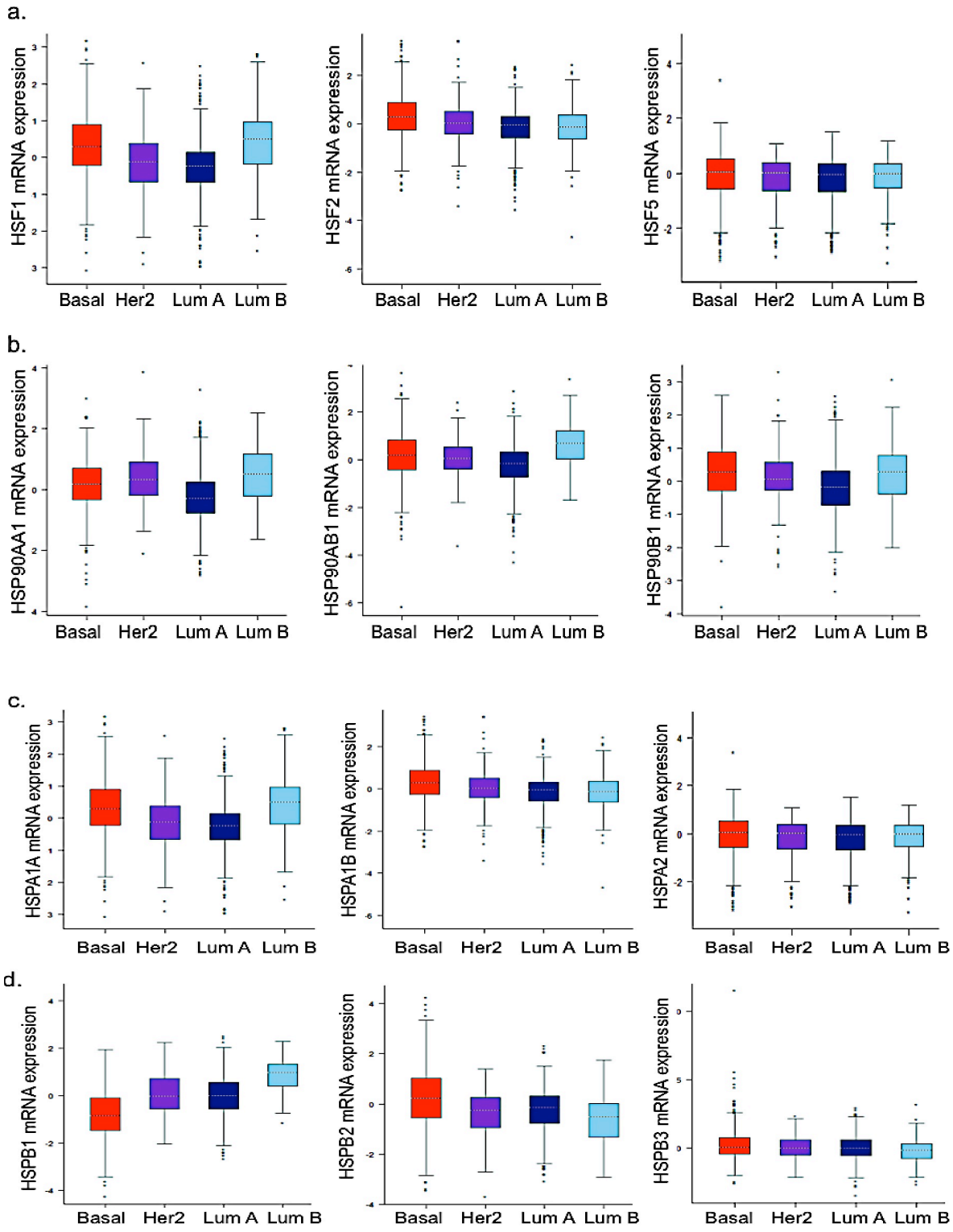


Figure 3.15: HSR pathway associated mRNA expression levels in BC subtypes.

Subtypes: (basal-like, HER-2 positive (HER-2), Luminal A (Lum A) and Luminal B (Lum B)); a. Box plot expression of HSF1, HSF2 and HSF5 mRNA in different BC subtypes; b. Box plot expression of HSP90AA1, HSP90AB1 and HSP90B1 along different BC subtypes; c. Box plot expression of HSPA1A, HSPA1B, HSPA2 mRNA indifferent BC subtypes; d. Box plot expression of HSPB1, HSPB2, HSPB3 mRNA in different BC subtypes.

- ***HSPB1 mRNA expression associates with poor prognosis in basal-like BC***

Patient samples were compared based on mRNA levels greater than the mean expression, significantly more patients with basal-like subtype express high mRNA levels of *HSF2* (51%) and *HSPB2* (46%), however less patients with basal-like subtype express high mRNA levels of *HSPA2* (9%) or *HSPB1* (10%) (Table 3.8). In addition, luminal A subtype has lowest percentage of patients expressing high levels of *HSP90AB1* mRNA (22%) and highest percentage of patients expressing *HSPA2* mRNA (54%), and luminal B subtype have highest percentage of patients expressing high mRNA levels of *HSP90AA1* (65%) and *HSPB1* (75%) (Table 3.8).

Table 3.8: Percentage patients expressing high mRNA of HSR pathway related genes.

High mRNA gene expression	% Patient Samples Molecular Subtype			
	Basal-like	HER-2	Luminal A	Luminal B
HSF1	50%	31%	20%	56%
HSF2	51%**	35%	28%	29%
HSF5	39%	32%	29%	28%
HSP90AA1	42%	49%	22%*	59%
HSP90AB1	44%	32%	23%	65%*
HSP90B1	48%	35%	24%	47%
HSPA1A	30%	24%	36%	61%*
HSPA1B	39%	33%	38%	40%
HSPA2	9%*	23%	54%*	37%
HSPB1	10%*	35%	31%	75%**
HSPB2	46%*	19%	22%	12%
HSPB3	37%	33%	30%	23%

*High levels of mRNA were considered as higher than mean. * indicates p-values less than 0.05 and ** indicates p-values less than 0.01as calculated by One-way Anova.*

Observing the impact of gene expression on AE-free survival, high mRNA levels of *HSPB1* ($p = 0.07$) and *HSF5* ($p = 0.02$) are associated with poor prognosis as determined by lower probability of AE-free survival in basal-like BC subtype (Figure 3.16a). In HER-2 BC, high mRNA levels of *HSPB2* ($p = 0.03$) and *HSPA1B* ($p = 0.01$) are associated with poor prognosis (Figure 3.16b), and high mRNA level of *HSPB1* ($p < 0.001$) was associated with poor prognosis in luminal A subtype; however, high levels of *HSF5* ($p < 0.01$) were associated with better prognosis in luminal A BC (Figure 3.16c).

Observing the impact of HSR associated genes expression on tumour size, grade and LN involvement, patient samples with mRNA expression higher than mean were analyzed and

presented in Figure 3.2.17. Significantly larger tumour size was observed in basal-like subtypes with high *HSF2* and *HSPB2* mRNA expression, and more patients with tumour grade 3 were associated with high *HSF1, HSPA1A* and *HSPB1* levels in basal-like subtype (Figure 3.17). In HER-2 subtype, no significant difference in tumour size was observed however, significantly high percentage of patients with grade 3 tumours were observed with high levels of *HSPA1B, HSPB2* and *HSPB3* (Figure 3.17). As for luminal A subtype, no significant impact of expression of genes encoding proteins of the HSR pathway was observed in any subtype, however, in luminal B subtype, high mRNA expression of *HSF1* and *HSPA1A* was associated with large tumour size, and high *HSP90AB1* and *HSPB3* mRNA expression were associated with more patients with tumour grade 3. Moreover, significantly less positive LN involvement was observed in basal-like BC subtypes with high *HSP90AB1* mRNA expression, and significantly high percentage of positive LN involvement was observed with high expression of *HSPB1* (Figure 3.17). In HER-2 subtype, significantly higher percentage of patients with LN involvement expressed high levels of *HSF5, HSP90B1, HSPA1A, HSPA1B* and *HSPA2*, and only *HSF2* had a significant impact on LN involvement in luminal A subtype (Figure 3.17). Finally, in luminal B subtype, high *HSF2, HSF5* and *HSP90AA1* had significantly increased LN involvement (Figure 3.17).

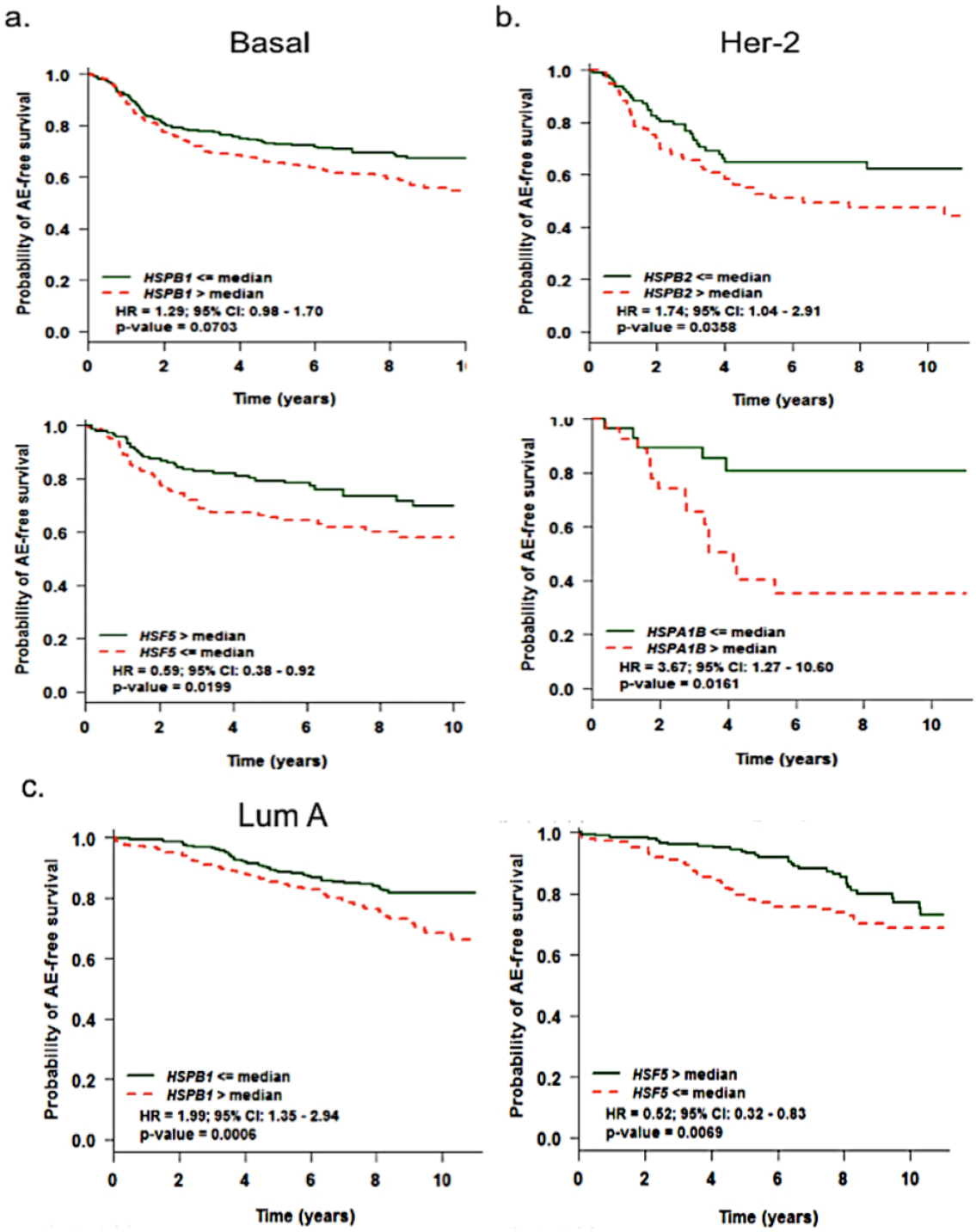


Figure 3.16: Impact of HSR pathway associated mRNA expression levels on BC subtypes survival.

Kaplan-Meier survival analysis of basal-like subtype based on mRNA expression level of HSPB1 and HSF5. Probability of adverse event (AE) free survival in different BC subtypes was stratified based on mRNA expression level relative to mean (higher than mean (> mean) or lower or equal to mean (≤ mean)); b. Kaplan-Meier survival analysis of based on HSPB2 and HSPA1B mRNA expression level in HER-2 subtype. Probability of adverse event (AE) free survival in different BC subtypes was stratified based on mRNA expression level relative to mean (higher than mean (> mean) or lower or equal to mean (≤ mean)); c. Kaplan-Meier survival analysis of Lum A subtype based on mRNA expression level of HSPB1 and HSF5 in

luminal B subtype. Probability of adverse event (AE) free survival in different BC subtypes was stratified based on mRNA expression level relative to mean (higher than mean (> mean) or lower or equal to mean (≤ mean)).

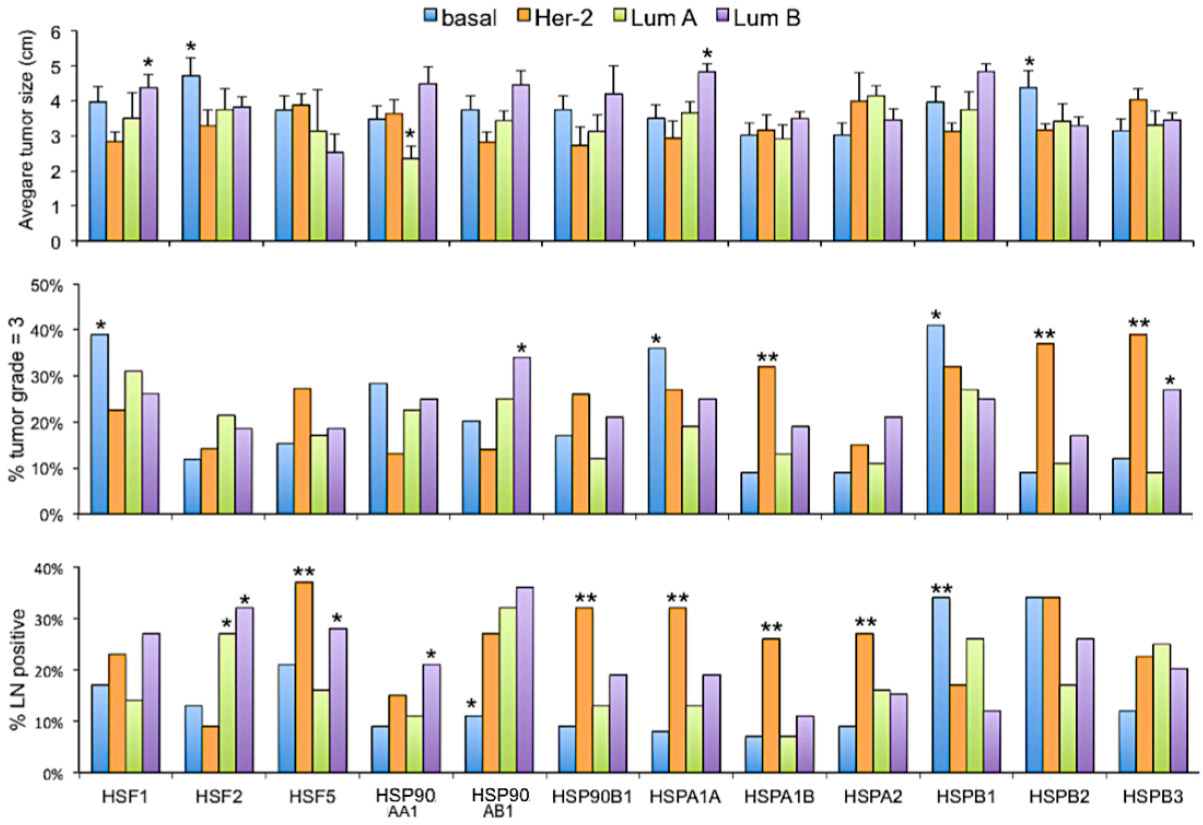


Figure 3.17: HSR pathway associated mRNA expression levels and BC subtypes prognosis.

*Impact of UPR pathway associated mRNA high levels on average (Av) tumour size, tumour grade 3 and LN involvement in different BC subtypes. * indicates p-values less than 0.05 and ** indicates p-values less than 0.01 as calculated by One-way Anova determining significant difference in expression among the subtypes.*

3.2.9. UPR in basal-like BC

- ***Expression of genes encoding proteins of the UPR pathway in BC subtypes***

The UPR pathway is reported to impact the development of cancer (Jager et al., 2012, Rutkowski and Kaufman, 2004). Role of the UPR in different BC subtypes has been studied based on the mRNA expression of the main components of the 3 UPR arms: the IRE1 pathway, the EIF2AK3, also known as PERK, pathway and the ATF6 pathway. *IRE1* mRNA expression level was similar in all BC subtypes, however, *XBP1* mRNA level was significantly lower in basal-like BC subtypes (Figure 3.18a). Analyzing expression of mRNA involved in PERK arm of the UPR pathway, *EIF2AK3* mRNA expression was slightly lower in basal-like subtype, and no difference in *EIF2A* and *ATF4* expression levels were observed (Figure

3.18b). As for the ATF6 arm of the pathway, higher expression of mRNA *ATF6* was observed in luminal A subtype (Figure 3.18c). mRNA level of *CHOP* was also analyzed since CHOP is downstream effector of the PERK arm of the UPR pathway; in addition, it is induced by ER stress and mediates apoptosis (Nishitoh, 2012). A slight decrease in *CHOP* expression was observed in luminal A BC subtype (Figure 3.18d).

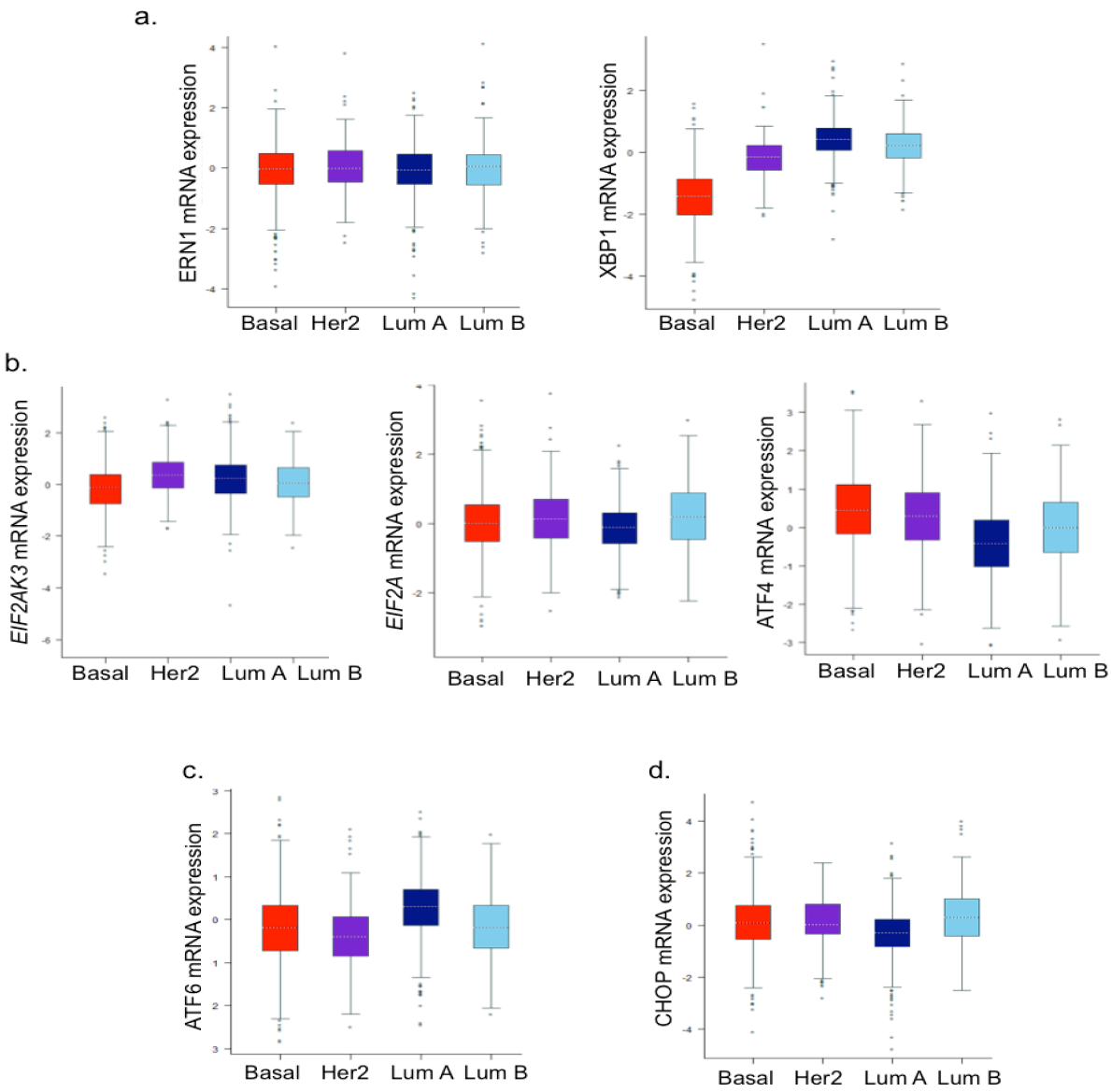


Figure 3.18: UPR pathway associated mRNA expression levels in BC subtypes.

Subtypes: (basal-like, HER-2 positive (HER-2), Luminal A (Lum A) and Luminal B (Lum B)); a. Box plot expression of IRE1, IRE2 and XBP1 mRNA along different BC subtypes; b. Box plot expression of EIF2AK3 (PERK), EIF2A and ATF4 mRNA along different BC subtypes; c. Box plot expression of ATF6 mRNA along different BC subtypes; d. Box plot expression of CHOP mRNA along different BC subtypes.

- ***Low levels of XBP1 mRNA expression in basal-like BC is associated with poor prognosis***

Comparing BC subtypes based on percentage of samples expressing mRNA levels higher than mean, only 2% of patients with basal-like subtype expressed high *XBP1* mRNA level which was significantly less than patients expressing high *XBP1* in other subtypes. In luminal A subtype, only 19% of patients express high *ATF4* mRNA levels which is significantly less than patients in other subtypes, while 51% of luminal A patients express high *ATF6* mRNA level which is significantly more in other subtypes. Finally, significantly more patients with luminal B subtype (47%) express high CHOP level in comparison to other subtypes (Table 3.9).

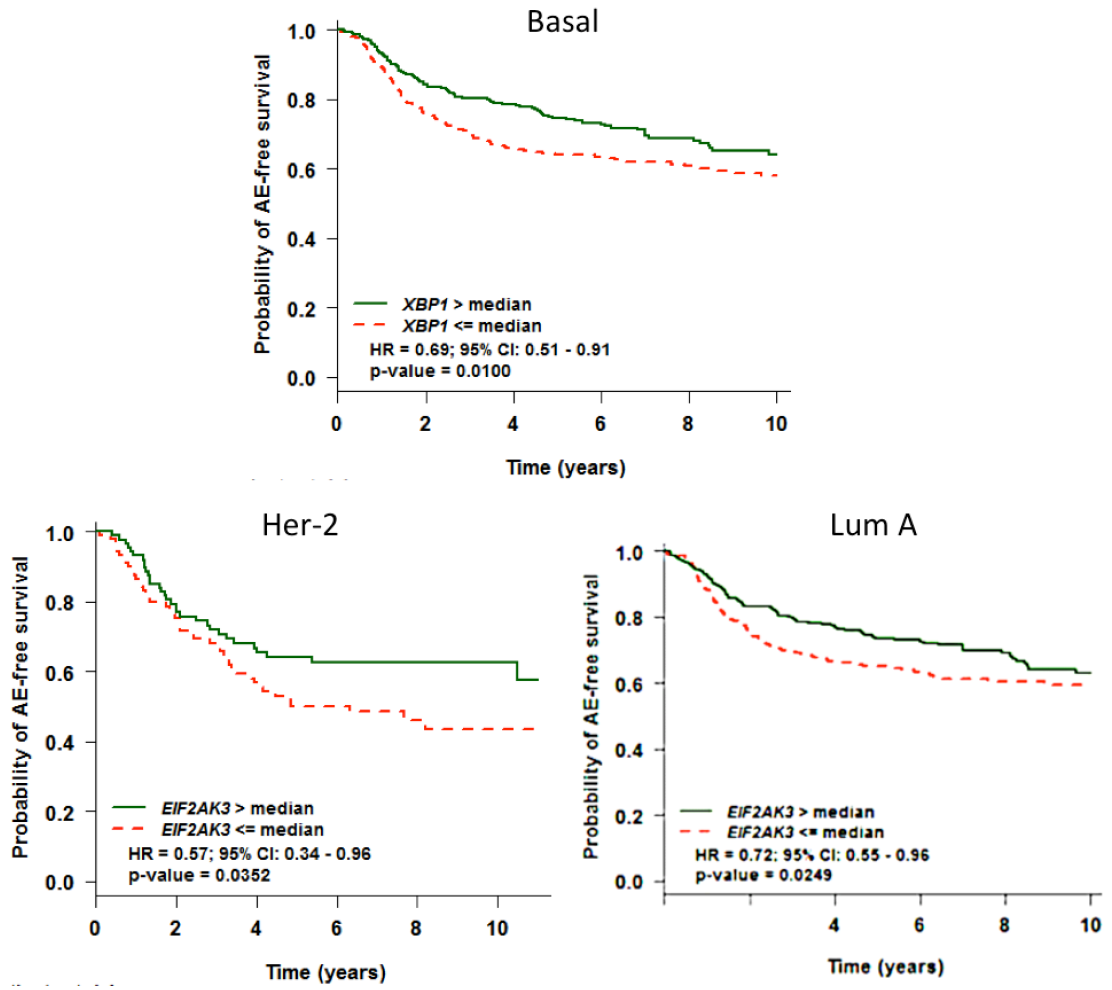
Table 3.9: Percentage patients expressing high mRNA of genes encoding proteins associated with the HSR pathway

High mRNA gene expression	% Patient Samples Molecular Subtype			
	Basal-like	HER-2	Luminal A	Luminal B
IRE1	33%	33%	32%	30%
XBP1	2%**	20%	59%	45%
EIF2AK3	27%	50%	43%	37%
eIF2A	35%	43%	26%	45%
ATF4	54%	44%	19%*	37%
CHOP	39%	35%	20%	47%*
ATF6	27%	18%	51%*	27%

*High levels of mRNA were considered as higher than mean. * indicates p-values less than 0.05 as calculated by One-way Anova.*

Observing the impact of gene expression on AE-free survival, high *XBP1* mRNA level in basal-like BC subtypes is associated with significantly better prognosis and higher probability of AE-free survival ($p = 0.03$), while in HER-2 and luminal A subtypes, high *EIF2AK3* mRNA is associated with better prognosis and higher AE-free survival ($p = 0.03$ and $p = 0.02$ respectively) (Figure 3.19a). Observing the impact of UPR associated genes mRNA expression on tumour size, grade and LN involvement, patient samples with mRNA expression higher than mean were analyzed and presented in Figure 3.15b. Significantly larger tumour size was observed in luminal B with high *EIF2AK3* mRNA expression and no significant impact was observed with other mRNA expression in different subtypes. More percentage of patients with tumour grade 3 was associated with high *IRE1* mRNA in luminal A and B subtypes, high *EIF2AK3* mRNA in HER-2 subtype and high *ATF4* mRNA in luminal B subtype (Figure 3.19b). Moreover, positive LN involvement was observed in luminal A BC subtype with high *XBP1* and *eIF2A* mRNA expression (Figure 3.19b).

a.


Basal
Probability of AE-free survival
1.0
0.8
0.6
0.4
0.2
0.0
XBP1 > median
XBP1 <= median
HR = 0.69; 95% CI: 0.51 - 0.91
p-value = 0.0100
0
2
4
6
8
10
Time (years)
Her-2
Probability of AE-free survival
EIF2AK3 > median
EIF2AK3 <= median
HR = 0.57; 95% CI: 0.34 - 0.96
p-value = 0.0352
Time (years)
Lum A
Probability of AE-free survival
EIF2AK3 > median
EIF2AK3 <= median
HR = 0.72; 95% CI: 0.55 - 0.96
p-value = 0.0249
Time (years)

b.

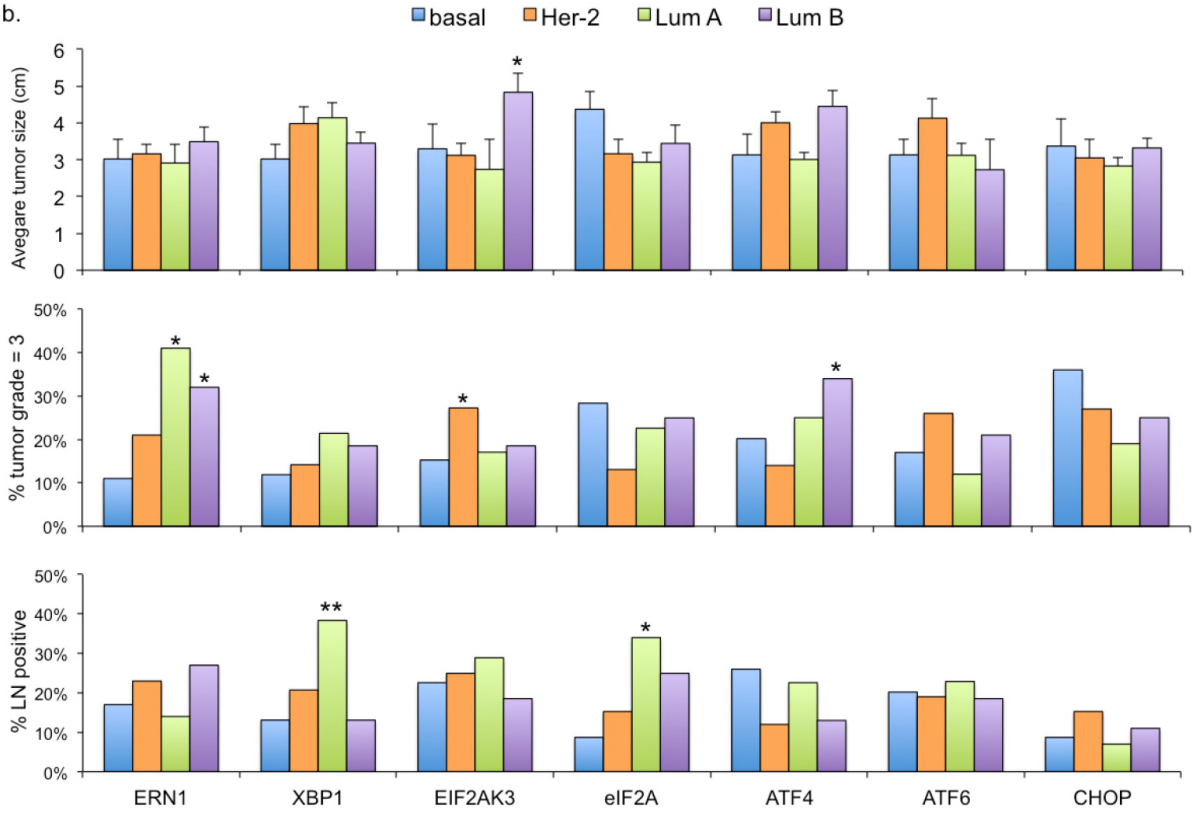

basal
Her-2
Lum A
Lum B
Avegare tumor size (cm)
% tumor grade = 3
% LN positive
ERN1
XBP1
EIF2AK3
eIF2A
ATF4
ATF6
CHOP

Figure 3.19: UPR pathway associated mRNA expression levels and BC subtypes.

*Kaplan-Meier survival analysis of basal-like subtype based on mRNA expression level of XBP1, HER-2 and Lum A subtype based on EIF2AK3 mRNA expression, and Lum B subtype based on IRE2 mRNA expression. Probability of adverse event (AE) free survival in different BC subtypes was stratified based on mRNA expression level relative to mean (higher than mean (> mean) or lower or equal to mean (≤ mean)); b. Impact of UPR pathway associated mRNA high levels on average (Av) tumour size, tumour grade 3 and LN involvement in different BC subtypes. * indicates p-values less than 0.05 and ** indicates p-values less than 0.01 as calculated by One-way Anova determining significant difference in expression among the subtypes.*

3.2.10. NF-κB1/2 and *Rel*-A/B genes are positively correlated with *TP53* and *MKI-67* gene expression

As mentioned earlier, p53 and Ki-67 are prognostic markers of basal-like BCs (Yadav et al., 2015, Li et al., 2015a). Correlation analysis was done and correlation data is presented in Table 3.12. Looking at correlation of pro-survival pathways related genes and *TP53* mRNA expression, significant correlation was observed with *AKT2*, however, correlation value is low (r = 0.05). No correlation was observed with any genes involved in the mTOR pathway. A highly significantly positive correlation was observed with *NF-κB1, NF-κB2, Rel-A* and *B* mRNA expression genes of the NF-κB pathway ($p < 0.01$), and a negative correlation was observed with NF-κB inhibitor *IKBKB;* a positive correlation in *NF-κB2, RelA* and *RelB* mRNA expression genes was also observed in luminal A BC subtype. For the HSR pathway, *HSF1* and *HSP90AB1* genes expression was highly correlated with *TP53* in basal-like, HER-2 and Luminal A BC subtypes. Finally, *CHOP* and *ATF4* mRNA expression were negatively correlated with *TP53* mRNA expression in basal-like and luminal A BC subtypes.

Looking at correlation expression with proliferation marker Ki-67, significant positive correlation of *MKI-67* mRNA expression was observed with *PIK3R2* in basal-like, HER-2 and luminal B subtypes, with *mTOR* in basal-like and luminal B subtypes, RPS6KB1 and EIF4EBP1 in basal-like subtype, *JAK1* and *PIAS2* in basal-like and HER-2 subtypes, *NF-κB2, Rel-A* and *Rel-B* in basal-like subtype, *HSP90AB1* in basal-like BC, HER-2, luminal A and luminal B subtypes; *HSF1, HSPA1A* and *HSPB1* in basal-like BC only. Finally significant negative correlation was obtained with *IKBKB, IKBKG*, *HSPB2* and *XBP1* mRNA expression in basal-like and HER-2 BC subtypes.

Table 3.10: Correlation of investigated genes expression with *TP53* and *MKI-67* mRNA expression in different BC subtypes

		TP53				***MKI-67***			
	gene	**basal-like**	**HER-2**	**Lum A**	**Lum B**	**basal-like**	**HER-2**	**Lum A**	**Lum B**
PI3K/AKT	*PIK3CA*	0.06	0.07	-0.11*	0.00	-0.05	-0.05	-0.01	0.02
	PIK3CG	0.01	0.11	0.00	0.11	-0.07	-0.10	0.05	0.02
	PIK3CD	0.02	0.07	0.04	-0.13	-0.05	-0.20*	0.17**	0.06
	PIK3CB	-0.03	0.02	-0.14*	-0.16	-0.07	0.00	-0.05	0.29**
	PIK3R1	0.03	0.03	-0.02	0.09	-0.01*	-0.03	-0.06	0.05
	PIK3R2	0.04	0.06	0.15**	0.06	0.16**	0.25**	0.06	0.26*
	PIK3R3	0.07	-0.04	-0.07	-0.08	0.11	0.12	0.03*	0.09
	AKT1	0.10	-0.09	0.05	0.02	0.09	0.15	0.03	0.03
	AKT2	0.05*	0.04	0.10	-0.01	0.21	0.06	-0.04	0.20**
	AKT3	0.00	0.09	-0.05	-0.08	-0.01	-0.15	-0.02	0.09
mTOR	*mTOR*	0.02	-0.10	-0.05	0.90	0.19**	0.08	0.08	0.25*
	RPTOR	0.01	0.01	0.20**	0.00	0.04*	0.03	0.07	0.02
	RICTOR	0.02	0.01	-0.15*	0.00	-0.07	-0.06	-0.12	0.07
	RPS6KB1	0.03	-0.17	-0.03	-0.05	0.14**	0.09	-0.01	0.08
	EIF4EBP1	-0.06	0.04	0.08	0.22*	0.17**	-0.03	0.06	0.03
	eIF4E	0.02	0.04	0.02	-0.01	-0.01	-0.01	0.01	0.05
	PRKCA	-0.05	0.02	-0.02	0.07	0.27**	0.06	0.16**	0.13
JAK/STAT	*STAT1*	0.13**	-0.02*	-0.10	-0.03	0.00	-0.18	0.19**	0.14
	STAT2	0.17**	0.04	0.07	0.01	-0.04	0.02	0.04	0.19
	STAT3	0.10*	0.04	0.25**	-0.12	0.07	-0.25*	0.03	0.06
	JAK1	-0.02	0.21*	0.01	0.04	0.14**	0.18*	-0.02	-0.09
	JAK2	0.08	-0.03	-0.03	0.07	-0.03	-0.13	0.02	0.09
	JAK3	0.10*	0.17*	0.16**	-0.10	0.09	-0.06	0.18**	-0.02
	PIAS1	0.04	-0.04	-0.07	-0.14	-0.01*	-0.04	-0.15	0.24*
	PIAS2	0.01	0.00	0.11	-0.02	0.16**	0.17*	-0.02	0.12
	PIAS3	-0.04	-0.06	-0.02	0.15	-0.06	0.01	-0.14*	0.02
NF-κB	*NFKB1*	0.11**	0.12	0.04	0.10	0.05	-0.06	0.10	0.12
	NFKB2	0.12**	0.05	0.20**	-0.05	0.11*	0.13	0.10	0.05
	Rel-A	0.16**	0.17	0.27**	0.06	0.17*	0.03	0.04	0.12
	Rel-B	0.21**	0.22	0.20**	0.12	0.12*	-0.12	0.08	-0.04
	IKBKB	-0.06*	0.15	0.04	0.11	-0.09*	-0.05	-0.01	0.03
	IKBKG	0.09	0.16	-0.01	0.02	-0.24*	0.02	0.15**	-0.08
	IKBKE	0.03	0.03	0.13	0.06	0.09	-0.04	0.07	0.04

Table 3.2.18 continued….

HSR	*HSF1*	0.24*	0.24**	0.18*	-0.09	0.20**	0.16	0.10	0.07
	HSF2	0.06	-0.12	0.12	0.11	0.05	-0.03	0.07	-0.10
	HSF5	0.09	0.02	0.06	-0.01	-0.11	0.15	0.09	-0.10
	HSP90AA1	-0.02	-0.24*	-0.03	-0.05	0.06	0.13	0.06	0.05
	HSP90AB1	0.17**	0.17*	0.19**	0.02	0.12**	0.19*	0.17**	0.29**
	HSP90B1	-0.03	-0.06	0.00	-0.09	0.01	-0.08	0.12*	0.16
	HSPA1A	0.05	0.06	0.08	-0.04	0.14**	0.02	-0.03	0.20
	HSPA1B	0.10	-0.12	0.23*	-0.12	0.18	-0.25	0.11	0.05
	HSPA2	0.04	-0.08	0.13*	0.17	-0.01	0.01	-0.04	0.09
	HSPB1	0.02	0.03	0.12*	0.17	0.21*	0.16	-0.03	-0.05
	HSPB2	-0.03	0.15	-0.01	0.14	-0.20*	-0.22*	-0.09	-0.17
	HSPB3	-0.04	0.02	0.04	0.03	0.06	0.03	-0.03	0.01
UPR	*IRE1*	0.05	0.02	-0.03	0.04	0.08	0.10	0.04	0.08
	IRE2	0.01	0.00	0.10	0.21	0.01	0.20*	0.10	0.06
	XBP1	-0.03	0.02	-0.02	-0.20	-0.22*	-0.30*	0.09	0.13
	EIF2AK3	-0.03	0.01	-0.09	0.01	-0.04	-0.13	0.10	-0.01
	eIF2A	0.03	-0.11	0.12	-0.21	-0.05	0.03	-0.02	-0.09
	ATF4	-0.13*	-0.10	-0.19*	-0.01	-0.02	0.08	0.04	0.03
	CHOP	-0.11*	-0.01	-0.18*	-0.06	-0.03	0.00	-0.03	-0.03
	ATF6	0.00	0.10	0.06	0.03	-0.07	-0.05	-0.02	-0.05

*Correlation (Rho) values with * indicating p < 0.05 and ** indicates p < 0.01, significant positive correlations are highlighted with blue and significant negative correlations are highlighted with yellow*

3.1. Discussion

BC heterogeneity and the inconsistency in clinical response to treatment have led to wide interest in understanding the molecular mechanisms of the different BC phenotypes. Currently, ER, PR and HER-2 are the most widely used markers for BC classification and implement the choice of treatment (Makki, 2015). Also, gene expression profiling has been widely used to understand the molecular mechanisms involved in disease progression (Kwei et al., 2010), metastasis (Ellsworth et al., 2008) drug metabolism and drug resistance (Normanno et al., 2005; Brennan et al., 2005). Here BC patient databases were analyzed for mRNA expression involved in pro-survival signaling pathways. mRNA expression of genes involved in survival pathways were compared among different subtypes in order to identify genes and pathways that contribute to the basal-like BC phenotype, and to further identify the mechanistic pathway involved in basal-like BC development, progression and treatment resistance. Genes' expression and impact on BC prognosis are summarized in Figure 3.20.

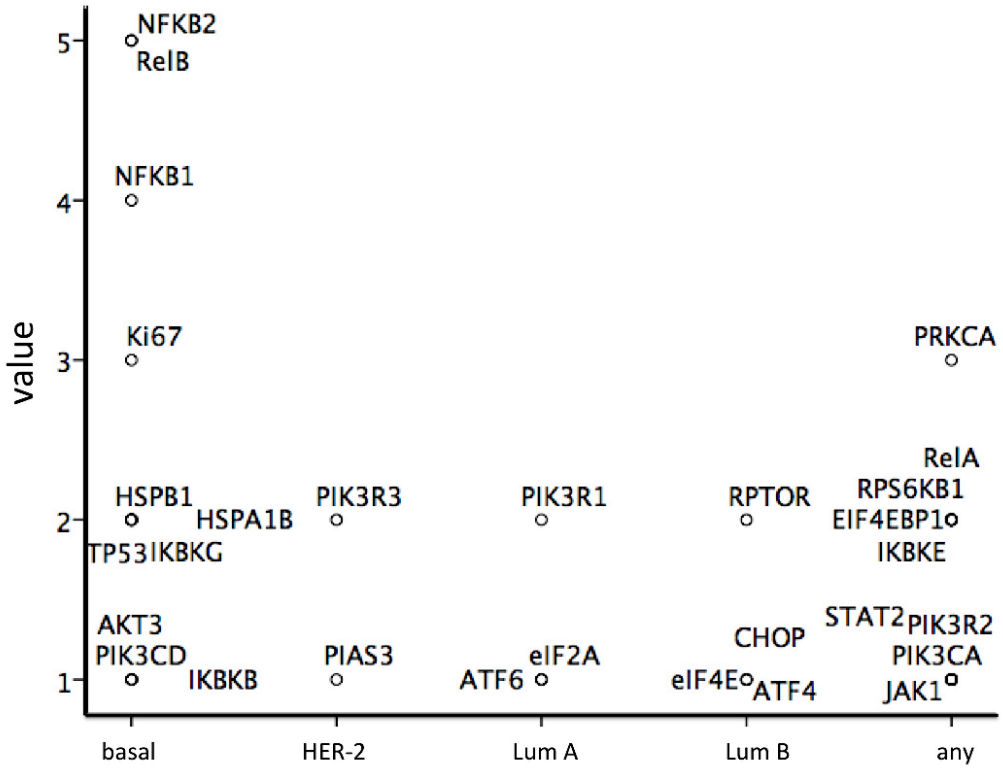


Figure 3.20: Summary of genes mRNA expression and their impact on BC prognosis based on BC subtypes.

Genes studied were given value (1-5) based on their association with BC. Each cancer related factor was given a value of 1 if it was associated with the disease within a subtype. Factors considered are: mRNA expression, AE free survival, tumour size, tumour grade, LN metastasis. "Any" refers to the genes associated with at least 2 BC subtypes and the higher score obtained in any of the subtypes is given.

First, analysis of *TP53* expression showed no difference in *TP53* expression between the different subtypes; however, high *TP53* gene expression is involved in worse prognosis in basal-like BC. *TP53* is known to be a tumour suppressor, however, similar observation was also reported in follicular lymphoma, where high expression of *TP53* was observed in high grade, oversized tumours and poor prognosis; in addition, this correlation was also observed in a subset of ductal carcinomas in situ, but not in atypical lesions (Chitemerere et al., 1996; Pennanen et al., 2008). This may be explained by the fact that wild-type p53 has a short half-life, however, *TP53* mutation leads to lack of p53 function and a subsequent increase in transcription and accumulation of mutated p53 in the cell (Alsner et al., 2008, Yamashita et al., 2006). Analysis of databases was further done based on *TP53* mutation status, since many different types of cancer show a high incidence of *TP53* mutations, leading to the expression of mutant *TP53* proteins. Worse prognosis, high-grade tumours and increased LN involvement were observed with mutant *TP53* in BC (Fig. 3.2). Several other mutations are reported in many cancers including *PTEN* and *Rb* genes, but these mutations were not reported in the databases used in this study. Therefore, in order to understand the role of proto-oncogenes or tumour suppressor genes in basal-like breast cancer development, extensive genetic mutational screening is required on patient samples in order to identify what mutations or polymorphisms occur in the genetic make-up that are common within patients of the same subtype. In addition, mutational impact can further be investigated in *in vitro* models in order to verify the role of different genes, understand the underlying molecular mechanisms and identify potential therapy targets. Such approach might help predict the burden of breast cancer, in addition to its subtype and effective therapy, if any.

Levels of pS6K (S6 kinase) and AKT pathway related genes might be biomarkers predicting clinical outcomes and response to mTOR pathway inhibitors. High levels of pS6K have been associated with worse prognosis, and the levels of pS6K and pAkt predict a good response to rapamycin or rapamycin analogs in BC cells lines and other tumours (Noh et al. 2004, 2008; O'Reilly and McSheehy, 2010; Meric-Bernstam et al. 2012). In this analysis, no significant difference in the expression of the four PI3K isoforms or the regulatory PI3K was observed among the different BC subtypes except for AKT1, which was significantly higher in HER-2 BC subtype. The impact of AKT related mRNA expression was significant in HER-2 and luminal B subtype; however, no significant impact of PI3K/AKT related genes were observed in basal-like BC subtype. As for mTOR related genes, 46% of patient samples with HER-2 subtype express higher levels of transcription factor *RPS6KB1*. Low levels of *RPS6KB1* had significantly poorer outcome in patients with HER-2 subtype ($p = 0.038$), while high levels of *RPS6KB1* had a significantly poorer outcome in patients with luminal A subtype ($p = 0.062$). Most literature report PI3K/AKT/mTOR impact on HER-2 and luminal BC,

however basal-like tumours demonstrate higher levels of AKT activation compared with other subtypes (Nahta and O'Regan, 2010). Therefore, mRNA profiling of basal-like BC samples would not give any valuable outcome on targeting AKT/mTOR pathways since further analysis on pathway activation and phosphorylation of proteins would be required. Similarly, no impact of mRNA expression of genes involved in the JAK/STAT pathway has been identified and a relationship with HER-2 or luminal BCs is suggested.

On the other hand, a significant impact of NF-κB pathway on basal-like BC has been observed. High levels of *Rel-B* mRNA are expressed in basal-like samples, and increased percentage of patients with basal-like phenotype express high *NF-κB2* and *Rel-B* levels and low levels of inhibitor; moreover, high levels of *NF-κB1*, *NF-κB2* and *Rel-B* are associated with poor prognosis and lower AE-free survival in basal-like subtype which is not prominent in other subtypes. In addition, NF-κB pathway genes (*NF-κB2* and *Rel-A/B*) showed a positive correlation with *TP53* and *MKI-67* expression in basal-like BC and not in other subtypes. Significantly, 49% of basal-like BC patients express high levels of IKBKE (Barbie et al., 2014). IKBKE, which is also known as IKKε, represents a link between inflammation and cancer. It is also amplified in approximately 30% of breast carcinomas inducing NF-κB pathway survival signaling (Barbie et al., 2014). Therefore, expression of *NF-κB1*, *NF-κB2* and *Rel-B* in BC patient samples might be specific in determining the basal-like BC subtype and diagnosis. On the other hand, NF-κB is not considered to be a good target for therapy, partly because of its integral role in immune signaling and because it can display both positive and negative effects on tumour progression (Perkins and Gilmore, 2006). Thus, further analysis and studies of genes involved in NF-κB pathway and their expression levels in basal-like BC should be done in order to identify a potential prognostic marker and therapeutic target.

Two other major pathways reported to be involved in cancer development, are the HSR and the UPR. These two pathways are activated upon stress and there is a potential for a crosstalk between the HSR and the UPR. For example, the HSR is induced by tunicamycin treatment in cells lacking IRE1, and heat stress can activate the UPR in mammalian cells via eIF2α phosphorylation and XBP1 splicing (Parfitt, 2014). In addition, Kennedy et al, demonstrated a molecular link between HSR and ER stress-induced apoptosis, which involves down regulation of BIM (Kennedy, 2014). In the database analysis, HSF1, HSPA1A and HSPB1 expression showed a positive correlation with *MKI-67* mRNA expression in basal-like BC only, and HSF1 and HSPA1A showed a significant increase in percentage of patients with tumour grade 3, which indicates a potential impact of these genes on cells proliferation in basal-like BC. Previous studies have shown that HSPB1 and HSPA1 increased expression enhances pro-survival by inhibiting multiple cell death pathways (Calderwood and Gong, 2012). In addition, low XBP1 expression was correlated with increased proliferation and

associated with worse prognosis and decreased AE-free survival. However, XBP1 results are quite unexpected since spliced form of XBP1 (XBP1s) is reported to be overexpressed in TNBC presenting an aggressive phenotype, and was shown to be required for a complete hypoxic response through direct binding to HIF1α in TNBC enhancing tumour growth (Chen et al., 2014, Romero-Ramirez et al., 2004). Moreover, Chen et al has shown that high levels of XBP1 mRNA expression have decreased relapse free survival in two separate databases, while in this study, XBP1 expression was analyzed from 15 databases and the clinical outcome was cumulative of relapse free survival, metastasis free survival and overall survival and defined as adverse effect (AE) free survival. Transcription of target genes that enhance protein folding in the ER and degradation of misfolded proteins plays an important role in XBP1 function (Ron and Walter, 2007). It was shown that splicing of XBP1 mRNA decreases after prolonged ER stress, while PERK signaling is sustained over time (Lin et al., 2007). In addition, inactivation of XBP1 splicing, or knock down of XBP1 or IRE1α, sensitizes cells to death after irreversible ER stress (Lisbona et al., 2009). Chen et al also stated that the ratio of spliced to unspliced XBP1 is higher in TNBC, however, in the databases analyzed, it was not clear whether XBP1 mRNA levels obtained represent the spliced or unspliced forms of XBP1, therefore, quantitative analysis of the spliced vs unspliced forms can help understand better the impact of XBP1 in basal-like BC.

Although mRNA profiling has been used to identify genes and markers of different diseases including cancer, still this approach does not give a comprehensive explanation of underlying molecular pathogenesis underlying a certain disease, especially cancer. In cancer, several factors contribute to tumour progression and profile such as interaction between different genes, activators and inhibitory factors (Hanahan & Weinberg, 2011). Moreover, epigenetic factors and gene mutations are essential for tumour development, but mRNA profiling can give a platform and direct towards important factors that might be important targets for cancer therapy.

Based on the mRNA expression analysis obtained in this study, the NF-κB pathway appears to have a significant impact on basal-like BC development. Therefore, it is important to identify other factors that might enhance NF-κB activity in basal-like BCs and induce its pro-survival signaling. Receptor interacting serine/threonine protein kinase 2 (RIP2) has been shown to be an activator of the NF-κB pathway (McCarthy et al., 1998). Several studies have also implicated the role of RIP2 in cell proliferation and pro-survival in myoblasts, keratinocytes and Schwann cells (Munz et al., 2002, Adams et al., 2010, Khursigara et al., 2001). Andersson et al. also demonstrated the role of RIP2 in modifying NF-κB induced VEGF signaling and vascular permeability in myocardial ischaemia (Andersson et al., 2015), and inhibition of RIP2 was also shown to interrupt NF-κB activity in lung tissues and identified as a

novel therapeutic target for the treatment of allergic asthma (Goh et al., 2013). Due to its confirmed NF-κB activation function, the role of RIP2 in basal-like BC development was investigated, and results are outlined in Chapter 4. Moreover, a significant correlation between HSF1, HSPA1 and HSPB1 expression with proliferation was obtained only in basal-like BC, therefore, the role and mechanism of HSPA1A and HSPB1 in TNBC was studied and described in Chapter 5, in order to help understand the function of the HSR and identify potential interventional pathway in basal-like BC. Currently, it is unclear how tumour cells adapt to long-term stress. Since the HSR and UPR can trigger pro-survival and pro-apoptotic signals, it is important to understand how these processes can contribute to carcinogenesis in basal-like BC. The up-regulation of HSR expression of HSPA1 and HSPB1 in basal-like BC may be beneficial to increase protein-folding capacity and prolong tumour cells survival.

Chapter 4

RIP2 enhances cell survival by activation of NF-κB in Triple Negative Breast Cancer

4.1. Introduction

RIP2, receptor interacting serine/threonine protein kinase 2 (known also as RIPK2, RICK and CARDIAK), is a 61-kDa-adaptor kinase composed of two main domains, a serine-threonine-tyrosine kinase and a caspase activation and recruitment domain (McCarthy et al., 1998). RIP2 is well known for its role in inflammation and immunity (McCarthy et al., 1998, Magalhaes et al., 2011) and is capable of inducing both NF-κB activation and cell death via association with the tumour necrosis factor receptor (TNFR) (Hasegawa et al., 2008, Khursigara et al., 2001). Recently, Singel and colleagues have revealed a role for RIP2 in BC and as a potential chemo-sensitizer (Singel et al., 2014).

NF-κB signaling has been shown to contribute to chemoresistance of BC cells to many treatments, including tamoxifen, doxorubicin and paclitaxel (Biswas et al., 2000, Tapia et al., 2014, Dai et al., 2009). Since NF-κB is not considered to be a good target for therapy, partly because of its integral role in immune signaling and because it can display both positive and negative effects on tumour progression (Kim et al., 2006), it is important to elucidate the upstream elements of the pathway and identify more effective targets. NF-κB is a dimeric transcription factor composed of members of the NF-κB/*Rel* family, including p50, p52, p65 (*Rel*A), *Rel*-B, and c-*Rel* (Caamano and Hunter, 2002). Activation of NF-κB is triggered by the IKK kinases phosphorylation of I-κB and its subsequent proteasomal degradation. NF-κB then translocates to the nucleus where it binds to κB consensus sequences and modulates the expression of numerous target genes (Hoffmann and Baltimore, 2006). A large body of evidence has demonstrated that NF-κB plays a protective role against apoptosis in most tissues and cell types (Giri and Aggarwal, 1998, Antoon et al., 2014). The protection by NF-κB is due to transcriptional regulation of a number of anti-apoptotic proteins such as c-FLIP, Bcl-2, Bcl-x_L, cIAP2, and A1/Bfl-2, hence blocking NF-κB activation can overcome resistance (Bours et al., 1994, Dai et al., 2009, deGraffenried, 2004, Kim et al., 2006).

The tumour microenvironment of BC consists of different types of cells (fibroblasts, endothelial cells, pericytes, immune cells) and different soluble factors (cytokines, chemokines). It plays a very important role in tumour progression and the development of distant metastases. It includes cytokines and growth factors that are secreted by the cancer cells and act on BC cells via paracrine effect, such as TNF-α, interleukins, CSF-1 and VEGF, enhancing their proliferation (Jiang and Shapiro, 2014, Goldberg and Schwertfeger, 2010). Tumour necrosis factor-α (TNF-α) was found to be significantly elevated in tumour cells in comparison to normal breast tissue what suggests progression-related roles TNFα in BC (Soria et al., 2011). Granulocyte- and granulocyte-macrophage colony-stimulating

factors (G- and GM-CSF) are known to be powerful regulators of proliferation, differentiation and maturation of hematopoetic progenitors and were found to have an important role in biology of BC. Elevated expression of CSF-1 and its receptor correlates with invasiveness of tumour cells and poor clinical prognosis (Sapi, 2004). The regulation of CSF-1 expression and its receptor, CSF-1R, occurs autocrine and paracrine mechanisms involving tumour cells (Patsialou et al., 2009). In addition, interleukins (IL), specifically IL-6 and IL-8, are both critical for growth of TNBCs but not ER-positive BC cells (Lukaszewicz et al., 2007). IL-6 and IL-8 are also critical for anchorage-independent colony formation, as well as resistance to apoptosis (Tawara et al., 2011); in addition, coordinated cytokine expression and signaling is critical for the growth of TNBCs and act in a paracrine fashion to enhance neovascularization and inflammation-dependent carcinogenesis (Kuilman et al., 2008). Moreover, further investigations revealed that the expression of IL-6 and IL-8 is critical for xenograft tumour growth and that combined overexpression of these genes correlates with poor prognoses in patients with BC (Hartman et al., 2013). Another player in the complex process of progression of the BC is TGF-β. TGF-β is a multifunctional regulator of cell growth, apoptosis, differentiation and migration and exists in three isoforms (Perera et al., 2010). TGF-β is one of the most potent metastatic inducers (Serra and Crowley, 2003) and a tumour promoter by eliciting an epithelial-mesenchymal transition (EMT) (Dumont and Arteaga, 2000). Vascular endothelial growth factor (VEGF) is also a potent angiogenic cytokine in normal tissues (Hoeben et al., 2004) and stimulates tumour proliferation in vitro and induces tumour angiogenesis *in-vivo* (Mor et al., 2004). Moreover, VEGF is overexpressed in BC tissue when compared to normal breast tissue and levels of VEGF in these tissues correlate with disease-free and overall survival (Byrne et al., 2007, Ali et al., 2011). The described cytokines are all targets of NF-κB transcription factor (Zhou et al., 2003, Son et al., 2008), therefore, it is important to determine the cytokine profile of TNBC cells upon RIP2 overexpression, confirm RIP2 as a possible factor of tumourigenesis and identify it as a potential target for TNBC therapy.

Aims of this study:

- Examine the expression of RIP2 and its association with tumourigenesis in basal-like or TNBC patients
- Determine the role of RIP2 in TNBC
- Investigate the impact of RIP2 on NF-κB pathway and NF-κB target genes
- Investigate the immunological profile of TNBC upon RIP2 activation

4.2. Results

4.2.1. RIP2 expression is upregulated in TNBC subtype and large tumours in patient samples

BC patient samples were obtained from University College Hospital Galway (UCHG) and RIP2 expression in samples was analyzed by quantitative PCR (qPCR). Patient samples with TNBC subtype have higher endogenous RIP2 levels in comparison to other luminal or HER-2 subtypes (Figure 4.1). The average fold-increase of RIP2 expression in 5 samples obtained was 8.89 ± 5.61, which is higher than all other subtypes as shown in Table 4.1. In addition, patients with TNBC subtype had a relatively larger tumour size indicating that RIP2 expression may affect tumour growth, but that was not significant due to the small sample size ($p = 0.21$). However, the difference in TNBC in comparison to other subtypes can be seen. Table 4.1 outlines patients' samples characteristics.

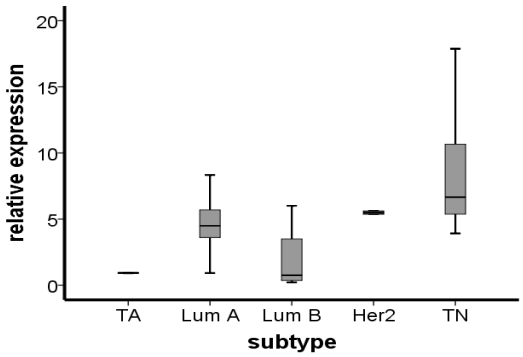


Figure 4.1: RIP2 expression associates with TNBC phenotype.

21 BC patient samples were obtained from University College Hospital Galway and RIP2 expression was analyzed by qPCR. Relative mRNA expression was expressed relative to TA (Tumour Associated). Lum A: Luminal A subtype, Lum B: Luminal B subtype, HER-2: HER-2+ BC, TN: Triple Negative.

Table 4.1: Characteristics of patients' samples.

Subtype	no	ER	PR	HER-2	average RIP2 fold increase	average tumour size (cm)
Luminal A	5	+	+	-	4.60 ± 2.7	21.40 ± 5.03
Luminal B	3	+	+	+	2.23 ± 1.63	32.33 ± 11.85
	1	-			1.00	18
HER-2	2	-	-	+	5.48	39.00
TN	5	-	-	-	8.89 ± 5.61	41.20 ± 17.03

Patient samples obtained from UCHG were grouped based on subtype. ER: estrogen receptor; PR: progesterone receptor; HER-2: human epidermal growth factor receptor. The mean is the average value of fold-increase in RIP2 expression ± SD; average tumour size shows tumour size among different subtypes ± SD.

4.2.2. RIP2 correlates with poor patient prognosis and associates with cancer subtype, tumour grade and size

Due to the limited availability of patient BC samples and in order to validate our observation from UCHG patient samples, online GEO NCBI patient databases were downloaded from NCBI Geo (Mizuno et al., 2009) as described in Chapter 3, and RIP2 expression effect on patient survival was analyzed in addition to its impact on tumour type, stage and size. A total of 4 databases with 595 patients that identify the basal-like subtype are described in Table 4.2; therefore, only these databases were considered for analysis in this chapter. Patient samples were compared as being basal-like or non-basal-like referred to as others which includes all other subtypes (Luminal A, B and HER-2); only one database GSE1456-GPL96 identifies samples as basal-like but as mentioned in Chapter 1, TNBC phenotype and basal-like subtype are often used interchangeably as almost 80% of molecular basal-like tumours are triple-negative (Badve et al., 2011). Compared to other BC subtypes, RIP2 expression is more in basal-like subtype and is associated with grade 3 tumours, stage 4 tumours and larger tumour size (Table 4.2). As shown in Table 4.2, in GSE19615 and GSE12276 databases, respectively, 78% and 64% of samples with tumour grade 3 are basal-like, 61% and 59% display tumour stage 4 with average tumour size 3.14 cm and 4.72 cm. Combining data from these databases, basal-like subtypes show significantly larger tumour size, and higher grade and stage of tumour ($p < 0.001$). Similar trend was observed in GSE1456-GPL96 with respect to tumour grade, 73% of basal-like while only 33% of non-basal-like samples have tumour grade 3 ($p < 0.01$). Tumour size and stage data were not available for this database. This trend was not significant in the E-TABM-158, which might be due to the small number of basal-like samples in this database.

Table 4.2: Patient databases with basal-like subtype and relative mean RIP2 expression, tumour grade, size and stage

database no	n	tumour type	n	mean RIP2	tumour grade = 3	tumour size (cm)	tumour stage = 4
GSE 19615	115	other	86	5.32	39%	1.72	16%
		basal-like	29	5.98	78%*	3.14**	61%**
E-TABM-158	117	other	96	7.29	42%	2.53	4%
		basal-like	19	8.15*	48%	2.91	39%**
GSE1456-GPL96	159	other	133	7.45	33%	NA	NA
		basal-like	26	7.99*	73%**		
GSE 12276	204	other	153	6.47	27%	2.19	20%
		basal-like	51	7.98**	64%**	4.72*	59%**

** p-value < 0.05 and ** p-value < 0.01. NA: not available*

Figure 4.2a shows the average RIP2 expression in the different BC cell lines. As shown in the boxplot, RIP2 expression is highest in basal-like BC with p(Wallis) < 0.0001. Moreover, comparing the samples as expressing high or low RIP2 level, where "high" represents samples in which RIP2 level is more than the mean in particular database and "low" represents samples in which RIP2 level is below the mean, 21% of samples have high RIP2 expression out of which 19% are basal-like, while 79% of samples have low RIP2 expression out of which only around 2% are basal-like ($p < 0.001$) and 60% of basal-like BC have high RIP2 expression while only 10% of other BC subtypes have high RIP2 expression (Figure 4.2b). Kaplan-Meier analysis showed that RIP2 over-expression in BC patients is associated with poor prognosis only in basal-like BC ($p < 0.05$) (Figure 4.2c). Moreover, RIP2 expression associates with higher tumour grade. In comparison between low and high RIP2 expressing samples, 78% of samples expressing high RIP2 are grade 3 tumours ($p < 0.01$) (Figure 4.2d).

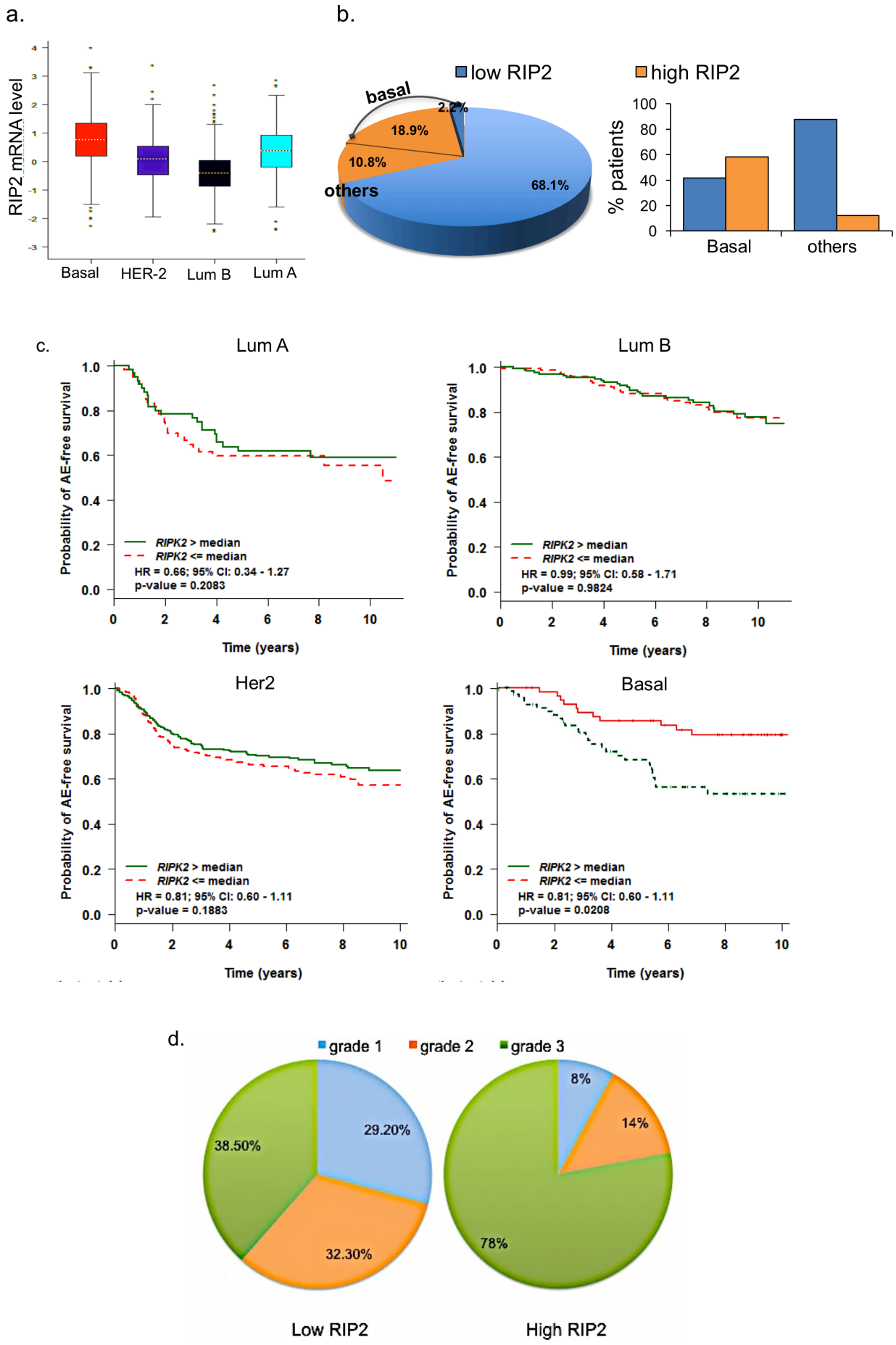

a.
RIP2 mRNA level
Basal
HER-2
Lum B
Lum A
b.
low RIP2
high RIP2
basal
2.2%
18.9%
10.8%
others
68.1%
% patients
Basal
others
c.
Lum A
Lum B
Her2
Basal
Probability of AE-free survival
Time (years)
RIPK2 > median
RIPK2 <= median
HR = 0.66; 95% CI: 0.34 - 1.27
p-value = 0.2083
HR = 0.99; 95% CI: 0.58 - 1.71
p-value = 0.9824
HR = 0.81; 95% CI: 0.60 - 1.11
p-value = 0.1883
HR = 0.81; 95% CI: 0.60 - 1.11
p-value = 0.0208
d.
grade 1
grade 2
grade 3
29.20%
38.50%
32.30%
8%
14%
78%
Low RIP2
High RIP2

Figure 4.2: RIP2 expression correlates to poor prognosis and associates to BC basal-like subtype, tumour grade and size.

a. Box plot showing RIP2 expression among different BC subtype; b. Pie chart showing samples with high RIP2 vs low RIP2 expression, and subsequent division into basal-like and other; c. Kaplan-Meier overall survival curves using RIP2 as a biomarker comparing high and low RIP2 expressions (dichotomized at the median) of public BC expression array datasets within the different subtypes; Statistical significance was performed using Kruskal-Wallis one-way analysis of variance; d. Pie chart showing distribution of BC tumour grades among patients with low RIP2 expression vs high RIP2 expression.

4.2.3. RIP2 correlates with *MKI-67* expression in basal-like BC subtype

Based on results in Section 4.2.2, and the impact of RIP2 on cancer size and grade, RIP2 effect on cancer growth and proliferation was assessed; patient databases were analyzed for *MKI-67* expression and correlated with RIP2 expression among different subtypes. *MKI-67*, also known as *Ki67*, is marker of proliferation and is used as a clinical diagnostic tool to assess tumour grade and a prognostic parameter in BC patients (Inwald et al., 2013). Based on correlation analysis, significant positive correlation r = 0.33 was observed in basal-like BC, while no significant correlation was found between RIP2 and *MKI-67* in other subtypes (Figure 4.3).

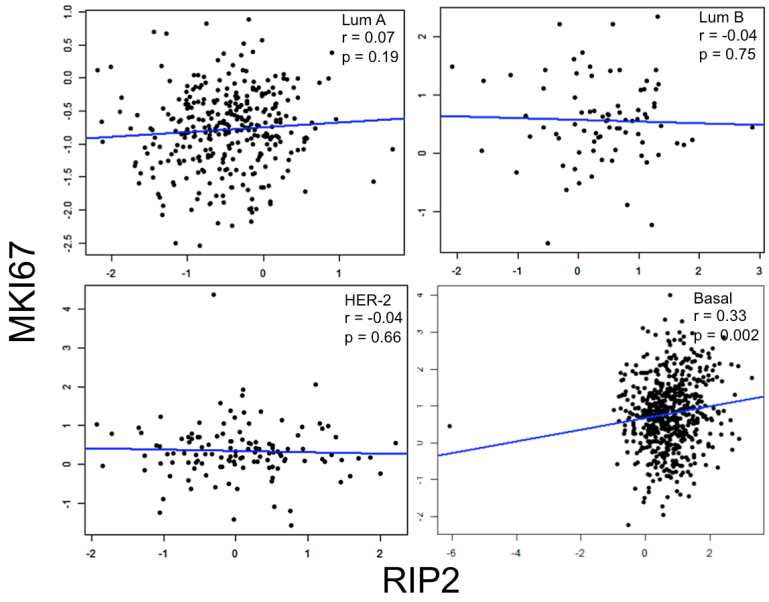


Figure 4.3: RIP2 correlates with *MKI-67* proliferation marker in basal-like BC.

Correlation graphs of RIP2 mRNA expression relative to MKI-67 mRNA in different BC subtypes. Correlation analysis was done using SPSS software.

4.2.4. RIP2 basal-like expression among BC cell lines

In order to understand the role of RIP2 in pro-survival signaling in BC and to reveal its impact on cancer development and prognosis especially basal-like, different BC cell lines

were screened for RIP2 expression. BC cell lines were cultured as per optimum conditions and harvested at 48 h post seeding and cell lysate was collected. Western Blot analysis shows that all TNBC cell lines except HCC1806 have higher RIP2 expression in comparison to non-TNBC and MCF10A control (Figure 4.4) which indicates a potential role of RIP2 in TNBC.

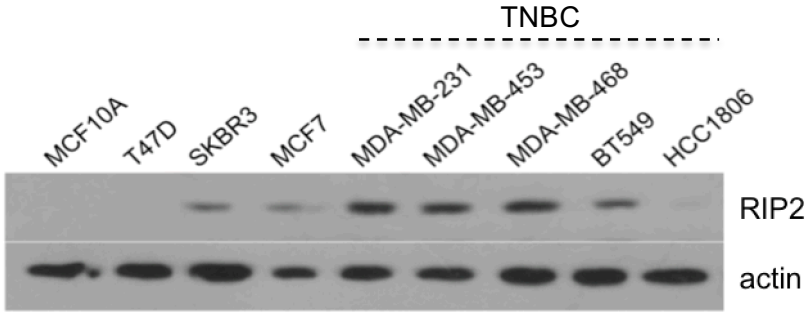


Figure 4.4: RIP2 expression among BC cell lines.

BC cell lines were harvested at 48 h post seeding and 30 ng of protein was loaded on 12% polyacrylamide gel. Membranes were probed for RIP2 and actin was used as loading control. Data shown are representative of 3 independent repeats of same experiment. MCF10A is a control breast tissue; T47D, SKBR3 and MCF-7 are luminal BC; MDA-MB-231, MDA-MB-453, MDA-MB-468, BT549 and HCC1806 are TNBC

4.2.5. RIP2 overexpression induces apoptotic cell death in MCF-7, T47D and SKBR3

In order to determine if RIP2 have any role in non-TNBC cell lines, wild-type RIP2, mutants and knockdown were used for transfection of Luminal A BC subtype MCF-7 and T47D, and HER-2 positive SKBR-3. The mutants used were the RIP2-K209R mutant where K209 is an essential ubiquitination site of RIP2 important for kinase domain activation, and CARD-only RIP2 also referred to as dominant-negative. shRNA against RIP2 was used for RIP2 knockdown. All mutants were cloned into pcDNA3.1 backbone, and hence pcDNA3.1 was used as plasmid control, and scrambled shRNA was used as control for shRNA-RIP2. Figure 4.5a shows the plasmids expression in the different indicated cell lines; actin was used as loading control. Wild-type RIP2 overexpression, RIP2 mutants overexpression, and RIP2 knockdown enhanced cell death among the three cell lines which was demonstrated by significantly reduced and a decrease cell count was observed in all cell lines used (Figure 4.5b). This observation might indicate a role of RIP2 in cell cycle control and requires further investigation.

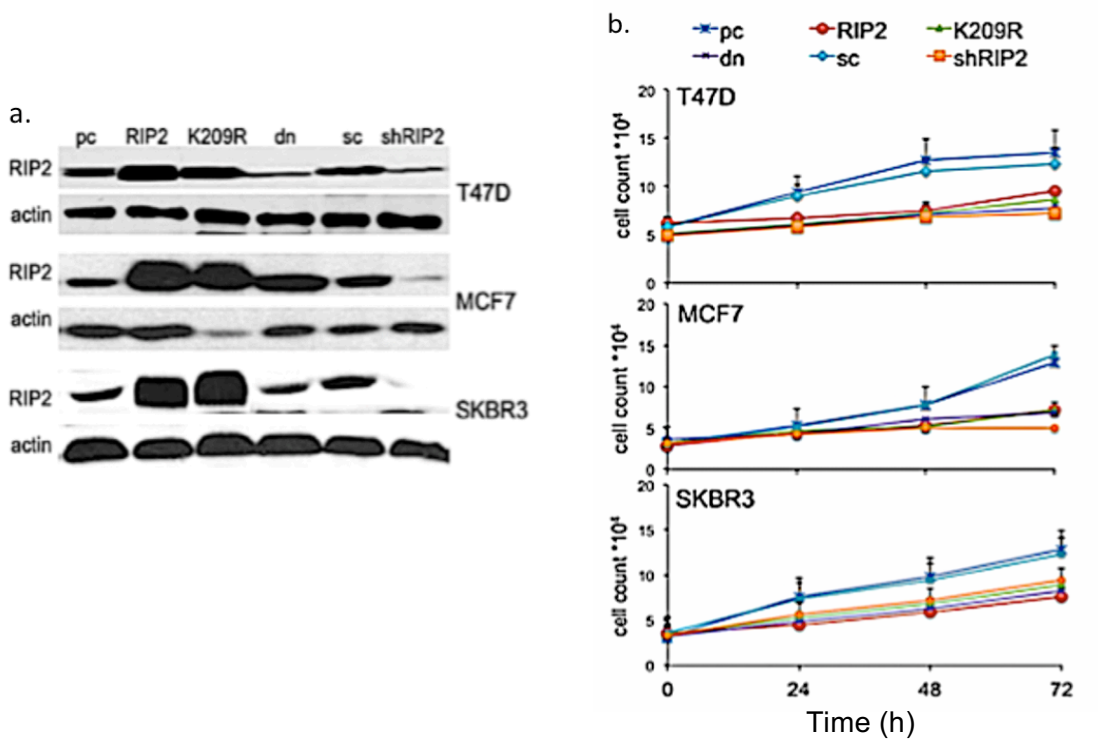


Figure 4.5: RIP2 overexpression induces cell death in MCF-7, SKBR3 and T47D.

Luminal BC cell lines, MCF-7, SKBR3 and T47D, were seeded at optimum seeding density in T25 flask. At 24 h post seeding cells were transfected with wild-type RIP2 (RIP2), RIP2-K209R (K209R) and CARD-only RIP2 (dn) and plasmid controls pcDNA3.1 (pc) and scrambled shRNA control (sc). a. Western blot analysis showing RIP2 expression 48 h post transfection. Cell lysates of transfected with the different RIP2 plasmids were loaded as indicated. Actin was used to control for loading; b Cell count at 48 h post transfection with control plasmids and RIP2 constructs. All data shown are representative of 3 independent repeats of the same experiments. Results are the mean of 3 separate experiments ± standard error of the mean.

4.2.6. RIP2 enhances survival in MDA-MB-231 TNBC

As shown in the previous section, RIP2 has no role in luminal and HER-2 BC subtypes; therefore, its role in cell proliferation and survival in TNBC with high endogenous RIP2 levels was determined (Figure 4.6). MDA-MB-231 cells were used as they are widely used in cell culture and represent a good TNBC model. MDA-MB-231 cells were seeded at $3x10^4$ cells/cm^2 cell density and transfected with control plasmids pcDNA3.1 and scrambled shRNA control and RIP2 plasmid constructs wild-type RIP2, RIP2-K209R, CARD-only RIP2 and shRNA-RIP2 for silencing RIP2 (described earlier). At various times post transfection the cells were observed and harvested to assess the impact of RIP2 on proliferation and survival using several assays including cell count, cell viability assay, loss in mitochondrial transmembrane potential and cell cycle analysis.

Upon overexpression of wild-type RIP2 in MDA-MB-231 cells higher cell density (Figure 4.6b) and increased cell count (Figure 4.6c) were observed in comparison to pcDNA3.1 control. On the other hand, RIP2-K209R mutant showed less cell density and cell number at 48 h indicating a role of ubiquitination in RIP2 function. In addition, a significant

decrease in cell number was observed with CARD-only RIP2 and RIP2 knockdown at 48 and 72 h post transfection ($p < 0.01$). Figure 4.1a shows relative RIP2 expression in MDA-MB-231 cells upon transfection with RIP2 plasmids. Arrows indicate the RIP2 protein at 60 kDa and 8 kDa that corresponds to the CARD-only domain of RIP2. Measuring cell survival by MTT and mitochondrial transmembrane potential by TMRE assays, a decrease in cell viability and mitochondrial membrane potential upon CARD-only RIP2 transfection and a significant decrease in cell viability and mitochondrial membrane potential at 48 h upon RIP2 knockdown in comparison to scrambled control were observed (Figure 4.6 d and e). RIP2 and K20R transfection did not show a significant change in cell viability or mitochondrial transmembrane potential change. However, cell cycle analysis at 48 h post transfection showed a slight increase in S and G2/M cells upon RIP2 transfection, which was not observed with RIP2-K209R and a significant increase in SubG1 cells and a decrease in G2/M cells upon CARD-only RIP2 expression and RIP2 knockdown compared to pc and scrambled controls (Figure 4.6f). Table 1 shows percentage of cells in different phases of cell cycle upon transfection with the different plasmid constructs.

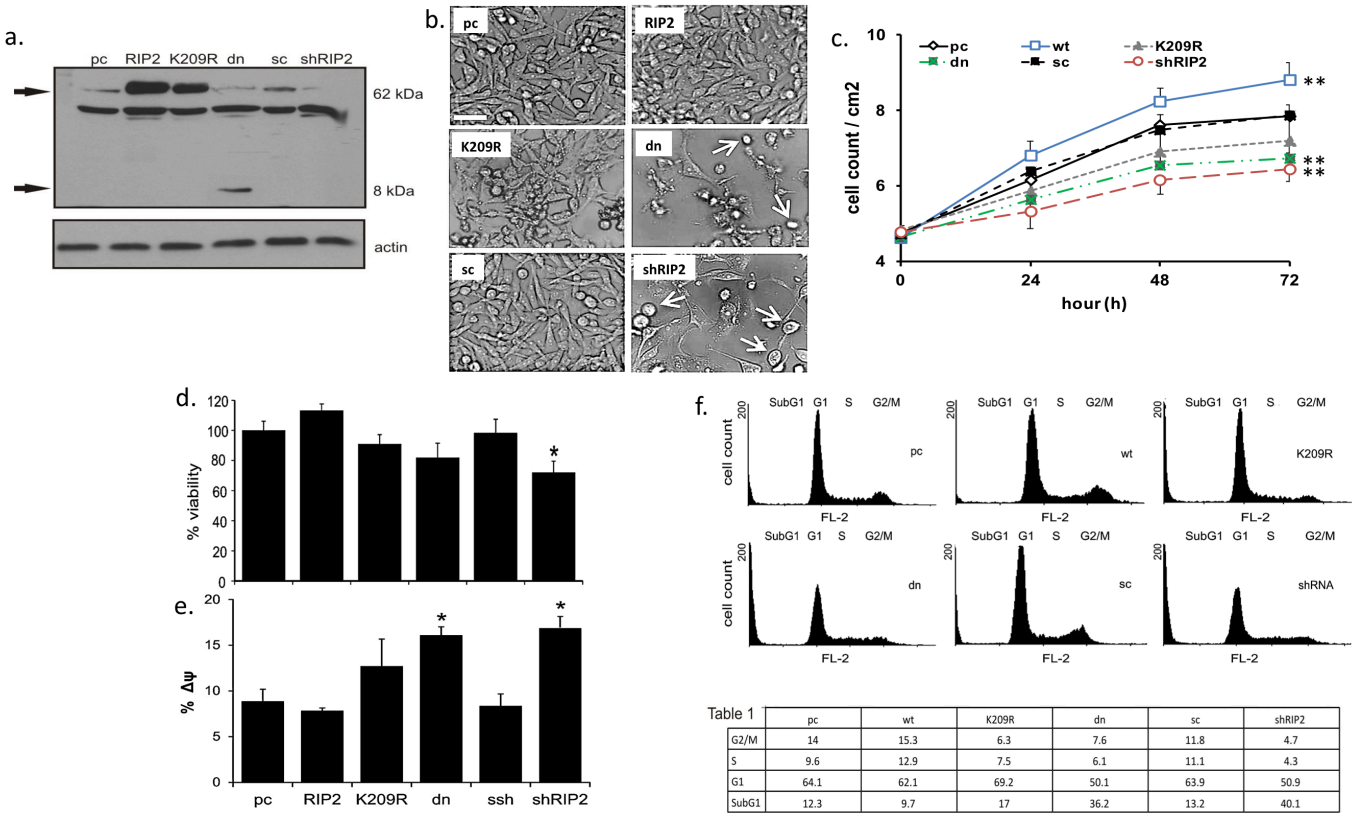


Table 1

	pc	wt	K209R	dn	sc	shRIP2
G2/M	14	15.3	6.3	7.6	11.8	4.7
S	9.6	12.9	7.5	6.1	11.1	4.3
G1	64.1	62.1	69.2	50.1	63.9	50.9
SubG1	12.3	9.7	17	36.2	13.2	40.1

Figure 4.6: RIP2 expression is important for MDA-MB-231 cell survival.

*MDA-MB-231 cells seeded at 3x10 cells/cm^2 and transfected 24 h post seeding with wild-type RIP2 (RIP2), RIP2-K209R (K209R) and CARD-only RIP2, shRNA-RIP2 and plasmid controls pcDNA3.1 (pc) and scrambled shRNA control (sc). a. Western blot analysis showing RIP2 expression 48 h post transfection. Cell lysates of MDA-MB-231 cells transfected with the different RIP2 plasmids were loaded as indicated. Arrows indicate RIP2 full length (60 kDa) and CARD-only RIP2 (8 kDa). * indicates a non-specific band. Actin was used to control for loading; b. Light microscope images of cells in 24-well plate at 48 h post transfection. Arrows indicate detaching round cells; c. MDA-MB-231 cell count at 24, 48 and 72 h post transfection; c.; d. MTT cell viability assay at 48 h post transfection with control plasmids and RIP2 constructs in 96-well plate; e. TMRE assay of MDA-MB-231 cells cultured in 24-well plate and harvested at 48 h post transfection with control plasmids and RIP2 constructs; f. Cell cycle analysis assay at 48 h post transfection with control plasmids and RIP2 constructs. The Table shows percentage of cells in different phases of cell cycle. All data shown are representative of 3 independent repeats of the same experiments. Results are the mean of 3 separate experiments ± standard error of the mean. Statistical significance was calculated by One-way and expressed in comparison to control (* $p < 0.05$; ** $p < 0.01$).*

4.2.7. RIP2 protects MDA-MB-231 cells against drug induced cell death

To determine whether RIP2 has a role in TNBC resistance to therapy, MDA-MB-231 cells were transfected with the different plasmids described and treated with a panel of drugs having different modes of activities and confirmed to induce cell death in BC. Cell survival was then measured by TMRE assay. A decrease in the loss of mitochondrial membrane potential was observed upon RIP2 overexpression and was significant with ceramide, taxol

and thapsigargin treatment ($p < 0.01$) (Figure 4.7a), while RIP2-K209R mutant, non-ubiquitinated RIP2 form, abolished protection observed with wild-type RIP2 indicating a role of ubiquitination in RIP2 enhanced drug induced cell death resistance. CARD-only RIP2 and RIP2 knockdown sensitized cells to treatments and a significant increase in the loss of mitochondrial potential was observed in all treatments ($p < 0.01$) (Figure 4.7a). The results of the assay show that RIP2 can protect MDA-MB-231 cells against ceramide, taxol and thapsigargin induced loss of mitochondrial potential, and mutant forms of RIP2 and knockdown could sensitize cells to drug induced mitochondrial potential loss.

To further confirm a role for RIP2 in drug resistance, ceramide and taxol were chosen for further cell survival assays due to their ability to induce apoptosis in MDA-MB-231 cells as shown by DAPI staining in Figure 4.7b, Arrows indicate apoptotic cells observed 24 h post ceramide and taxol treatment. In comparison to pcDNA3.1 control, RIP2 significantly increases cell viability upon treatment with ceramide and taxol ($p < 0.01$), while CARD-only RIP2 significantly sensitized cells to death as shown by decreased cell viability ($p < 0.01$), and RIP2-K209R mutant, in contrast to wild-type RIP2, did not show protection against ceramide and taxol induced cell death (Figure 4.7c). Similar to CARD-only RIP2, RIP2 knockdown significantly decreased cell viability in comparison to scrambled control hence sensitizing cells to death ($p < 0.001$). DEVDase assay shows a decrease in caspase-3/-7 like activity in wild-type RIP2 overexpressing cells ($p < 0.01$), and a significant increase in caspase-3/-7 like activity in cells expressing CARD-only domain or RIP2 knockdown upon treatment with ceramide and taxol (Figure 4.7d), which indicates that RIP2 has an anti-apoptotic role in MDA-MB-231 cells.

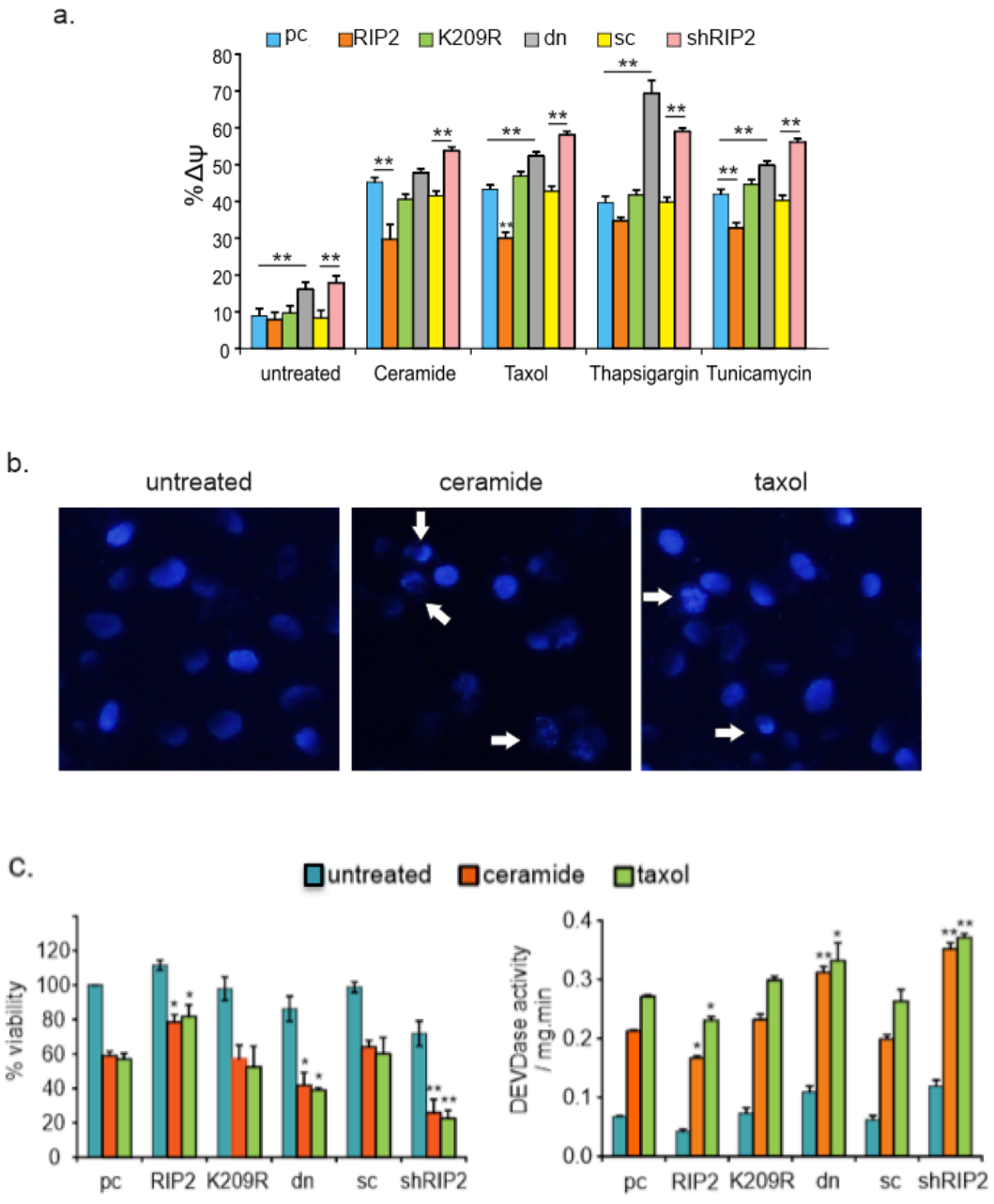


Figure 4.7: RIP2 expression affects cell response to treatments.

a. TMRE analysis upon treatment of pcDNA3.1 (pc), wild-type RIP2 (wt), RIP2-K209R (K209R), CARD-only RIP2 (Henstridge et al., 2014), scrambled shRNA control (sc) and shRNA-RIP2 (shRNA) transfected MDA-MB-231 cells with drugs (ceramide 30 μM, taxol 1 μM, thapsigargin 3 μM, tunicamycin 10 μg/ml); b. DAPI staining of MDA-MB-231 cells upon treatment of ceramide and taxol showing apoptotic cell morphology (indicated arrows); c. Cell viability MTT assay and d. Caspase-3/-7 like assay in MDA-MB-231. Cells were transfected with RIP2 plasmids and treated with ceramide (30 μM) and taxol (1 μM) 24 h post transfection and then harvested 24 h post treatment for indicated assays. All results shown above are representative data of 3 separate experiments. Mean values are shown ± standard error. Statistical significance is expressed in comparison to treated control ($p < 0.05$; ** $p < 0.01$).*

4.2.8. RIP2 enhances long term clonogenic survival in MDA-MB-231 cells

In order to verify that RIP2 protective effect against drug induced cell death is not a transient experimental effect, clonogenic survival assay was performed to further confirm the effectiveness of RIP2 on cell survival and proliferation. MDA-MB-231 pcDNA3.1 and wild-type RIP2 transfected cells were seeded in 6-well plates (1000 cells per well) and allowed to adhere. At 24 h post seeding, cells were treated with 30 μM ceramide and 1 μM taxol and left for further 24 h at which time the media was replaced with fresh media. Cells were left in culture and media was replaced every 2 days. After 15 days, media was removed and cells were stained by crystal violet. Colonies containing at least 50 cells were considered as a single colony. As shown in Figure 4.8a, it is very clear that more colonies are observed in wild-type RIP2 transfected cells in comparison to pcDNA3.1 transfected cells and a significant increase in number of colonies was observed in RIP2 overexpressing cells post ceramide and taxol treatment (Figure 4.8b) which confirms the pro-survival role of RIP2 in TNBC.

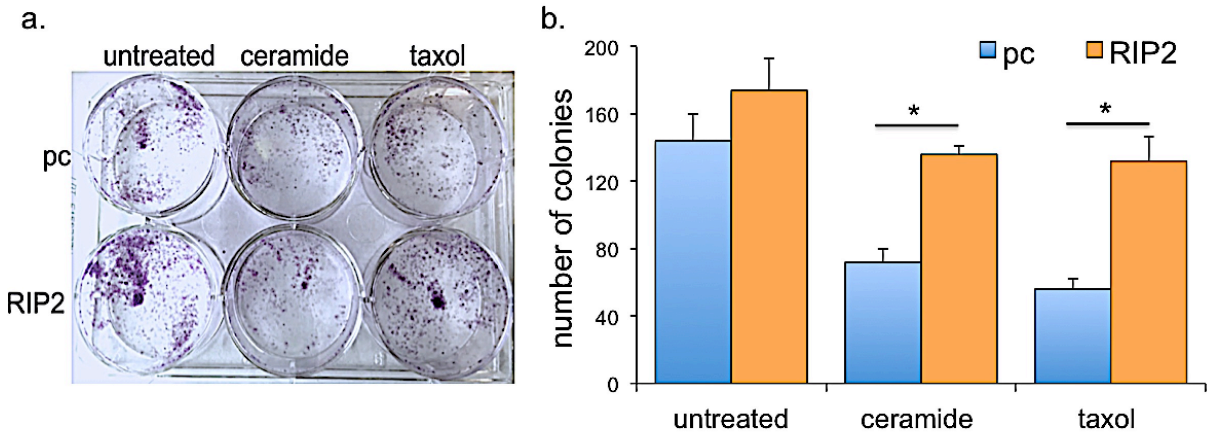


Figure 4.8: RIP2 promotes clonogenic survival of MDA-MB-231 cells

*a. Clonogenic assay MDA-MB-231 cells transfected with pcDNA3.1 (pc) or wild-type RIP2 (RIP2) were seeded in 6-well plate and treated with ceramide (30 μM) and taxol (1 μM) and and left for 15 days. Cells were then stained with crystal violet. b. Number of colonies counted at 15 days post treatment. All data shown above are representative data of 3 separate experiments. Results are mean values of 3 independent experiments ± standard error and * indicates significance level with $p < 0.05$.*

4.2.9. RIP2 activates NF-κB pathway and enhances survival in MDA-MB-231 TNBC cells

Several studies have reported the role of RIP2 in NF-κB activation and pro-survival signaling (McCarthy et al., 1998, Singel et al., 2014). We wanted to investigate the impact of RIP2 on NF-κB in MDA-MB-231 BC cells. MDA-MB-231 luciferase cells reporting NF-κB activity were used. Cells were transfected with the different control RIP2 plasmids and NF-κB activity was measured by luminescence assay using Vector plate reader. Wild-type RIP2

overexpressing cells showed 1.6 and 1.8 fold increase in NF-κB activity at 24 h and 48 h respectively post transfection in comparison to control vector as determined by relative light unit, RLU ($p < 0.05$) while RIP2-K209R mutant showed no increase. Moreover, RIP2 knockdown by shRNA decreased NF-κB activity significantly at 24 and 48 h post transfection ($p < 0.05$ and $p < 0.01$ respectively) and CARD-only RIP2 showed a significant decrease in NF-κB at 48 h ($p < 0.01$) (Figure 4.9a).

In order to determine the role of NF-κB in RIP2 pro-survival activity, NF-κB activity was targeted via treatment with SN50, an NF-κB cell permeable inhibitory peptide, or transfected cells with NF-κB super repressor plasmid (SR) (Bansal et al., 2014), which has a mutated site that inhibit IκB phosphorylation and degradation, what prevents NF-κB downstream signaling. Cells transfected with wild-type RIP2 and co-treated with SN50 or co-transfected with SR plasmid, exhibited a significant decrease in RLU as compared to wild-type alone which indicates that inhibiting NF-κB can impact RIP2 function (Figure 4.9b). To further determine whether RIP2 pro-survival activity in MDA-MB-231 cells is mediated through NF-κB SN50 treatment and co-transfection of wild-type RIP2 with the SR reversed the RIP2 protective effect which was observed upon ceramide treatment (Figure 4.9c) indicating that RIP2 pro-survival effect is mediated through NF-κB.

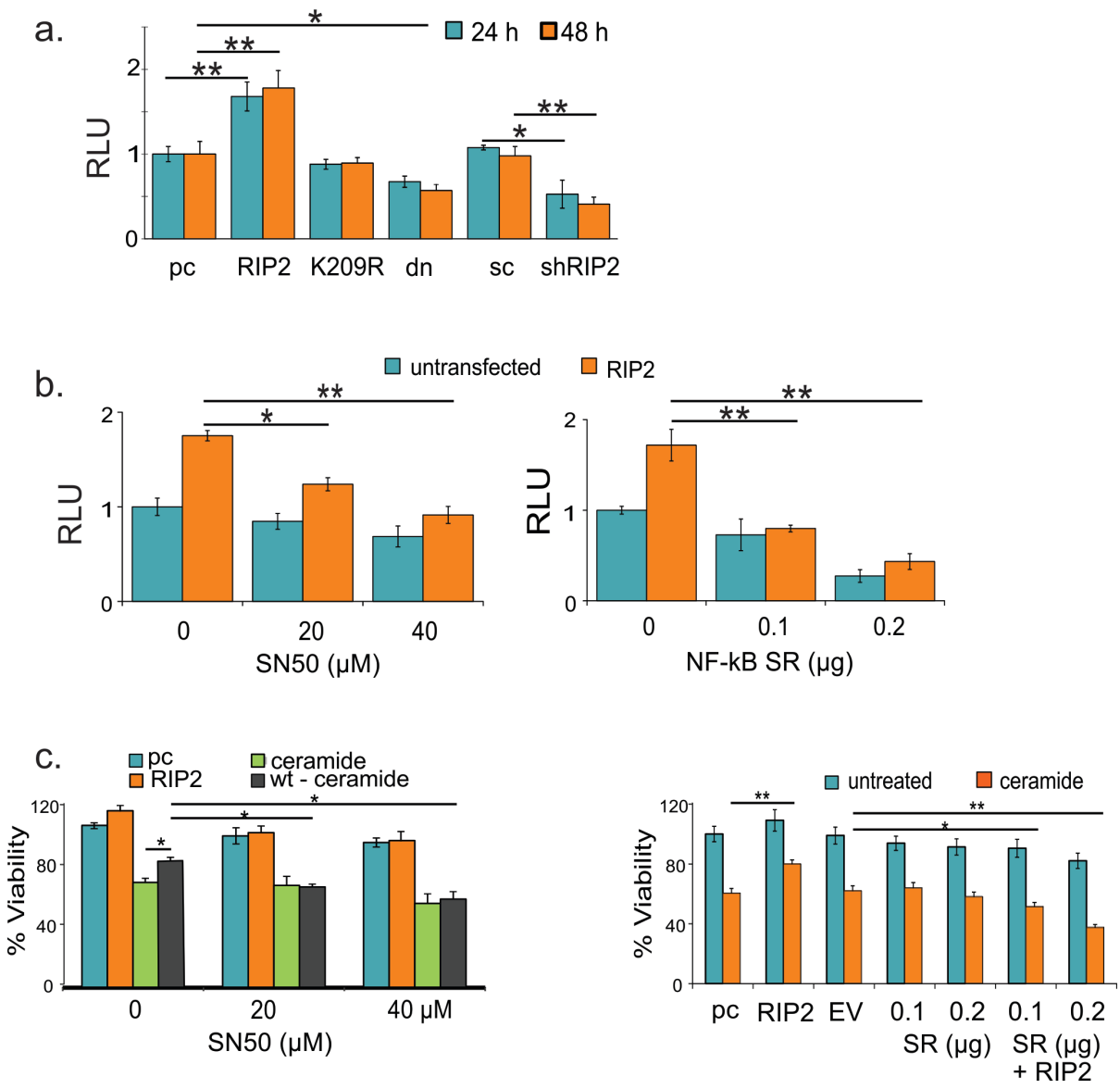


Figure 4.9: RIP2 enhances cell survival and drug resistance by increasing NF-κB activity.

*a. MDA-MB-231 NF-κB luciferase reporter cells were seeded in 96-well plates and transfected with the different plasmids described previously. Luciferase assay was performed and Vector plate reader was used to measure relative luciferase activity. Normalized luciferase values derived relative to pcDNA3.1 control. All luciferase reporter assays were performed in triplicate and error bars represent the standard error between the three data points. (*p < 0.05,**p < 0.01 using One-way Anova analysis). b. MDA-MB-231 luciferase reporter cells were transfected with RIP2 and co-treated with SN50 or co-transfected with NF-κB super-repressor plasmid (SR) (Bansal et al., 2014) and plasmid empty vector control (EV) (Antoon et al., 2014). NF-κB activity was determined by luminescence on Vector plate reader. c. MTT assay upon treatment of MDA-MB-231 cells with 30 μM ceramide. MDA-MB-231 reporter cells were transfected with RIP2 and co-treated with SN50 or co-transfected with NF-κB super-repressor plasmid (SR) and plasmid empty vector control (EV). Wild-type RIP2 indicated as wt and as RIP2 when co-transfected with SR (last 4 bars). Results were average data of 3 separate experiments ± SE. Statistical significance is expressed in comparison to treated control (*p < 0.05, **p < 0.01).*

RIP2 gene expression is associated with increased NF-κB activity but has not been shown to be a direct target of it (Matsuda et al., 2003). Here we show that RIP2 gene expression is directly affected by NF-κB activity. Inhibiting NF-κB pathway by SR expression or using pathway specific inhibitor SN50) decreased RIP2 levels in cells. MDA-MB-231 cells were transiently transfected with NF-κB SR plasmid (0.1 and 0.2 µg) or treated with SN50 inhibitor (20 µM) and RIP2 expression was detected by western blotting at 24 h. As mentioned in Chapter 2, the NF-κB SR plasmid contains the human IκB gene where the phosphorylation sites (Serine 32 and 36) have been mutated into Alanine residues. In this study treating MDA-MB-231 cells with SN50 decreased NF-κB activity (Figure 4.9). SN50 is reported to inhibit NF-κB complex translocation to the nucleus (Nie et al., 2012, Lin et al., 1995), but upon treating cells with SN50 a decrease in IκB phosphorylation was also observed (Figure 4.10). IκB phosphorylation is important for the NF-κB activation mediated by translocation of NF-κB complex subunits to the nucleus (Matsuda et al., 2003). Therefore, RIP2 expression decreases with decrease in phosphorylated IκB and decreased NF-κB activity (Figure 4.9)

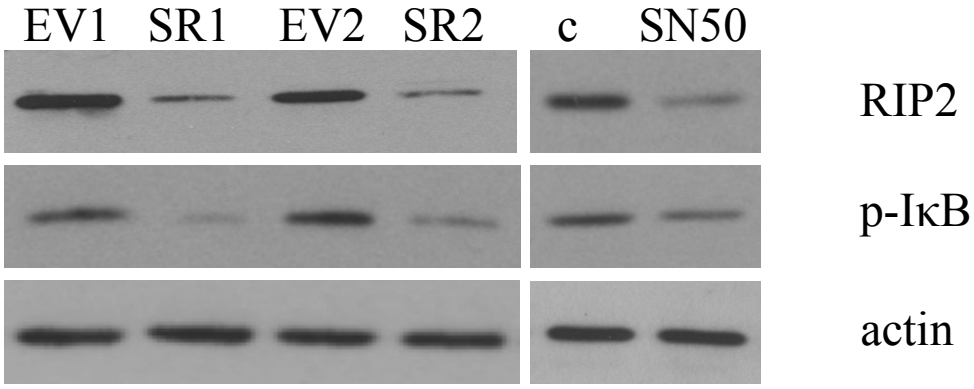


Figure 4.10: RIP2 levels decrease with inhibition of NF-κB.

Western blot analysis showing relative p-IκB and RIP2 expression upon SR plasmid transfection and SN50 treatment for 24 h cell were seeded in T25 flasks and 24 h post seeding, cells were transfected with NF-κB super-repressor plasmid (SR), plasmid empty vector control (EV) at 0.1 µg (EV1 and SR1) and 0.2 µg (EV2 and SR2); control (c) or treated with SN50 (20 µM). Endogenous levels of RIP2 were detected at 24 h post transfection or treatment. Actin was used as loading control. Data shown are representative of 3 separate experiments.

4.2.10. Phosphorylation of RIP2 affects its stability and alters its downstream signaling and drug resistance effect

PP2, a Src family kinase inhibitor, inhibits RIP2 kinase activity and auto-phosphorylation (Windheim et al., 2007). Treatment of MDA-MB-231 cells with PP2, decreased p-RIP2 endogenous levels reducing RIP2 expression at 24 and 48 h post treatment in a dose dependent manner as shown by western blot (Figure 4.11a), which indicates that phosphorylation, as reported in the literature (Nembrini et al., 2009), is

important for RIP2 stability in cells. Moreover, PP2 treatment decreased NF-κB activity in MDA-MB-231 cells as shown by decreased IκB phosphorylation (Figure 4.11a) and a dose dependent decrease in NF-κB activity at 8 and 24 h post PP2 treatment (Figure 4.11b) which indicates the robust importance of RIP2 kinase activity in NF-κB enhancement. Also PP2 dose treatment decreased gradually cell viability at 24 h (Figure 4.11b). In addition, treating MDA-MB-231 cells with PP2 (1 and 5 μM) sensitized MDA-MB-231 to ceramide induced cell death showing similar effect to knocking down RIP2 (Figure 4.11c). This effect might be attributed to the decrease in RIP2 stability rather than the kinase activity.

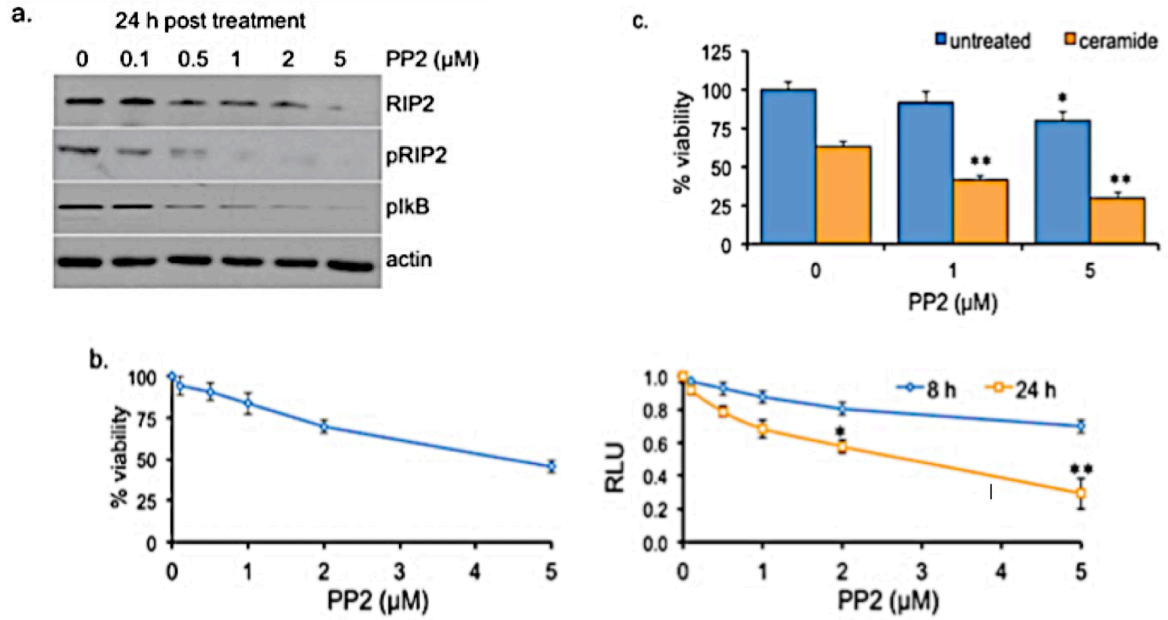


Figure 4.11: RIP2 kinase activity is essential for its stability and activity.

*MDA-MB-231 cells were seeded in T25 flasks and treated with PP2 inhibitor at increasing concentration (0, 0.1, 0.5, 1, 2 and 5 μM). Cells were harvested at 24 h post treatment and lysed for western blot. Membrane was probed for endogenous RIP2, p-RIP2 and p-IkB. Actin was used as a loading control. b. Cell viability (24 h) and NF-κB activity (8 and 24 h) of MDA-MB-231 cells post PP2 dose treatment measured. Cell viability was measured by MTT assay. Luciferase assay was performed and Vector plate reader was used to measure relative luciferase activity. Normalized luciferase values derived relative to pcDNA3.1 control. All luciferase reporter assays were performed in triplicate and error bars represent the standard error between the three data points. (*p < 0.05, **p < 0.01 using One-way Anova analysis). c. MTT assay of MDA-MB-231 co-treatment cells with PP2 (1 and 5 μM) and 30 μM ceramide or 1 μM taxol. Results were average data of 3 separate experiments ± SE. Statistical significance is considered in comparison to control (0 μM PP2 untreated or ceramide) (*p<0.05, **p<0.01).*

4.2.11. RIP2 enhances the expression of anti-apoptotic proteins in MDA-MB-231 through NF-κB activation

In order to understand the molecular mechanism by which RIP2 drives cell survival and resistance to drugs, western blot was performed in order to detect the expression of pro- / anti- apoptotic proteins upon RIP2 expression and knockdown. Proteins that are known to be targets of NF-κB transcription factor were chosen and probed on western blot membrane. Upon RIP2 overexpression, an increase in anti-apoptotic proteins like Bcl-2, Bcl-x_L, XIAP, cIAP1 and cIAP2 was observed, while a decrease in these proteins' expression was observed upon RIP2 knockdown (Figure 4.12a). Densitometry of blots of 3 separate experiments was done and bands were quantified by ImageJ software. A significant increase in anti-apoptotic Bcl-2, Bcl-x_L and XIAP was observed upon wild-type RIP2 overexpression ($p < 0.05$) (Figure 4.12b).

To confirm that RIP2 enhances expression of the anti-apoptotic proteins through the NF-κB activation, NF-κB super-repressor plasmid was co-transfected along with RIP2 into MDA-MB-231 cells along with proper controls and cells were then harvested and analyzed for protein expression. Inhibiting NF-κB by SR decreased the expression of the anti-apoptotic proteins and was not rescued upon RIP2 co-expression, which indicates that RIP2 enhances anti-apoptotic protein expression upon NF-κB activation (Figure 4.12c).

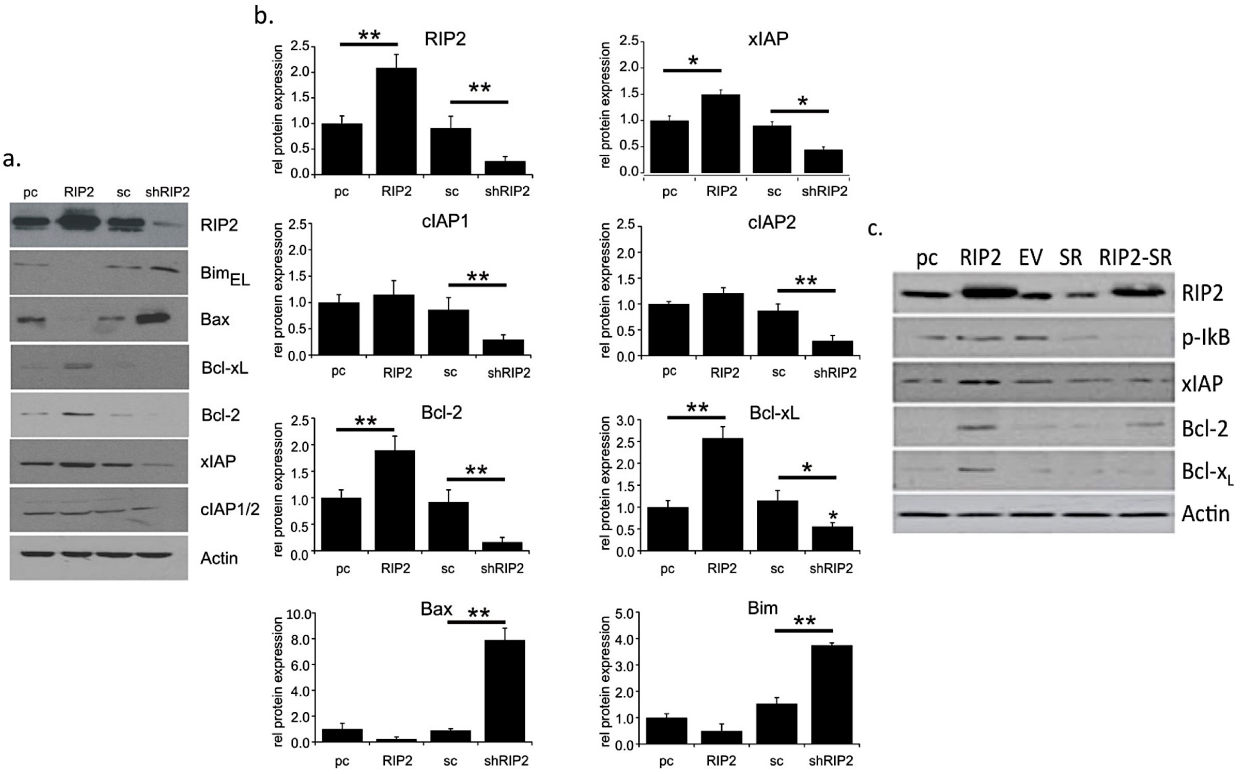


Figure 4.12: RIP2 enhances expression of anti-apoptotic proteins in MDA-MB-231 via NF-κB.

a. Whole cell lysates from MDA-MB-231 cells transfected with pc, RIP2, sc and shRNA-RIP2 (shRIP2) were probed using RIP2, Actin, Bcl-2, Bcl-x_L, XIAP, cIAP1, cIAP2 Bim and Bax primary antibodies (1:1000) and then with corresponding secondary antibody for 2 h; b. Relative protein expression of various proteins in MDA-MB-231 cells. Densitometric analysis was performed on each protein expression and actin, using the software Image J. Percentage protein density was found using ImageJ and then relative protein expression was calculated relative to actin and normalized to pc control; c. Protein expression of anti-apoptotic proteins Bcl-2, Bcl-x_L, XIAP and cIAP1/2 was detected upon co-transfection of MDA-MB-231 cells with RIP2 and NF-κB super-repressor. p-IκB primary antibody was used in order to verify the activity of the SR. All results shown above are representative data of 3 separate experiments. Mean values are shown ± standard error. Statistical significance is expressed in comparison to treated control (p < 0.05; ** p < 0.01).*

4.2.12. RIP2 expression correlates with anti-apoptotic protein expression in BC patient databases

Confirming the significance of RIP2 in TNBC, expression of RIP2, Bcl-2, Bcl-x_L, XIAP, and cIAP1/2 in patient BC database described in Section 3.2.1 was analyzed. RIP2 expression in BC patients positively correlates with Bcl-x_L, cIAP1/2 and XIAP expression but not Bcl-2 (Table 4.3). This correlation is more significant when analyzed based on the BC subtype where higher correlation value was observed in TNBC / basal-like BC (Table 4.4). Furthermore, comparing the samples with low protein expression to samples with high protein expression, samples with high RIP2 have significantly high levels of Bcl-2, Bcl-xL, XIAP, and cIAP1/2 (Table 4.4). As shown in bar graphs, around 49% of samples with high

RIP2 have high Bcl-2 while 36.8% of samples with low RIP2 have high Bcl-2 (p = 0.037), 59.6% of samples with high RIP2 have high Bcl-xL while 34.5% have low RIP2 and high Bcl-xL (p = 0.012), 42.18% of samples have high RIP2 and high cIAP1 while 31.25% have low RIP2 and high cIAP1 (p = 0.003), and 59.32% have high RIP2 and high cIAP2 while only 28.95% of low RIP2 samples have high cIAP2 (p < 0.001). Finally, 47.4% of patients with high RIP2 have high XIAP levels and 34.2% of patients with low RIP2 have high XIAP levels (p = 0.028).

Table 4.3: RIP2 correlation values against pro-apoptotic protein expression in all patients.

Data-base no	GSE19615	E-TABM-158	GSE1456-GPL96	GSE12276
Bcl-2	0.009	-0.002	-0.038	0.009
Bcl-xL	0.122	0.183*	0.059	0.122
XIAP	0.374*	0.221*	-0.058	0.374*
cIAP1	0.286*	0.393*	0.121	0.286*
cIAP2	0.462*	0.322*	0.082	0.262*

*Data shows correlation analysis relative to RIP2 expression in all BCs. * indicates p < 0.05 and ** indicates p < 0.01.*

Table 4.4: RIP2 correlation values against pro-apoptotic protein expression in all patients.

Data-base	GSE19615		E-TABM-158		GSE1456-GPL96		GSE12276	
	other	basal-	other	basal-	other	basal-	other	basal-
Bcl-2	-0.02	0.04	0.04	-0.07	0.02	0.20	-0.02	0.29*
Bcl-xL	0.15	0.20*	0.17	0.24**	0.04	0.62**	-0.14	0.20*
XIAP	0.14	0.58**	0.14	0.39**	-0.09	0.11*	0.14	0.26*
cIAP1	0.21	0.37**	0.31*	0.58**	0.07	0.28	0.21	0.42**
cIAP2	0.45*	0.58**	0.26*	0.41**	0.02	0.18*	0.15	0.39**

*Data shows correlation analysis relative to RIP2 expression in BC basal-like subtype and other subtypes. * indicates p < 0.05 and ** indicates p < 0.01.*

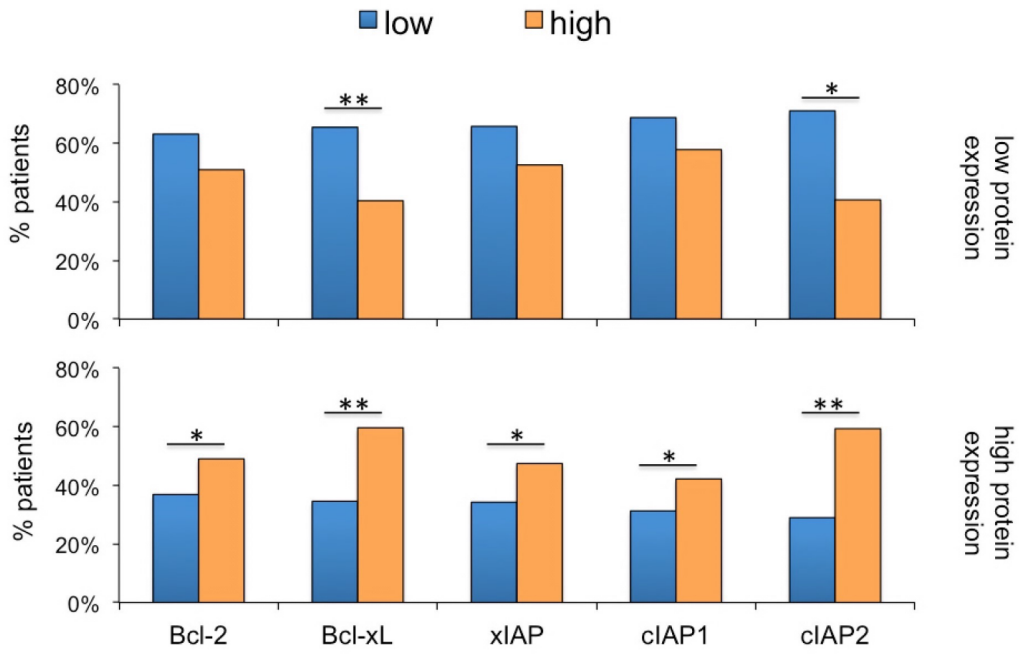


Figure 4.13: Bar charts showing percentage of patient samples with low/high RIP2 vs low/high anti-apoptotic proteins.

*Low and high values were determined relative to mean, lower or higher than mean value of samples. p-value was calculated by One-way Anova. * indicates $p < 0.05$ and ** indicates $p < 0.01$.*

4.2.13. Pro-survival RIP2 effect in other TNBC cell lines

To further confirm the role of RIP2 in TNBC BC, other TNBC cell lines (MDA-MB-453, MDA-MB-468, HCC1806 and BT549) were transfected with wild-type RIP2 and shRNA-RIP2 and observed at 24 and 48 h post-transfection. A decrease in cell viability was observed in MDA-MB-453 (Figure 4.14a) upon knockdown of RIP2, in a trend similar to MDA-MB-231, slight decrease in cell viability was observed in MDA-MB-468 (Figure 4.14b) and BT-549 (Figure 4.14c) and almost no change was observed in HCC-1806 (Figure 4.14d). Cells were also treated with ceramide (30 μM) and taxol (1 μM) in order to determine if RIP2 has any role in protecting these cells against drug induced cell death. 24 h post treatment, cell viability was measured via MTT and an increase in cell viability was observed in all cell lines upon RIP2 overexpression and most prominently in MDA-MB-453 (Figure 4.14a) and BT-549 (Figure 4.14c); with RIP2 knockdown using shRNA-RIP2 decrease in cell viability upon treatment was observed in all cell lines except HCC1806 (Figure 4.14d).

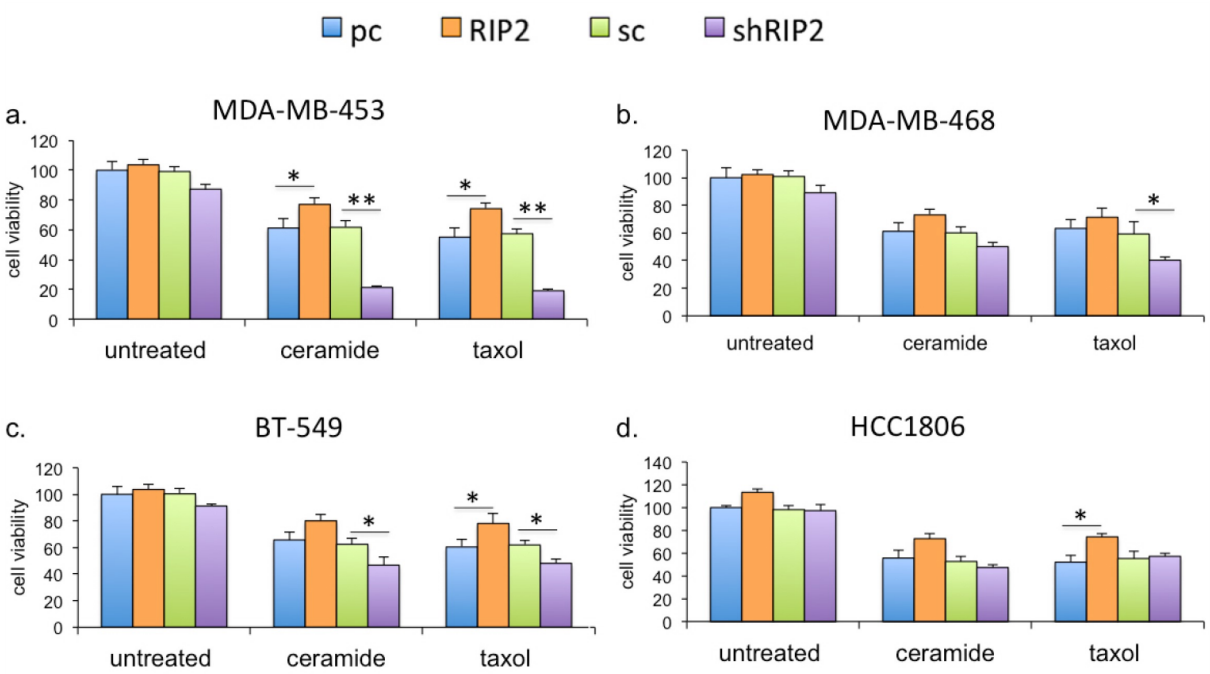


Figure 4.14: RIP2 expression affects TNBC cell lines' response to treatments.

MTT assay upon of MDA-MB-453, MDA-MB-468, BT-549 and HCC1806 transfected with pcDNA3.1 (pc), wild-type RIP2 (wt), scrambled shRNA control (sc) and shRNA-RIP2 (shRNA-RIP2) and treated with ceramide 30 μM or taxol 1 μM. All results shown above are representative of 3 separate experiments. Mean values are shown ± standard error. Statistical significance is expressed in comparison to treated control ($p < 0.05$; ** $p < 0.01$)*

4.2.14. BC cell lines characteristics and mutational information

In order to better understand the differences observed upon RIP2 expression in TNBC and non-TNBC cell lines, information about mutations in genes implicated in cancer was collected based on COSMIC cell line project http://cancer.sanger.ac.uk/cell_lines/cbrowse/all (Table 4.5). BC genes 1 and 2 (BRCA1 and BRCA2) are known to be mutated in BC, around 55% of women who inherit BRCA1 mutation and around 45% of women who inherit a harmful BRCA2 mutation will develop BC by age 70 years (Antoniou et al., 2003, Chen and Parmigiani, 2007). However, in the BC cell lines only MDA-MB-468 have mutated BRAC2 gene. Also, *TP53* is known to be mutated in around 50% of cancer (Alsner et al., 2008), and based on cell line mutational data all TNBC cell lines have *TP53* mutation, and have relatively higher number of mutations in cancer implicated genes in comparison to non-TNBC. Moreover, BC patients with *TP53* mutation have higher RIP2 expression level (Figure 4.15). No specific trend in PTEN or KRAS mutations was observed between the different BC cell types. Interestingly, MDA-MB-231 and MDA-MB-453 have high number of mutations (22 and 25 genes) including the caspase-

8 gene. Caspase-8 is important in the extrinsic apoptotic pathway; hence, it has a role in cancer cells response to drugs.

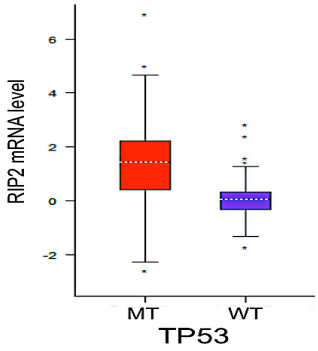


Figure 4.15: RIP2 expression relative to *TP53* status

Box plot showing RIP2 expression across patients samples based on TP53 status (mutated: MT or wild-type: WT); p < 0.001.

Table 4.5: Characteristics of different BC cell lines.

Cell Line	TN	No of mutated genes	BRCA1	BRCA2	*TP53*	PTEN	KRAS
BT549	Y	9	WT	WT	MT	MT	WT
HCC1806	Y	8	WT	WT	MT	WT	WT
MCF-7	N	12	WT	WT	WT	WT	WT
MDA-MB-231	Y	22*	WT	WT	MT	WT	MT
MDA-MB-453	Y	25*	WT	WT	MT	WT	WT
MDA-MB-468	Y	19	WT	MT	MT	MT	WT
SKBR3	N	5	WT	WT	WT/?	WT	WT
T47D	N	9	WT	WT	MT	WT	WT

*TN: triple negative, Y: yes, N: no, MT: mutated, WT: wild-type, * caspase-8 is mutated*

4.2.15. RIP2 induces the release of extracellular factors in MDA-MB-231 cells

The role of NF-κB in enhancing cancer cells proliferation is well reported (Rayet and Gelinas, 1999, Khan et al., 2013), and correlation of NF- κB genes with *MKI-67* is positively correlated in basal-like BC subtypes as shown in Chapter 3. As mentioned earlier, RIP2 enhances NF-κB activation, which leads to transcription of NF-κB target genes that stimulate proliferation and survival in TNBC. In order to determine whether RIP2 can lead to transcription and hence release of growth factors and cytokines through NF-κB, the presence of extracellular factors in culture media collected from RIP2 overexpressing MDA-MB-231 was studied. In general, normal medium growth conditions containing 10% serum is rich in growth factors and could mask the effects of extracellular factors that may be secreted to media and hence RIP2 pro-survival effect would not be profound, therefore, cells were grown in 4% FBS media and tested whether media collected from wild-type RIP2

overexpressing cells could also affect cell survival under reduced nutrient conditions. Cells were transfected with wild-type RIP2 and plasmid controls (pc) for 48 h at which point culture medium was collected from transfected cells and centrifuged at high speed to remove debris. Collected media were then stored at -20 ^{o}C and will be referred to as RIP2 or pc-conditioned media. MDA-MB-231 cells were transfected with shRNA-RIP2, at 4 h post transfection the culture medium was removed and replaced with wild-type RIP2 and pc-conditioned media along with fresh media. Cells were counted 4 h post transfection which was indicated as indicated as 0 time point, and then counted again at 24, 48 and 72 h post transfection to determine if RIP2 was inducing the release of extracellular factors which affect the proliferation and survival of cells. It was observed that RIP2 knockdown using shRNA-RIP2, caused an increase in cell death as seen under the microscope and reduced cell number compared to scrambled control. Upon addition of wild-type RIP2 conditioned media to cells transfected with shRNA-RIP2, cell death was reduced and the cell number was increased in comparison to pc-conditioned media (Figure 4.16 a and b). Therefore, this enhanced survival might be attributed to the presence of extracellular factors in RIP2-conditioned media.

This potential role of RIP2 in inducing the release of extracellular factors was further confirmed. Loss of mitochondrial membrane potential was used to measure the ability of RIP2 conditioned media to enhance cell survival in MDA-MB-231 post RIP2 knockdown. shRNA-RIP2 transfected cells with wild-type RIP2 conditioned media had significantly less decrease in mitochondrial membrane potential in comparison to shRNA-RIP2 transfected cell in pc-conditioned media (Figure 4.16c). Moreover, clonogenic assay of shRNA-RIP2 transfected cells harvested at 15 days post media change showed an increase in number of colonies upon addition of RIP2-conditioned media in comparison to pc-conditioned media (Figure 4.16d). To test the effect of RIP2 conditioned media on drug resistance, MDA-MB-231 cells were cultured in 4% serum conditioned for 24 h, after which the medium was changed to pc- or wild-type RIP2 conditioned media and treated with/without ceramide or taxol in 4% serum conditions for 48 h. Cell viability was then measured by MTT. It was evident that wild-type RIP2 conditioned media almost completely inhibited the induction of cell death by ceramide or taxol (Figure 4.16e). Moreover, cells treated with ceramide or taxol and in the presence of conditioned media, were cultured in 24 well plate left for 15 days, after which they were stained with crystal violet to assess colony formation. Significantly more colonies were observed with wild-type RIP2 conditioned media wells in comparison to pc-conditioned media (Figure 4.16f).

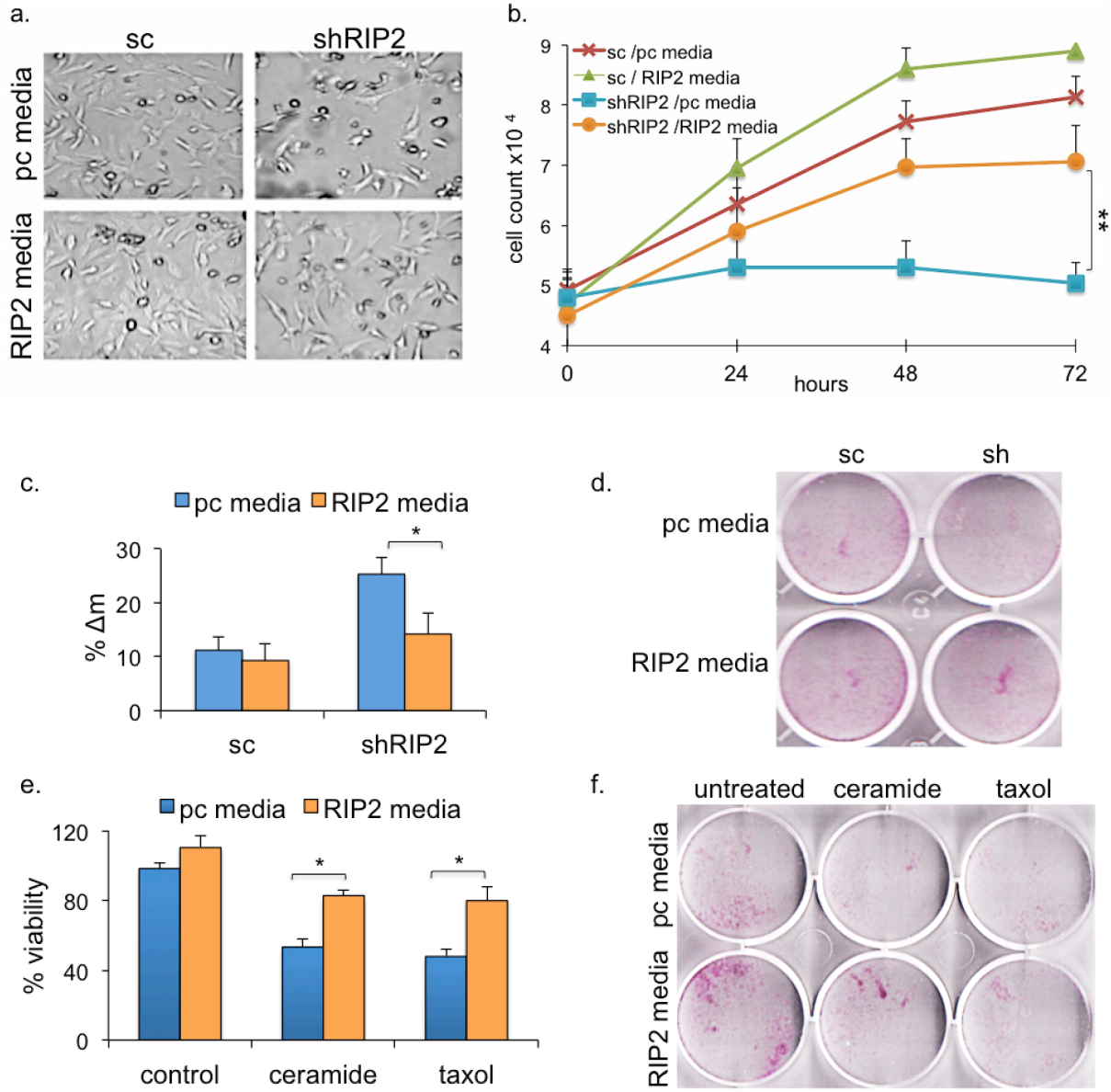


Figure 4.16. RIP2 pro-survival activity is elicited via extracellular factors.

*a. Images of MDA-MB231 transfected with shRNA-RIP2 (shRIP2) or scrambled control and treated with conditioned media show that death induced by RIP2 knockdown can be rescued in the presence of RIP2 co addition of RIP2 conditioned media; b. Cell count in MDA-MB-231 transfected with shRNA-RIP2 and control at 0, 24, 48 and 72 h in RIP2 or pc-conditioned media; c. TMRE assay measuring loss of mitochondrial transmembrane potential (%Δm) showing effect of RIP2 conditioned media in shRNA-RIP2 (shRIP2) MDA-MB-231 transfected cells. d. Clonogenic assay of MDA-MB-231 transfected with shRNA-RIP2 or scrambled control and cultured in pc or RIP2 conditioned media showing that addition of RIP2 conditioned media can protect cells from RIP2 knockdown induced cell death; e. Cell viability of MDA-MB-231 treated with ceramide (30 μM) and taxol (1 μM) for 48 h in different media conditions; f. Clonogenic assay of MDA-MB-231 15 days post treatment showing that death induced by drugs can be rescued by addition of conditioned supernatant. All experiments shown above are representative of 3 independent experiments' repeats. All values shown represent mean ± standard error with significance level * $p < 0.05$ and ** $p < 0.01$ obtained by One-way Anova.*

4.2.16. Delineating the effect of RIP2 on extracellular factors

In order to determine whether extracellular protein factors within the wild-type RIP2 conditioned media were responsible for rescuing cells from cell death as observed in Section 4.2.15, the supernatants collected from MDA-MB-231 cell culture were denatured by heating at 55 ^{o}C for 30 min. Heat denatured supernatants (HS) were then added to MDA-MB-231 cells and the capability of the HS to rescue cells from cell death was assessed. RIP2 knockdown by shRNA in MDA-MB-231 showed an increased loss of mitochondrial transmembrane potential when cultured in pc media in comparison to RIP2 media. However, heat denaturing of RIP2 conditioned media abolished the effect observed with RIP2 conditioned media as determined by increase in mitochondrial transmembrane loss (Figure 4.17a). Similarly, RIP2 conditioned media was able to protect MDA-MB-231 cells against ceramide and taxol induced cell death, as shown by difference in cell viability of cells incubated with pc media in comparison to RIP2 media and discussed in Section 4.2.15. However, denaturing the RIP2 conditioned media decreased the cell viability observed with RIP2 conditioned media (Figure 4.17b). This reveals that RIP2 conditioned media contains factors that induce survival in MDA-MB-231 cells and are denatured upon heating.

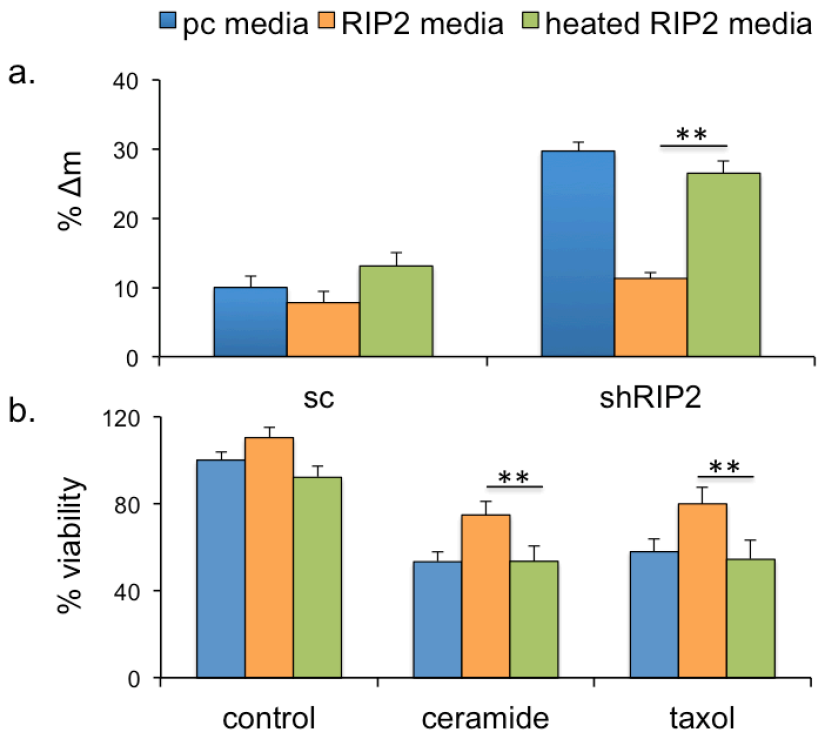


Figure 4.17: Heat denaturing supernatants abolished RIP2 enhanced cell protection.

RIP2 conditioned media was heated at 55 ^{o}C for 30 min. Denatured conditioned media was added to a. shRNA-RIP2 (shRIP2) transfected and b. ceramide (30 μM) or taxol (1 μM) treated MDA-MB-231 cells and compared to unheated conditioned media. All values shown represent mean of three independent experiments ± standard error with significance level * $p < 0.05$, ** $p < 0.01$ obtained by One-way Anova.

4.2.17. Identification of extracellular targets from RIP2 overexpressing cells

Based on previous described data, RIP2 may be enhancing survival in MDA-MB-231 cells through modulating the release of extracellular factors. Therefore, determining extracellular factors released upon RIP2 expression is important. To identify these factors a cytokine array was performed on conditioned media from MDA-MB-231 cells transfected with wild-type RIP2 and pcDNA3.1 in 4% serum and collected 48 h post transfection. To avoid repeating the cytokine arrays and to ensure that the supernatants selected were representative of previous observations, RIP2 expression of transfected cells was detected and the supernatants used for the array were assessed to determine if a similar pro-survival effect described earlier was observed. Confident that the supernatants generated were representative of previous experiments, a cytokine array analysis was performed (Figure 4.18a). Six cytokines were recognized as being differentially up-regulated in RIP2 conditioned media namely Angiopoietin-2, VEGF and VEGF-c involved in angiogenesis, and GM-CSF, IL-8, PIGF involved in cell growth and development (O'Byrne, 2015, Yadav et al., 2015, Hoeben et al., 2004, Mor et al., 2004). Moreover, a few genes were down regulated upon RIP2 overexpression including insulin like growth factor binding protein 1 (IGFBP-1), a tumour suppressor, and pentaxrin-3 (PTX3) that is a modulator of tumour-associated inflammation (Zhang and Yee, 2002, Bonavita et al., 2015). A summary of cytokines that are detected in the array are presented in Table 4.18 and quantitative measurement of targets that were identified showing a fold change relative to control was done by ImageJ software (Figure 4.18b). In addition, the proteins in the array were defined as being NF-κB target genes or not, since we previously established the association between RIP2 and NF-κB activation. Target genes were first identified using the online platforms http://www.bu.edu/nf-kb/gene-resources/target-genes/ and http://bioinfo.lifl.fr/NF-KB/, and then the references sited were confirmed. Interestingly, all proteins modulated upon RIP2 expression were found to be NF-κB target genes as shown in Table 4.6

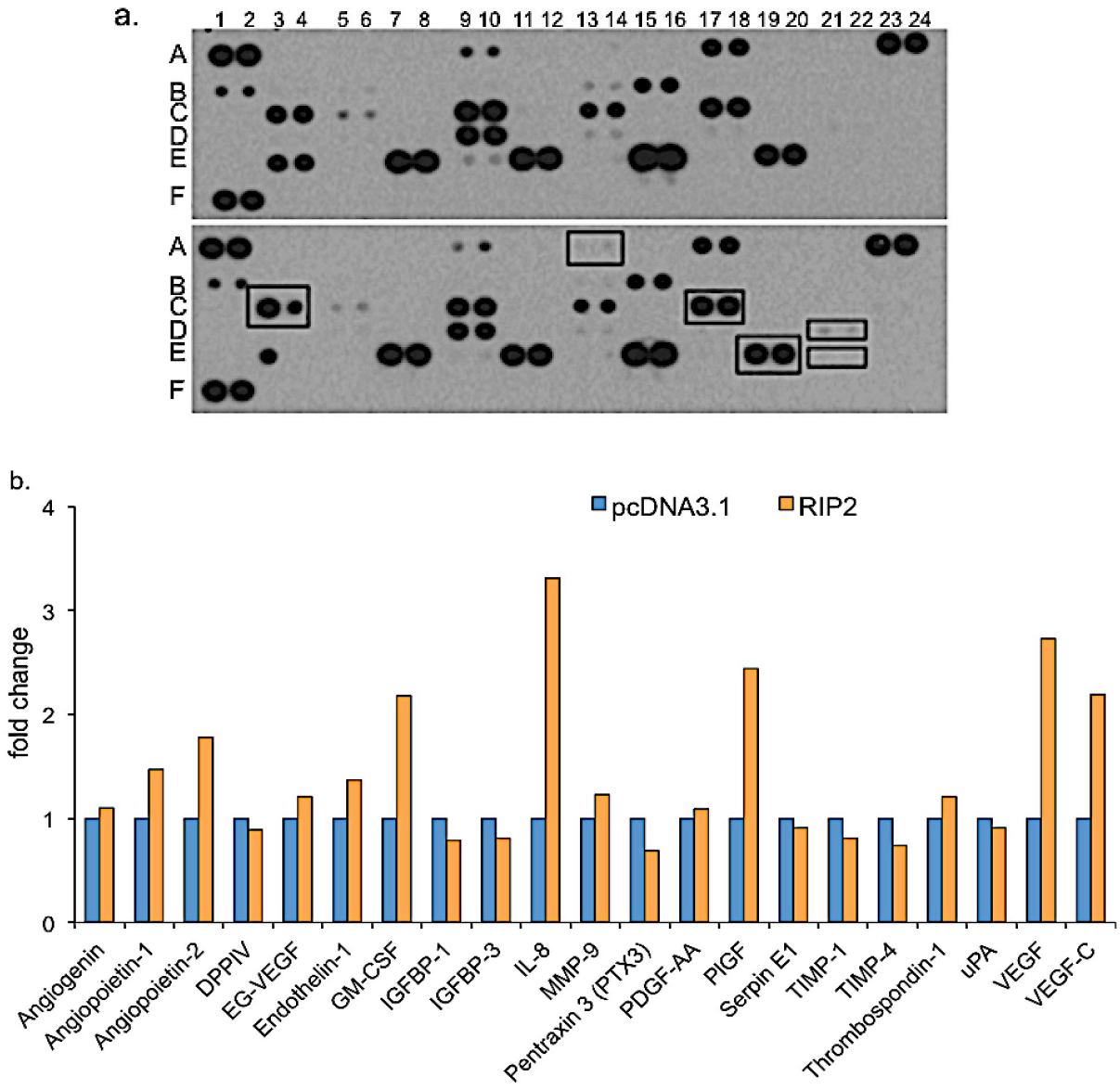


Figure 4.18: RIP2 is responsible for the release of extracellular factors.

a. Cytokine array (R&D) systems. Conditioned media from MDA-MB-231 pcDNA3.1 or RIP2 transfected cells were collected as described before at 48 h post transfection and 1 ml media was added to cytokine membrane and experiment was performed as per manufacturer's protocol. Boxes indicate the spots for Angiopoietin-2, VEGF and VEGF-c, GM-CSF, IL-8, PIGF. All other spots are outlined in Table 4.6. b. Quantification of array measured by Image J software. Values presented are RIP2 average of the repeated spots and normalized to pcDNA3.1 control average of spots.

Table 4.6: Cytokine array table showing the different cytokines detected.

	Target/Control	Fold change	NF-kB target	Function
A1	Reference			
A5	Activin A	-	yes	produced in the gonads, pituitary gland, placenta
A7	ADAMTS-1	-	no	disintegrin and metalloproteinase
A9	Angiogenin	1.1	no	a potent stimulator of new blood vessels
A11A	Angiopoietin-1	1.47	yes	vascular development and angiogenesis
A13	Angiopoietin-2	1.78	yes	vascular development and angiogenesis
A15	Angiostatin/	-	no	angiogenesis inhibitor
A17	Amphiregulin	-	no	autocrine growth factor as well as a mitogen
A19	Artemin	-	no	neurotrophin in the glial cell line-derived neurotrophic
A23	Reference			
B1	Coagulation	-	no	cell surface glycoprotein
B3	CXCL16	-	no	small cytokine belonging to the CXC chemokine family
B5	DPPIV	0.89	no	serine protease
B7	EGF	-	no	epidermal growth factor
B9	EG-VEGF	1.21	no	
B11	Endoglin	-	yes	endothelial cell membrane glycoprotein
B13	Endostatin/	-	no	20-kDa C-terminal fragment derived from type XVIII
B15	Endothelin-1	1.37	yes	predicts BC recurrencepeptide/mitogen
B17	FGF acidic	-		
B19	FGF basic	-		
B21	FGF-4	-		
B23	FGF-7	-		
C1	GDNF	-	no	Glial cell-derived neurotrophic factor
C3	GM-CSF	2.18	yes	colony stimulating factor
C5	HB-EGF	-	yes	Heparin-binding EGF-like growth factor
C7	HGF	-	yes	Hepatocyte growth factor
C9	IGFBP-1	0.79	yes	Insulin-like growth factor binding protein-1
C11C	IGFBP-2	-		
C13C	IGFBP-3	0.81	yes	tumour suppressor
C15C	IL-1β	-	yes	
C17C	IL-8	3.31	yes	chemokine
C19C	LAP (TGF-β1)	-		
C21C	Leptin	-		
C23C	MCP-1	-	yes	
D3	MMP-8	-	yes	
D5	MMP-9	1.23	yes	matrix metalloprotinease
D7	NRG1-β1	-	yes	neuroregulin
D9	Pentraxin 3	0.69	yes	a modulator of tumour-associated inflammation
D11	PD-ECGF	-	no	
D13	PDGF-AA	-	no	
D15	PDGF-AB/	-	no	
D17	Persephin	-	no	
D19	Platelet Factor	-	no	
D21	PlGF	2.44	yes	a key molecule in angiogenesis and vasculogenesis

D23	Prolactin	-	no	
E1	Serpin B5	-	no	
E3	Serpin E1	-	no	Plasminogen activator inhibitor-1
E5	Serpin F1	-	no	
E7	TIMP-1	0.81	no	
E9	TIMP-4	0.74	no	
E11	Thrombospond	1.21	yes	
E13	Thrombospond	-	yes	
E15	uPA	0.91	no	
E17	Vasohibin	-	no	
E19	VEGF	2.73	yes	vascular endothelial growth factor
E21	VEGF-C	2.19	yes	member of VEGF family acts on lymphatic endothelial
F1	Reference			
F23F	Negative			

Table shows the different cytokines detected in the array with the reference spot, their function. In addition, proteins were identified whether they are targets of NF-κB transcription. Blue color indicates higher expression in pcDNA3.1-conditioned media. Red indicates higher expression in wild-type RIP2 conditioned media. (-) indicates no detection in cytokine array.

4.2.18. RIP2 expression correlates with cytokine and growth factor protein expression in BC patient databases

The effect of RIP2 on cytokine and growth factors release was validated in patient BC databases for RIP2 expression correlation with expression of relevant cytokines and growth factors. Positive correlation was observed between mRNA expression of RIP2 and mRNA expression of IL-8 and VEGF (Table 4.7). Moreover, correlation of RIP2 with mRNA expression of IL-6, TGFβ1 and CXCL1 were also analyzed in all patients. These factors are NF-κB targets and reported in the literature to associate with BC progression and development (Lukaszewicz et al., 2007, Serra and Crowley, 2003, Acharyya et al., 2012). A significant correlation between RIP2 and IL-6, TGFβ1 and CXCL1 were also analyzed in all patients (Table 4.7). These factors are NF-κB targets, and their correlation with RIP2 expression was more significant when patient data were stratified based on TNBC subtype as shown in Table 4.8. This supports the role of RIP2 in immunological profile of BC

Table 4.7: RIP2 correlation values against selected cytokine expression in all patients

Data-	GSE19615	E-TABM-158	GSE1456-	GSE12276
IL-6	0.078	0.247	-0.121	0.297
IL-8	0.122	-0.073	-0.105	0.117
VEGF	0.713*	-0.052	0.257**	0.574*
TGFβ1	0.091	0.287*	0.213	-0.193
CXCL1	0.336*	0.082*	0.276	0.196

*Data shows correlation analysis relative to RIP2 expression in all BCs. * indicates $p < 0.05$ and ** indicates $p < 0.01$.*

Table 4.8: RIP2 correlation values against selected cytokine expression in BC subtypes

Data-base no	GSE19615		E-TABM-158		GSE1456-GPL96		GSE12276	
	other	basal-like	other	basal-like	other	basal-like	other	basal-like
IL-6	-0.12	0.39**	0.12	0.57**	-0.18	0.19	0.26	0.37**
IL-8	0.15	0.44**	-0.24	0.13	-0.31	0.29**	-0.03	0.22*
VEGF	0.64*	0.76**	-0.19	0.57**	-0.09	0.11*	0.34*	0.67*
TGFβ1	-0.11	0.18	0.21*	0.48**	0.07	0.35**	-0.07	0.23**
CXCL1	0.27*	0.54**	0.02	0.21**	0.19*	0.39**	0.17	0.32*

*Data shows correlation analysis relative to RIP2 expression in BC basal-like subtype and other subtypes. * indicates p < 0.05 and ** indicates p < 0.01.*

4.2.19. Quantitative analysis of IL-6, IL-8 and GRO-α upon RIP2 expression

Secreted levels of IL-6, IL-8 and CXCL1 (GRO-α) were examined via ELISA in MDA-MB-231 cells transfected with RIP2 plasmids constructs (RIP2, K209R, dn), RIP2 knockdown via shRNA-RIP2 and appropriate controls (pcDNA3.1 and scrambled shRNA). RIP2 overexpression significantly increased the secreted levels of IL-6, IL-8 and GRO-α, while with RIP2 mutants RIP2-K209R and CARD-only RIP2 slight reduction of IL-6 and IL-8 was observed (Figure 4.20). However, reduced levels of IL-6 and IL-8 were observed with RIP2 knockdown, while no detectable changes were observed with GRO-α since basal-like levels are almost not detectable (Figure 4.19).

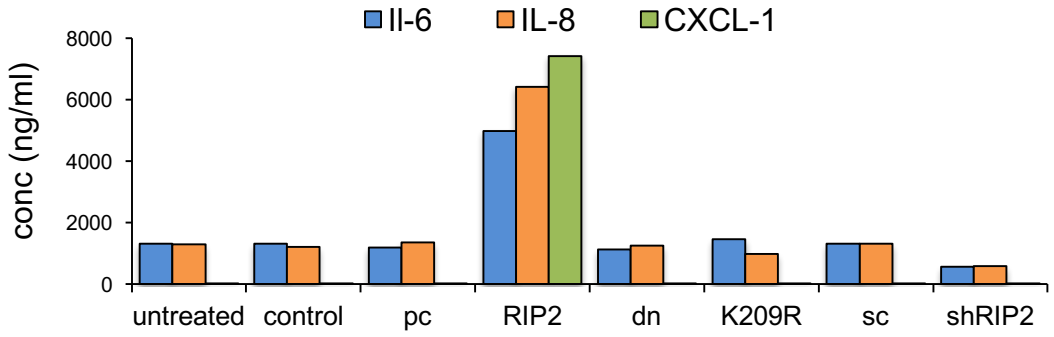


Figure 4.19: Quantitative analysis of IL-6, IL-8 and CXCL1 (Gro-α) upon transfection with RIP2 plasmids' constructs.

ELISA analysis of secreted IL-6, IL-8 and CXCL1in MDA-MB-231 cells grown in 4% serum show increased levels upon wild-type RIP2 transfection. (n=1, with 3 experimental replicates)

4.2.20. IL-6 and IL-8 partially modulate RIP2 pro-survival signaling

To examine the functional effects of these cytokines, neutralizing antibodies against IL-6 and IL-8 were added to conditioned media. In MDA-MB-231 cells neutralizing antibodies against IL-6 and IL-8 could reduce wild-type RIP2 mediated pro-survival effect (Figure 4.20).

Wild-type RIP2 conditioned media increased cell survival of MDA-MB-231 cells treated with ceramide and taxol; however, when wild-type RIP2 conditioned media was incubated with IL-6 and IL-8 neutralizing antibodies, a significant decrease in survival was observed in ceramide treated cells (Figure 4.20). Moreover, upon taxol treatment, a significant decrease in MDA-MB-231 viability was observed when wild-type RIP2 culture medium was pre-incubated with IL-6, IL-8 and IL-6/IL-8 neutralizing antibodies in comparison to wild-type RIP2 culture media alone. Therefore, IL-6 and IL-8 secreted to the media have a potential role in cancer survival and resistance.

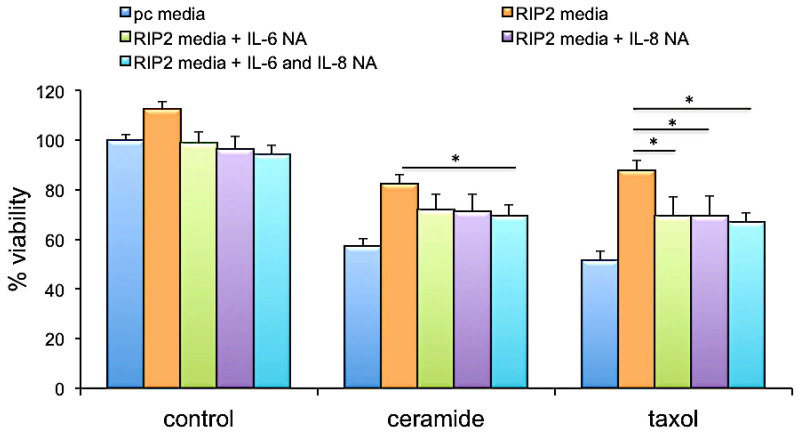


Figure 4.20: IL-6 and IL-8 slightly modulate RIP2 effect.

*MDA-MB-231 cells were seeded in 96-well plate in 4% serum conditions. At 24 h post-seeding media was replaced by pc or RIP2 conditioned media or conditioned media with IL-6/IL-8 NA, and cells were treated with ceramide ceramide (30 μM) or taxol (1 μM). Cell viability was measured by MTT 24 h post treatment. All values shown represent mean of three independent experiments ± standard error with significance level *p<0.05, obtained by One-way Anova.*

4.2.21. RIP2 is required for TNF downstream signaling and induction of NF-κB

TNF (tumour necrosis factor) is one of the known factors that induces NF-κB and enhances the expression of pro-survival signaling in cells which mainly depend on phosphorylation and degradation of IκB proteins (Wajant et al., 2003). Moreover, BC cells express high levels of TNF which is reported to induce cancer growth and metastasis (Yu et al., 2013). Analyzing patient BC databases revealed a significant positive correlation between RIP2 and TNF mRNA ($r = 0.16$, $p < 0.01$), which was more significant in basal-like subtype observed upon database stratification. Positive correlation was observed in basal-like BC where $r = 0.28$ ($p < 0.001$) and luminal A ($r = 0.16$, $p < 0.01$), while negative correlation was observed in HER-2 ($r = -0.09$, $p = 0.21$) and Luminal B ($r = -0.12$, $p = 0.03$) (Table 4.9).

In order to determine whether RIP2 has any role of TNF induced NF-κB pro-survival signaling, and further elucidate RIP2 involvement in pro-survival signaling in BC cells, MDA-MB-231 cells were treated with TNF (200 ng/ml). TNF treatment induces NF-κB activation through increase in IκB phosphorylation, which is inhibited upon treatment with PP2 (Figure 4.21a). PP2, as mentioned earlier, inhibits RIP2 auto-phosphorylation and reduces RIP2 stability. Additionally, PP2 treatment alone decreases MDA-MB-231 cells viability, however, as measured by MTT, co-treatment with TNF further decreases cell viability in comparison to PP2 alone (Figure 4.21b). Moreover, treatment of cells with PP2, or RIP2 knockdown via shRNA-RIP2 transfection, decreases significantly TNF induced NF-κB activity as determined by NF-κB reporter luciferase assay which indicates a potential role of RIP2 in TNF downstream signaling (Figure 4.21c). Therefore, TNF activation of NF-κB is dependent on RIP2, and inhibiting RIP2, activates TNF induced death pathways.

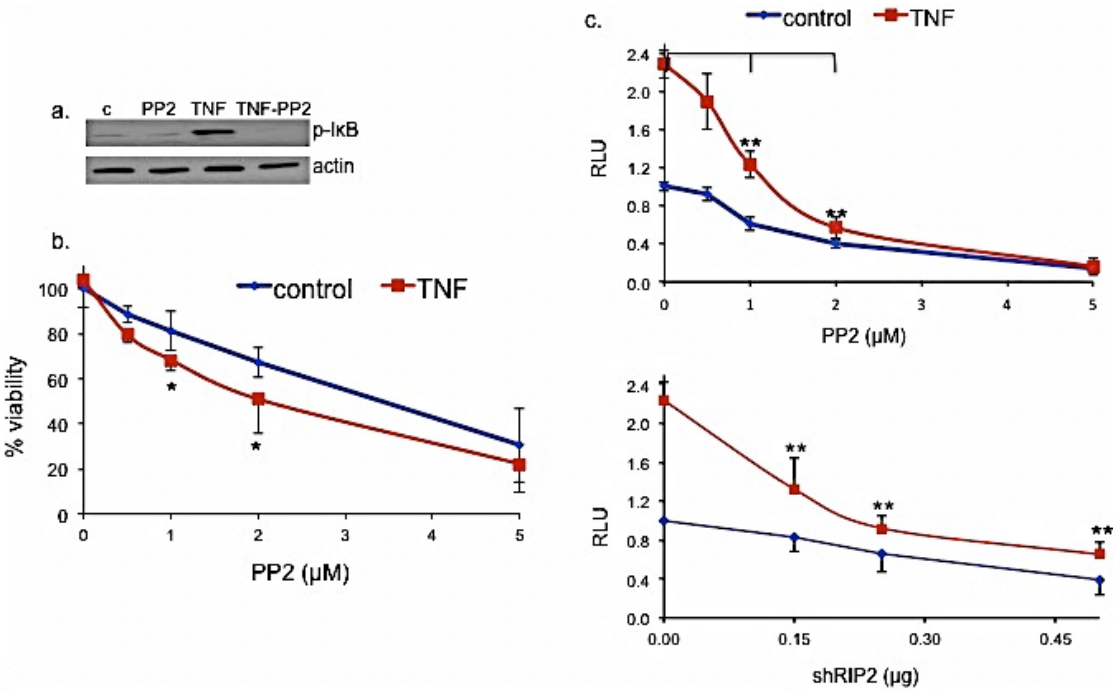


Figure 4.21: RIP2 is required for TNF dependent NF-κB activation.

a. Western blot showing pIκB levels in MD-MB-231 upon treatment with TNF (200 ng/ml) and/or PP2 (1 μM). Actin is used as loading control. b. MDA-MB-231 cells were seeded in 96-well plate and treated with PP2 dose with or without TNF (200 ng/ml) for 24 h, and cell viability was measured by MTT assay relative to control untreated cells. c. Luciferase assay measuring NF-κB activity in MDA-MB-231 cells upon PP2 dose shRNA-RIP2 (shRIP2) dose transfection with or without TNF (200 ng/ml).

Table 4.9: RIP2 correlation values against TNF expression in BC patients

All patients	Basal-like	HER-2	Lum A	Lum B
0.16**	0.28**	0.16	-0.09**	-0.12*

*Data shows correlation analysis relative to RIP2 expression in BC basal-like subtype and other subtypes. * indicates p < 0.05 and (**) indicates p < 0.01.*

4.3. Discussion

In this study, we identified RIP2 as an activator of NF-κB pro-survival signaling in BC cells. RIP2 knockdown in TNBC cells MDA-MB-231 showed a decreased cell number and sensitized cells to drug induced cell death. In addition, RIP2 knockdown decreased NF-κB activity and as a result significantly lowered the expression of Bcl-2, Bcl-x_L, cIAP1/2 and XIAP anti-apoptotic proteins. In addition, BC patient samples with elevated RIP2 expression show increased virulence and metastasis as reported (Singel et al., 2014) and contribute to proliferation and resistance to treatment as shown in this study. Comparing levels of RIP2 expression in different BC patient subtypes, patient samples that express higher RIP2 levels have higher tumour grade and size, also RIP2 expression levels in basal-like BC is correlated with Ki67 expression, what indicates the role of RIP2 in inducing cell proliferation in these cells. . The role of RIP2 in cell proliferation and cancer development was further investigated in MDA-MB-231 cell line. High levels of RIP2 have a role in cell survival and proliferation, since the knockdown induces cell death. Moreover, levels of RIP2 mutant forms, the non-ubiquitinated RIP2 mutant (RIP2-K209R) and CARD-only RIP2, have negative impact on survival, growth and drug resistance indicating that ubiquitination at lysine 209 residue and the kinase domain are both important for RIP2 function. Moreover, inhibiting phosphorylation of RIP2 kinase domain by PP2, decreased RIP2 stability as shown by reduction in RIP2 expression, and hence a decrease in cell growth and viability was observed. However, investigating the role of kinase domain, by overexpressing RIP2 kinase domain only in TNBC, would be important for understanding RIP2 function and activity of RIP2 kinase in enhancing cell growth and tumour development.

High constitutive NF-κB activity was previously described in BC and was associated with resistance and tumourigenesis (Cogswell et al., 2000, Nakshatri et al., 1997). The role of RIP2 in cell proliferation and NF-κB activation was also previously described in myogenesis and cutaneous wound repair (Adams et al., 2010, Munz et al., 2002). Increased expression of wild-type RIP2 maintains myoblast proliferation in differentiation medium, and NF-κB activity is high in myoblasts transduced to express high levels of RIP2 (Munz et al., 2002). In addition, Adams et al showed that RIP2 is induced by scratch wounding and is essential for wound repair (Adams et al., 2010). Due to its proven role in cell proliferation and cancer development, and since its expression in BC patients correlates to poor prognosis and survival, RIP2 is an attractive novel target for the majority of TNBC and selected BCs that have higher RIP2 expression. Moreover, NF-κB signaling has been shown to contribute to chemoresistance of BC cells to many treatments, including tamoxifen, doxorubicin and paclitaxel (deGraffenried, 2004, Weldon et al., 2001), and is activated in

number of cancers including BC, prostate, colorectal and esophageal cancers (Cogswell et al., 2000, Nakshatri et al., 1997). However, NF-κB is not considered to be a good target for therapy, because of its integral role in immune signaling and can display both positive and negative effects on tumour progression (Perkins and Gilmore, 2006), it is important to identify RIP2, an upstream element of the pathway, as a useful therapy target. The role of RIP2 in activation of NF-κB pathway upon NOD2 induction is well established in the literature (Wertz and Dixit, 2010, Hasegawa et al., 2008); however, its mechanism of activation of NF-κB in cancer cells is not known, and understanding this mechanism can help in identifying therapeutic targets in cancers that express RIP2 and have high NF-κB activity

In MDA-MB-231 cells model, RIP2 overexpression showed significant protection against ceramide, taxol and thapsigargin drug induced cell death. Ceramide has been shown to decrease Bcl-x_L splicing by regulating Bcl-x alternative splicing (Chalfant et al., 2002) and Paschall et al showed that Bcl-x_L protein level is dramatically decreased by ceramide in metastatic human colon cancer cells, and in the metastatic BC cells (Paschall et al., 2014). In this study, a significant resistance to ceramide upon RIP2 expression was observed, which might be due to increased Bcl-x_L expression induced by RIP2 through NF-κB activation. Moreover, RIP2 knockdown and CARD-only RIP2 expression sensitizes cells to death against a range of cytotoxic and genotoxic drugs. This is explained by the direct effect of RIP2 on NF-κB activation and hence transcription induction of anti-apoptotic proteins such as Bcl-2, Bcl-x_L and IAPs. Higher expression of these proteins was previously described to have a role in BC resistance to treatment (Emi et al., 2005, LaCasse et al., 2008, Bouchalova et al., 2015). Interestingly, patient samples having higher RIP2 levels express higher expression of NF-κB target proteins and show poorer prognosis and lower AE-free survival rate. Therefore, based on the analysis of publically available databases, RIP2 impact on disease development and drug resistance might be explained by NF-κB activation and expression of anti-apoptotic genes. In our model, high levels of RIP2 in MDA-MB-231 may mask the impact of RIP2 overexpression in these cells, and maximizing the RIP2 effect, while the importance of RIP2 was more profound and significant upon RIP2 knockdown. According to Wang et al, MDA-MB-231 cells have higher Bcl-x_L mRNA level compared to MCF-7 cells (Wang et al., 2005). As shown earlier, MDA-MB-231 cells have higher RIP2 expression than MCF-7 cells, and therefore, higher Bcl-x_L mRNA levels might be due to higher transcription levels induced by RIP2 enhanced NF-κB activation in MDA-MB-231 cells. Therefore, overexpressing RIP2 in cells that have low basal-like levels of RIP2 might give a clearer understanding to the role of RIP2 in drug resistance and tumour

development and it would be important to further investigate the significance of high levels of RIP2 in BC.

The role of RIP2 in BC was also supported by RIP2 expression in different cell lines. BCs cell lines derived from metastatic and high-grade tumour, as described by ATCC and Lehmann et al, MDA-MB-231, MDA-MB-453 and MDA-MB-468 show high RIP2 expression in comparison to HCC1806 and BT549 that are derived from primary carcinoma (Lehmann et al., 2011). Moreover, due to caspase-8 mutation in MDA-MB-231 and MDA-MB-453 cell lines, extrinsic apoptosis is not activated (McIlwain et al., 2013); therefore, RIP2 through induction of NF-κB might inhibit the intrinsic pathway and resist drug induced cell death. On the other hand, RIP2 pro-survival effect was lower in MDA-MB-468 and BT-549, as shown in Figure 4.14, which might be due caspase-8 presence in these cells, activating the extrinsic apoptotic pathway (McIlwain et al., 2013). However, in HCC-1806, that express low RIP2 levels, overexpression of RIP2 was able to significantly protect these cells from drug induced cell death, which might be explained by increased expression of pro-survival Bcl-2 signaling enhanced by RIP2 activated NF-κB transcription. However, the role of RIP2 in these cells and its impact on pro-survival signaling need to be further characterized.

Through cytokine array analysis of conditioned media obtained from pcDNA3.1 or RIP2 MDA-MB-231 transfected cells, IL-8, GROα, GM-CSF alongside VEGF were identified as possible targets for RIP2 mediated pro-survival signaling. IL-6 and IL-8 have previously been reported as key regulators of TNBC development and are known to be NF-κB transcription targets (Libermann and Baltimore, 1990, Hartman et al., 2013). Here it is shown that RIP2 inhibition could dramatically reduce secreted levels of IL-6 and IL-8, which suggests that RIP2 plays an important role in the regulation of these cytokines in BC. Inhibition of secreted IL-6 and IL-8 by neutralizing antibodies could reverse the rescue observed with RIP2 conditioned medium, and reduce RIP2 dependent resistance to ceramide and taxol, suggesting that IL-6 and IL-8 play a role in BC cell survival. Moreover, it has been previously reported that impairment of NF-kappa B activation could suppress GROα gene expression (Ohtsuka et al., 1996), and this work is the first to link RIP2 signaling to GROα (CXCL1) regulation and release in TNBC. GROα is a chemokine associated with lung relapse in breast tumours (Minn et al., 2005) and increasing the aggressiveness of circulating tumour cells (CTC) (Kim et al., 2009). Interestingly this study by Kim et al also identified IL-6 and IL-8 as tumour attractants. In 2012, Acharyya et al reported that GROα can promote metastasis and chemoresistance in BC via recruitment of myeloid cells inducing a paracrine signaling loop that confers resistance to chemotherapy and enhances metastatic potential (Acharyya et al., 2012). Furthermore, RIP2 enhanced TGFβ release in MDA-MB-231 cells was observed. TGFβ is also linked to BC metastasis with reports

indicating that TGFβ plays an important role in EMT of BC (Xu et al., 2009). Contradictory reports of the function of TGFβ in cancer exist; TGFβ is reported to play both tumour inhibiting and enhancing effects (Zugmaier et al., 1989, McEarchern et al., 2001). However, whether RIP2-induced pro-survival signaling in TNBC is mediated through cytokines release needs to be further studied

Analyzing BC databases further validated the in-vitro observations and role of RIP2 in pro-survival signaling. As mentioned earlier, RIP2 is highly associated with TNBC profile and poor disease prognosis, which might be explained by the mechanism of RIP2 action in TNBC described below. As summarized in Figure 4.3 RIP2 activates NF-κB leading to transcription of essential genes involved in cell proliferation and pro-survival, in addition to vascularization and angiogenesis. Interestingly, RIP2 gene expression is associated with increased NF-κB activity but has not been shown to be a direct target (Matsuda et al., 2003); however, here it is shown that RIP2 expression is decreased upon NF-κB inhibition, which might indicate that RIP2 is an NF-κB target and that RIP2/NF-κB exhibit a feed forward mechanism, in which RIP2 induces NF-κB activity and then NF-κB increases RIP2 expression to further enhance the pro-survival effect through expression of anti-apoptotic proteins. These expressed proteins include Bcl-2, Bcl-xL and IAPs function partly as intracellular inhibitors of apoptosis, and increase resistance to induced cell death, and might explain the resistance of BC to drug therapy. Additionally, through NF-κB activation, RIP2 also induces the production of released factors in basal-like BC, specifically IL-6, IL-8, VEGF, TGF and Groα as shown by correlation analysis. IL-6 and IL-8 are known to have an autocrine and paracrine effect on cells (Hartman et al., 2013). Moreover, there is high correlation observed in BC patients between RIP2 expression and the expression of the factors identified in Section 4.19. The role of these factors released with RIP2 overexpression need to be further studied to investigate their impact on autocrine survival signaling of TNBC, and whether they have a role in RIP2 activation and enhanced expression.

The impact of RIP2 on TNFα induced NF-κB activation was also studied. TNFα is known to induce NF-κB activity triggering cell proliferation (Widera et al., 2006a). Moreover, TNFα is also an NF-κB target gene (Shakhov et al., 1990, Collart et al., 1990) but its role in cancer is contradictory. TNFα is able to kill some tumour cell lines that are resistant to secreted TNF-mediated cytotoxicity; in contrast, transmembrane TNFα expressed by tumour cells protects them from apoptosis by inducing constitutive activation of NF-κB (Zhang et al., 2008, Yan et al., 2009). Upon inhibition of RIP2 phosphorylation via PP2, or by knocking down RIP2, TNFα induced NF-κB activity was reduced which indicates that RIP2 acts downstream of TNFα. Also PP2 treatment inhibits NF-κB activation upon TNFα treatment

decreasing cell viability, which might be due to activation of TNFα death pathways. Therefore, TNF may induce RIP2 enhancing NF-κB activity what leads to increased transcription of genes, including TNF, which further acts via autocrine signaling on cells, activating RIP2 again, and hence inducing cell growth, resistance and tumour development Further studies are required in order to identify other released factors that would also activate RIP2 in TNBC.

It is interesting that RIP2 overexpression and knockdown induce cell death in non-TNBC cell lines, which is also verified in the negative association between RIP2 expression and non-basal-like BC in patients. Moreover, as discussed in Figure 4.15, RIP2 expression is associated with *TP53* mutation in BC patients, and therefore, tumour suppressor genes might regulate RIP2 expression and keep it in optimum required levels. Therefore, RIP2 is an interesting therapeutic target in TNBC since its impact on growth and pro-survival signaling is validated, however, being able to understand the mechanism of its activation that leads to RIP2 elevated expression in TNBC would be beneficial to help establish effective targeted therapy.

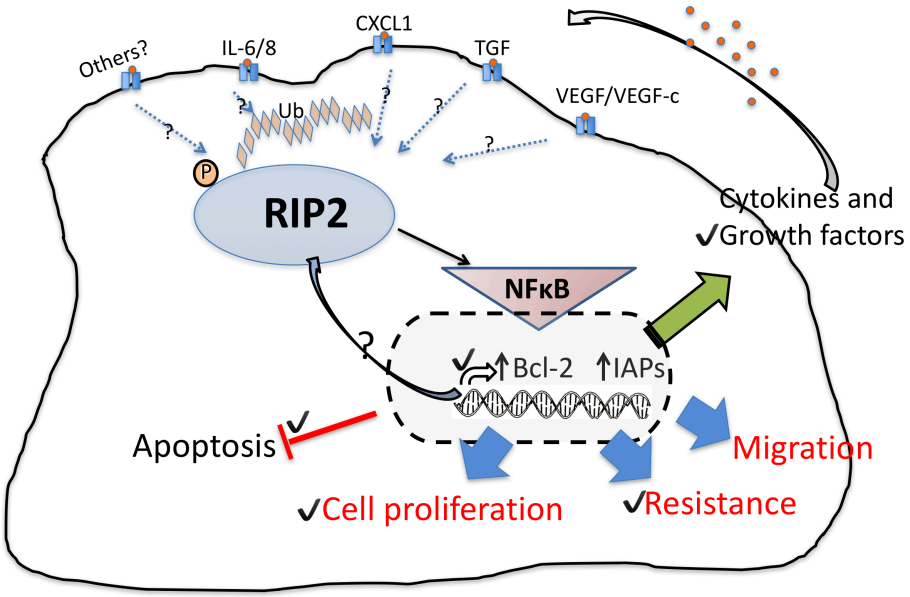


Figure 4.3: Suggested mechanism of action elicited by RIP2.

RIP2 upon phosphorylation (P) and subsequent ubiquitination (Magalhaes et al., 2011) will activate NF-κB and lead to the transcription of anti-apoptotic proteins (Bcl-2 family and IAPs) that inhibit apoptosis and the release of cytokines and growth factors that have autocrine activity and leads to further activation of pro-survival signaling and enhance survival, resistance and cell migration. The mechanisms annotated by (√) sign, i.e transcription of anti-apoptotic proteins, enhance cell proliferation and resistance, and release of cytokines and growth factors, are described for the first time in TNBC cells as a result of this project. Further studies are required (dotted lines "?") in order to validate whether these factors activate RIP2 which enhances NF-κB transcription activity.

Chapter 5

HSPA1 and HSPB1 in Triple Negative Breast Cancer

5.1. Introduction

This study investigates the role of HSPA1 and HSPB1 in TNBC. Heat shock proteins have been associated with survival of cancerous cells and resistance to therapeutic agents (Sherman and Multhoff, 2007). Overexpression of HSPB1 and HSPA1 has been shown to be essential in the survival of BC cells (Calderwood and Gong, 2012). Therefore, down-regulation of HSPs may be of critical importance in the treatment of breast cancers.

HSPA1 and HSPB1 are induced by a variety of stresses such as heat, oxidative stress, or anticancer drugs. In normal and unstressed cells, HSPA1 and HSPB1 are expressed at low levels (Kregel, 2002). Upon stress induction, HSPA1 and HSPB1 affect aggregation and folding of other proteins; moreover, they can directly modulate the apoptotic signaling pathway by inhibiting different steps of the apoptotic pathways to save cells from stress-induced damage that might trigger undesirable cell death (Gupta et al., 2010, Garrido et al., 2006). In contrast to normal cells, cells from a wide range of tumour cells express high levels of HSPA1 and/or HSPB1, which increase the tumourigenic potential of cancer cells as demonstrated by experimental rodent models (Ciocca and Calderwood, 2005, Garrido et al., 2006). This effect on tumourigenesis may be due to HSPs function as molecular chaperones. This chaperone activity supports cancer cells to fold proteins, such as the cell cycle machinery, kinases and several proteins that enhance tumour cells growth. HSPA1 and HSPB1 tumourigenic role has also been attributed to their anti-apoptotic properties. HSPA1 and HSPB1 were proven to have an anti-apoptotic activity in different cell lines. For example, HSPB1 interacts with cytochrome-c upon its release from the mitochondria, while HSPA1 or HSP90 interact with Apaf-1, which inhibits apoptosome complex formation and impedes caspase activation (Garrido, 1999, Garrido, 2006). In addition, HSPA1 and HSPB1 can modulate the apoptotic intrinsic pathway via inhibiting BAX activation and hence modulating MOMP. HSPA1 could also interact with AIF inhibiting caspase-independent apoptosis (Dudeja et al., 2009, Sherman and Multhoff, 2007, Rashmi et al., 2004,).

HSPA1 family of chaperone proteins has been also associated with metastasis and a more aggressive form of BC and therefore worse prognosis (Meng et al., 2011). It has been reported that inhibition of HSPA1 expression can lead to BC cell death (Nylandsted et al., 2000). Moreover, HSPA1 and HSPB1 have been shown to associate with other proteins. HSPA1 has been reported to function as a chaperone protein for both c-Myc oncogene and the p53 gene suggesting that up-regulated HSPA1 could play a role in tumour formation (Conroy and Latchman, 1996). It has also been reported that mutations in *TP53* lead to change in p53 protein conformation, and hence formation of p53-HSPA1 complex detected

in the nucleus of cancer cells (Davidoff et al., 1992). Also HSPB1 was described to modulate PTEN tumour suppressor gene expression, suggesting an interaction between PTEN and HSPB1 in MCF-7 BC cell line (Cayado-Gutierrez et al., 2013). Moreover, phosphorylation of HSPB1 is a characteristic of tumour cells (Jolly and Morimoto, 2000). Recently, Ruan et al showed that phosphorylated HSPB1 is involved in the crosstalk between Jun N-terminal kinases (JNKs), a type of MAP kinases and the NF-κB signaling pathway (Ruan et al., 2015). JNK is known to possess both cell survival and pro-apoptotic activity, but the mechanism describing its action in both pathways remains to be elucidated (Ruan et al., 2015). However, the crosstalk between JNK and NF-κB is reported to regulate cell survival by modulating HSPB1 phosphorylation (Ruan et al., 2015). HSPB1 is phosphorylated at serine residues 15, 78 and 82 by mitogen-activated protein kinases associated protein kinases (Stokoe et al., 1992, Rouse et al., 1994). It has been shown to decrease the size of the oligomers, and phosphorylation at Ser-82 residue causes dissociation of HSPB1 oligomers (Theriault et al., 2004). In addition, HSPB1 phosphorylation occurs in response to cellular stress (Ito et al., 2005). Protein kinase-C mediated HSPB1 phosphorylation at serine 15 protects against iron-dependent apoptosis by reducing iron-mediated production of lipid reactive oxygen species (Sun et al., 2015). Therefore, HSPB1 phosphorylation status and structural organization have a protective role against cell death (Arrigo and Gibert, 2013).

Lately, there has been an increased interest in studying extracellular HSPs (eHSPs) and their role in cancer therapy (Schmitt et al., 2007). HSPD1 (HSP60), HSPA1 and GRP78 have been found to stimulate many cells in the innate and adaptive immunity and are biologically plausible and likely candidates to serve as alarmins (Pockley et al., 2000, Pockley and Muthana, 2005). Tumour cells may exploit such anti-inflammatory effects, as the dampening of the immune response would permit tumour progression and metastasis (O'Byrne, 2015). Increased serum levels of HSPB1 have been associated with worse outcome in women with breast, ovarian, and uterine cancer (De and Roach, 2004) and antibodies to HSPB1 have been identified in the blood of women with BC and other gynecologic cancers (Korneeva et al., 2000, Korneeva et al., 2002). Higher levels of anti-HSPB1 serum antibodies improved BC survival (Conroy et al., 1998). Increased tumour expression of HSPB1 is associated with shorter cancer free periods and advanced cancer staging, possibly due to the inhibition of tumour cell apoptosis (Thor et al., 1991).

Apart from the HSR, the unfolded protein response (UPR) is another pathway activated in response to stress and is important to maintain proper protein folding in the endoplasmic reticulum (ER) (Jager et al., 2012), discussed in Section 1.6.1. Moreover, it was recently discovered that HSPA1 plays an important role in inhibiting ER stress-mediated

apoptosis (Gupta et al., 2010). However, the mechanisms by which HSPB1 protects eukaryotic cells against cell death under ER stress conditions remain to be explored.

Both the HSR and UPR are essential mechanisms required to maintain proper protein folding upon cellular stress and therefore are vital for cell survival. Several studies have investigated interactions between the HSR and UPR. In yeast, a connection between the two pathways was shown via Sir2 de-actylase protein necessary for HSF1 activation (Weindling and Bar-Nun, 2015). HSF1 is conserved between human and yeast and is responsible for regulating the HSR by interacting with heat shock elements (HSEs). Similarly, Hou et al suggested that crosstalk between the UPR and HSR is due to oxidative stress response and that the HSR may actually contribute to the cell's resistance to conditions of ER stress, and other stress responses, by its role in protein folding (Hou and Taubert, 2014). Understanding the mechanism of interaction between the HSR and UPR might reveal the mechanisms of proteo-toxic stress responses and help identify new targets in cancer therapy.

The aim of this study was to:

- Identify the role of HSPB1 and HSPA1 in TNBC
- Examine crosstalk between the HSR and UPR in TNBC
- Detect release of HSPB1 or HSPA1 to extracellular medium and identify their role

5.2. Results

5.2.1. Basal-like expression of HSPB1, pHSPB1 and HSPA1 in BC cell lines

A panel of BC cell lines was screened for HSPB1, pHSPB1 and HSPA1 expression. All BC cell lines and non-transformed mammary epithelial cell line MCF10A, have high basal-like HSPB1 expression but only MDA-MB-468 and MCF10A have high basal-like pHSPB1. On the other hand, HSPA1 was expressed in all BC cell lines with lower expression levels in MDA-MB-453 and BT-549, and was negligible in MCF10A (Figure 5.1).

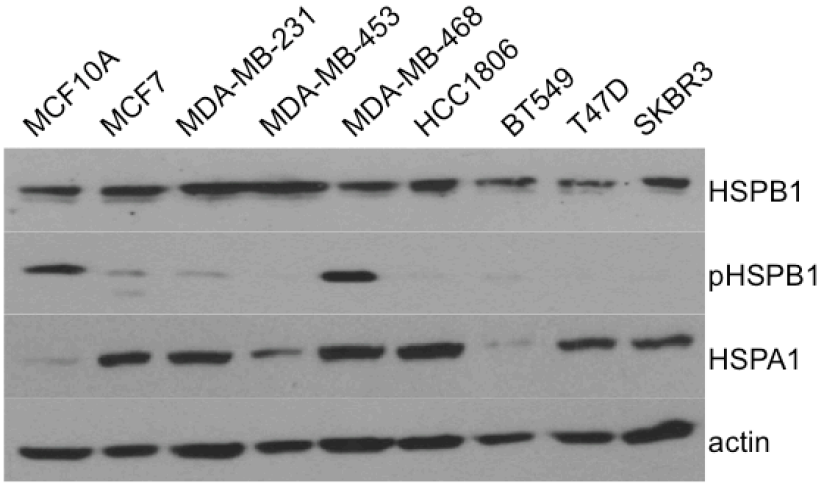


Figure 5.1 pHSPB1, HSPB1 and HSPA1 basal-like expression in BC cell lines.

Western blot detecting basal-like expression of p-HSPB1, HSPB1 in MCF-7, SKBR-3, T47D, MDA-MB-231, MDA-MB-453, MDA-MB-468, HCC1806 and BT549 BC cells. MCF-10A was used as breast control cell line. Cells were seeded based on optimized cell number (Table 1) and harvested at 48 h and lysed. Actin was used as loading control. Data shown are representative of 3 independent experimental repeats.

5.2.2. HSPB1 silencing induces cell death in MDA-MB-231 cells

Overexpression of HSPB1 has been reported in the progression of BC, therefore the effect of HSPB1 inhibition was determined in TNBC cells. MDA-MB-231 cells were transfected with siRNA pool against HSPB1. HSPB1 silencing was confirmed by western blot at 48 h post transfection (Figure 5.2a). Significant cell death was observed at 48 h post transfection in comparison to siRNA control (Figure 5.2b). We confirmed the siHSPB1 induced apoptotic cell death by DAPI staining and caspase-3/-7 like assay (Figure 5.2 c and d). However, we were not able to confirm that the observed effect is due to HSPB1 silencing only as we also observed a decrease in HSPA1 expression upon HSPB1 knockdown (Figure 5.2a).

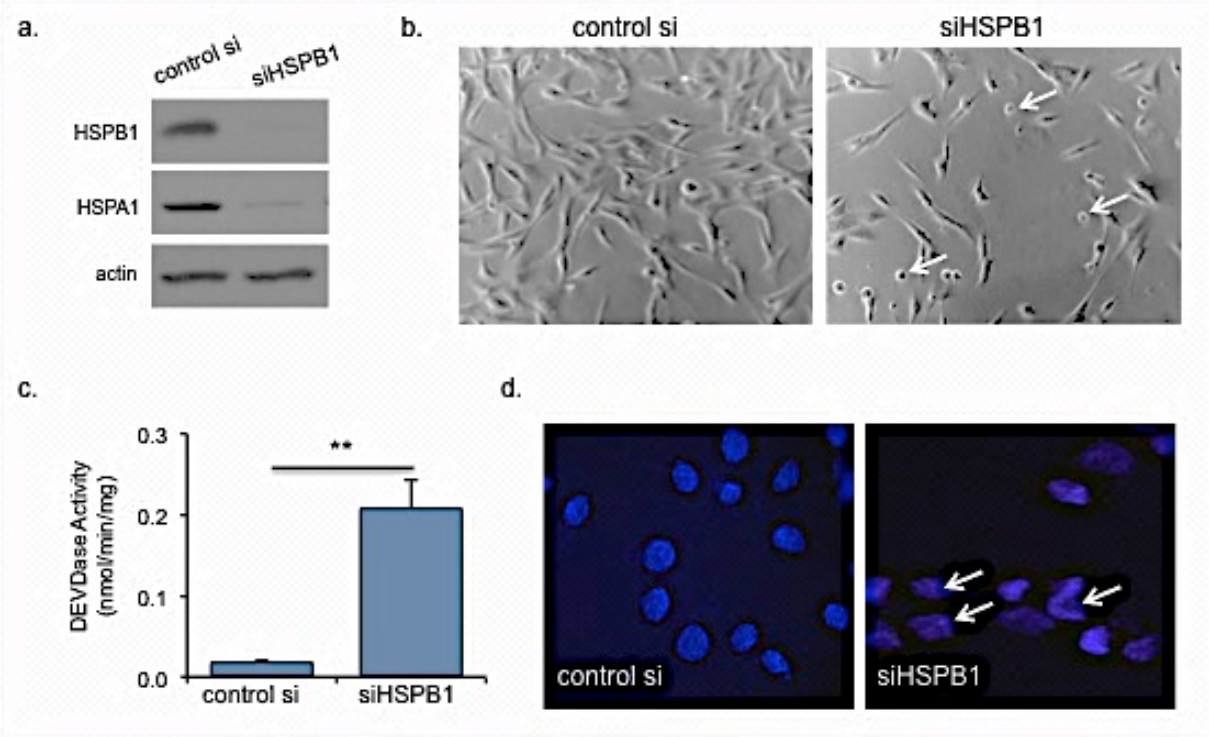


Figure 5.2 HSPB1 silencing induces cell death.

*a. Western blot for HSPB1 expression post siRNA HSPB1 transfection, membrane was probed for HSPB1 and HSPA1 at 48 h post transfection. b. Microscopic images of MDA-MB-231 cells transfected with siRNA against HSPA1. c. Caspase-3-/7 like activity measured by DEVDase assay of MDA-MB-231 cells 48 h post siHSPB1 transfection. d. DAPI staining of MDA-MB-231 cells showing apoptotic nuclei (arrows) upon transfection of MDA-MB-231 with siHSPB1. All data shown above are representative of 3 independent experimental repeats. Values shown are mean of 3 independent experiments ± standard error. p-values were calculated relative to control using One-way Anova and represented by * indicates $p < 0.05$ and ** indicates $p < 0.01$.*

5.2.3. Heat Shock Factor Inhibitor KNK437 induces cell death and sensitizes MDA-MB-231 cells to Taxol and Doxorubicin

To further determine the role of basal-like HSPB1 in TNBC, we treated MDA-MB-231 with heat shock factor-1 (HSF1) inhibitors. HSF1 transcription factor regulates HSPB1 and HSPA1 expression (Whitesell and Lindquist, 2009). KNK437 inhibits stress-induced synthesis of HSP through inhibition of transcription activity of HSF-1 (Yokota et al., 2000). Upon treating MDA-MB-231 cells with KNK437, a dose dependent decrease in expression of HSPB1 and HSPA1 was observed (Figure 5.3a). Moreover, cells treated with KNK437 exhibited significant cell death at 100 μM KNK437 as determined by MTT assay 48 h post treatment, and apoptotic cell death was further confirmed by PARP cleavage (Figure 5.3a and b). Figure 5.3a shows the expression of HSPB1, HSPA1 and PARP cleavage upon KNK437 treatment. A decrease in HSPB1 and HSPA1 expression was observed at high concentrations of KNK437 (75 and 100 μM) where an increase in PARP cleavage is also

observed indicating apoptotic cell death. Therefore, KNK437 can reduce HSPB1 and HSPA1 expression levels in MDA-MB-231 cells leading to cell death.

To test the biological effect of inhibition of HSF1 on MDA-MB-231 cells and to determine whether this inhibition that causes reduction in levels of HSPB1 and HSPA1 sensitizes cells to drug induced cell death, MDA-MB-231 cells were co-treated with HSF1 inhibitor and taxol or doxorubicin. No significant difference in response to treatments was observed unless using KNK437 at high dose where reduction in cell viability was observed upon co-treatment of KNK437 and taxol (1 μM) or doxorubicin (100 nM) (Figure 5.3c).

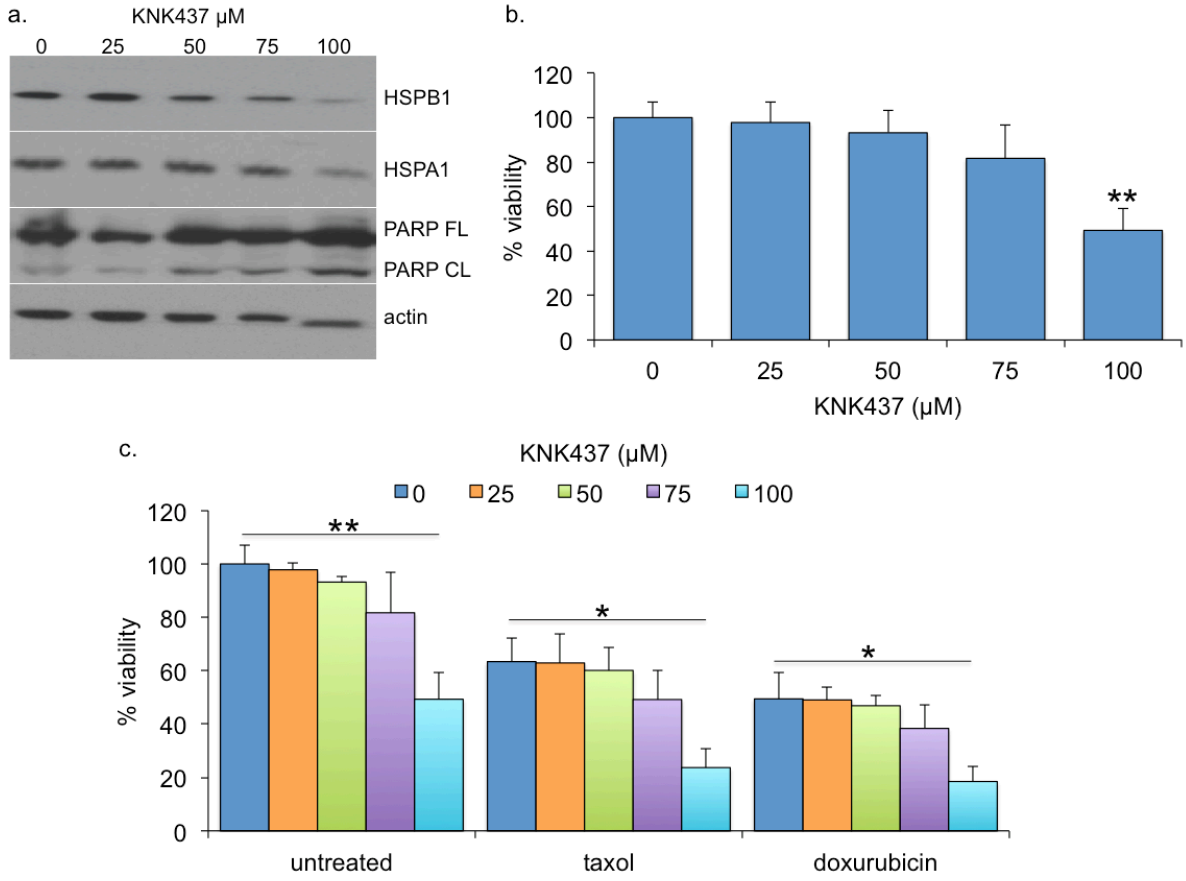


Figure 5.3: Effect of HSF1 inhibitors treatment of MDA-MB-231 cells.

*MDA-MB-231 cells were treated with KNK437 dose at 25, 50, 75 and 100 μM. DMSO was used as negative control. All data shown are representative of 3 independent experimental repeats. a. Western blot showing expression of HSPB1, HSPA1 and PARP full length (FL) and cleaved (CL) at 48 h; b. Cell viability of MDA-MB-231 cells 48 h post dose treatment with KNK437 measured by MTT; c. Cell viability of MDA-MB-231 cells upon co-treatment with KNK437 and taxol (1 μM) or doxorubicin (100 nM) at 48 h post drug treatment as measured by MTT. Values shown are mean ± standard error. p-values were calculated relative to control using One-way Anova and represented by * indicates $p < 0.05$ and ** indicates $p < 0.01$.*

5.2.4. HSPB1 and HSPA1 are not induced by ER stress in TNBC

In-vitro experiments show that thermal and chemical stresses induce expression of heat shock proteins especially HSPB1 and HSPA1 in cancer cells (Multhoff, 1997, Andrieu et al., 2010, Buzzard et al., 1998), and a few studies have reported crosstalk between the HSR and the UPR (Weindling and Bar-Nun, 2015, Hou et al., 2014). Moreover, HSPB1 is phosphorylated in response to ER stress (Ito et al., 2005). In order to determine if HSR is induced by ER stress in TNBC, MDA-MB-231 and MDA-MB-468 cells were treated at 24 h post seeding with different ER stressors. p-HSPB1, HSPB1 and HSPA1 expression was determined by western blot 6, 12 and 24 h post treatment with ER stress drugs thapsigargin (Drukker et al., 2013) and tunicamycin (Tm). The expression of HSPs upon ER stress varied between different experiments and no remarkable change in HSPB1 or HSPA1 was recognized between control and Tg or Tm treated cells (Figure 5.4a). Protein expression was quantified using ImageJ software and combined from 3 different experiments, average results are represented in Figure 5.4b, no significant difference between control and Tg or Tm treated cells was observed.

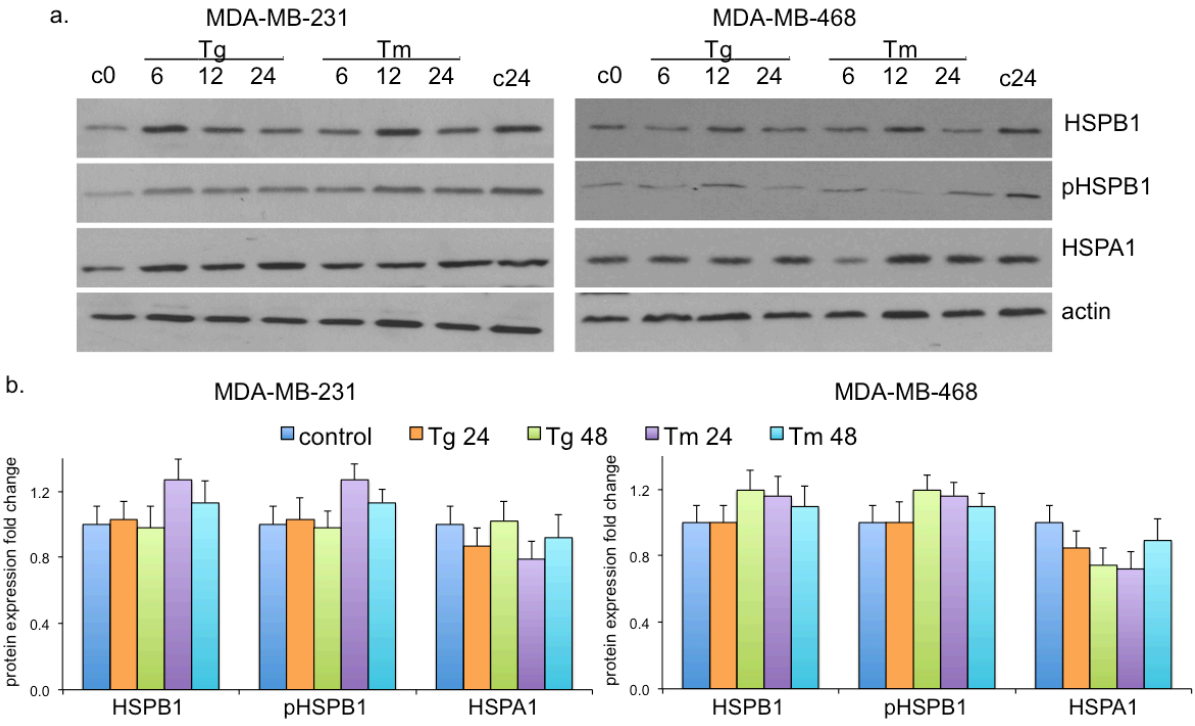


Figure 5.4 HSPB1 and HSPA1 were not inducible upon ER stress in TNBC cells.

a. Western blot for ER stress treated cells were probed for HSPB1, pHSPB1, HSPA1 at 24 h, 48 h and 72 h post treatment with thapsigargin (Tg) (1 μM) and tunicamycin (Tm) (5 μM) in comparison to control at 0 h (c0) and 24 h (c24). Actin was used as loading control. b. Densitometry showing quantitative measurement of HSPB1, pHSPB1 and HSPA1 upon treatment with ER stress drugs. All data shown above are representative of 3 independent

experimental repeats. Values shown are mean of 3 independent experiments ± standard error.

5.2.5. IRE1 and PERK inhibitors do not affect HSPB1 and HSPA1 expression in TNBC

The role of ER stress and UPR is reported to impact cancer progression and resistance (Sherman and Multhoff, 2007, Tameire et al., 2015). In order to further investigate the interaction between HSR and ER stress response in TNBC, we targeted the IRE1 arm and PERK arm of the UPR by treating MDA-MB-231 cells with ER stress inhibitors and detected HSP expression levels post treatment. To inhibit the UPR, 20 μM IRE1 inhibitor MKC-8866 (MannKind Corp) or 300 nM PERK inhibitor GSK797800 (Toronto Research Chemicals) were added to cells for 24 and 48 h. MKC-8866 and GSK797800 concentrations used were optimized previously and confirmed for IRE1 and PERK inhibition (Cleary 2015, unpublished data). No change in p-HSPB1, HSPB1 or HSPA1 expression was observed post treatment with inhibitors (Figure 5.5a). This was further confirmed by quantitative measurement of protein expression using ImageJ software. No significant difference in fold change of protein expression was observed (Figure 5.5b). In order to confirm the activity of MKC-8866 inhibition of IRE1, XBP1 primary antibody was used to detect spliced XBP1 in treated control and inhibition of splicing upon treatment with inhibitor (Figure 5.5c). However, the PERK inhibition was not verified for this experiment but the concentration used was previously optimized to inhibit PERK activity in MDA-MB-231 cells.

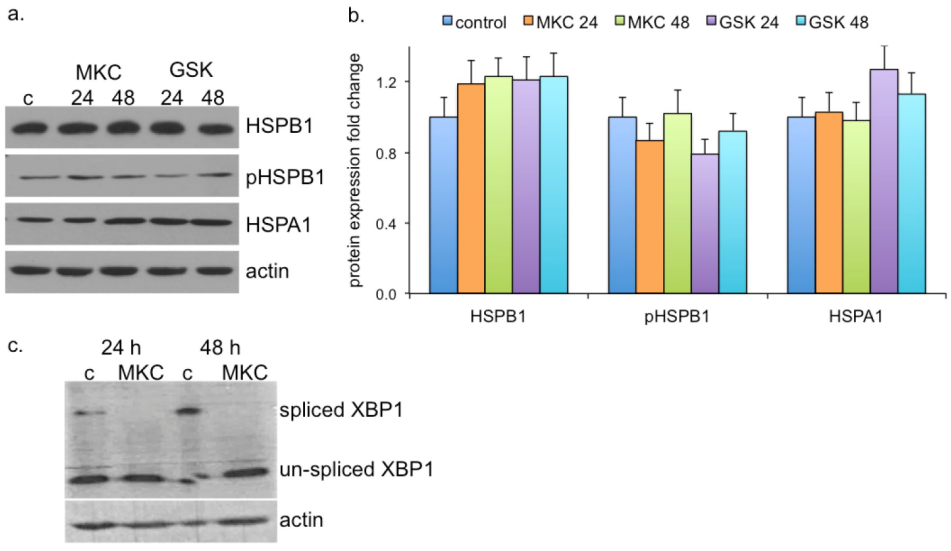


Figure 5.5 HSPB1, HSPA1 expression is not affected upon inhibition of UPR specific pathways in TNBC cells.

a. Western blot of MDA-MB-231 probed for HSPB1, p-HSPB1, HSPA1 at indicated time-points post treatment with 20 μM IRE1 inhibitor MKC-8866 (MKC) and 300 nM PERK inhibitor GSKG797800 (GSK). Actin was used as loading control; b. densitometry showing the relative protein expression of HSPB1, pHSPB1 and HSPA1 upon treatment with MKC

and GSK; c. XBP1 splicing inhibition upon treatment with MKC-8866 in MDA-MB-231 cells. All data shown above are representative of 3 independent experimental repeats.

5.2.6. HSPB1 is released into media in MDA-MB-231 and MDA-MB-453 cells

Several HSPs are reported in the literature to be released to extracellular space as mentioned in tne Introduction (Section 1.5). Serum HSPB1 has been suggested as a prognostic marker of tumour malignancy because HSPB1 serum levels are elevated in cancer patients (Liao et al., 2009, Huang et al., 2010). Therefore, we investigated HSPB1 release in BC cell lines. Culture media at 48 h post seeding was collected and centrifuged at high speed for 10 min to get rid of any live cells. Media was then concentrated using concentration column. 100 µl of Lamelli buffer was added to 500 µl of media concentrate and 50 µl of media was loaded for western blot analysis. eHSPB1 was detected in media collected from MDA-MB-231 and MDA-MB-468 but not from MCF-7 cells, while HSPA1 was not detected in culture media (Figure 5.6). In addition, no actin was observed in culture media indicating that proteins detected in extracellular space are due to release of proteins and not due to cellular debris or presence of live cells in culture media.

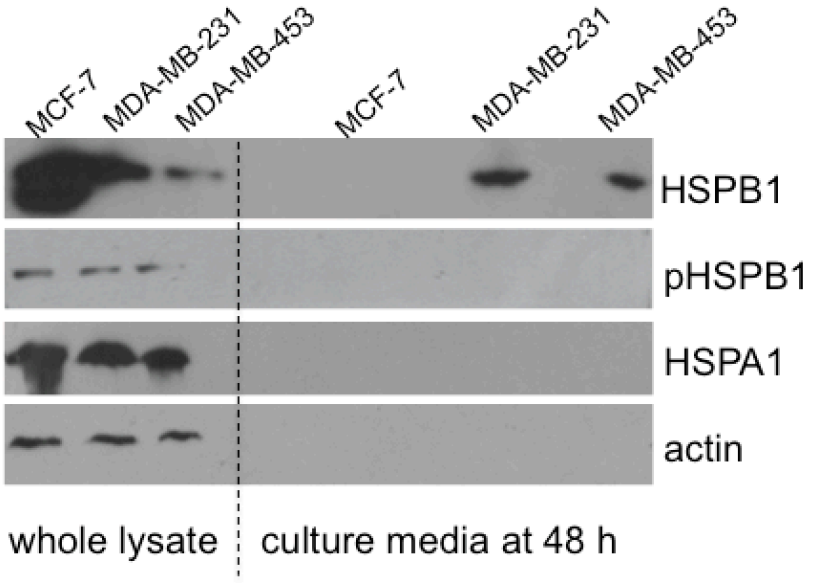


Figure 5.6: HSPB1 is released into media of TNBC.

Western blot showing HSPB1 expression in culture media of MDA-MB-231 and MDA-MB-468 cells but not in MCF-7 cells. Membrane was also probed for p-HSPB1 and HSPA1. Whole cell lysates of MCF-7, MDA-MB-231 and MDA-MB-453 were also run for reference. Actin was used as control for intact cells. Results shown are representative of 3 repeats of same experiment.

5.2.7. eHSPB1 does not induce cell survival

Preliminary experiments were done to determine the role of eHSPB1 in BC. HSPB1 primary antibody was used as an antagonist to bind to HSPB1 present in media. In order to determine if eHSPB1 has any effect on proliferation, MDA-MB-231 cells were seeded in 6-

well plate and cells were counted at 24, 48 and 72 h post seeding. No difference in cell count was observed between control media and media with anti-HSPB1 (Figure 5.7a). Moreover, MDA-MB-231 cells were seeded in 96-well plate and conditioned media was added with/without HSPB1 antibody at 24 h post seeding to determine cell viability. MDA-MB-231 cell viability was measured using MTT at 24 and 48 h post treatment. No difference in cell viability was observed between treated and non-treated MDA-MB-231 cells (Figure 5.7b). In addition, anti-HSPB1 in media did not affect cell response to drugs. MDA-MB-231 cells seeded in 96-well plate and treated with ceramide (30 μM) and taxol (1 μM) showed no difference in cell viability between cells grown in control media or anti-HSPB1 treated media (Figure 5.7c).

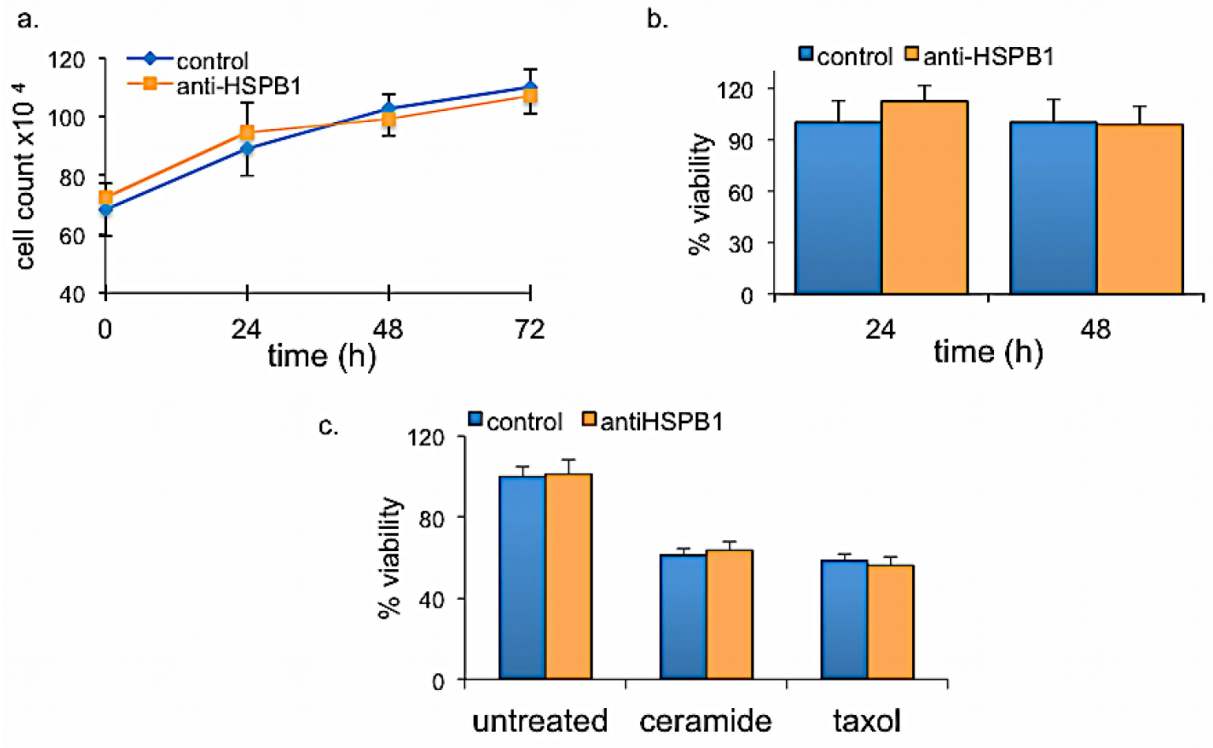


Figure 5.7: eHSPB1 does not induce cell survival.

MDA-MB-231 cells were seeded at 5x10^4 cells/cm^2. MDA-MB-231 culture media collected at 48 h was incubated with anti-HSPB1 antibody (5 μg/ml) for 2 h. MDA-MB-231 culture media control and media incubated with anti-HSPB1 was added to cells in culture 24 h post seeding. a. Cell count of cells at 24 h, 48 h and 72 h post media change. b. MTT assay measuring cell viability. c. MTT viability assay of MDA-MB-231 treated with ceramide (30 μM) and taxol (1 μM) with culture media or culture media with anti-HSPB1 antibody. All values shown are mean of 3 independent experiments ± standard error.

5.2.8. eHSPB1 has no impact on cell migration

HSPB1 phosphorylation by VEGF activation of protein kinase-D induces endothelial migration indicating the potential role of HSPB1 in VEGF-dependent angiogenesis; moreover extracellular HSPB1, was shown to regulate VEGF-mediated angiogenesis

through direct interaction (Lee et al., 2012, Evans et al., 2008). Therefore, the role of eHSPB1 in migration of BC cells was investigated using scratch assay. Cells were seeded in 6-well plate and allowed to be confluent after which a 3x3 scratches were performed in each well. Media was changed and anti-HSPB1 was added to media. No difference in wound healing was observed at 24, 48 and 72 h post scratching. Figure 5.8a shows MDA-MB-231 cells post-scratch at 48 h. Moreover, scratch width was measured at 24, 48 and 72 h post seeding and expressed as percentage healing. No significant difference in wound healing was observed in cells cultured in normal media versus cells in media treated with anti-HSPB1 (Figure 5.8b).

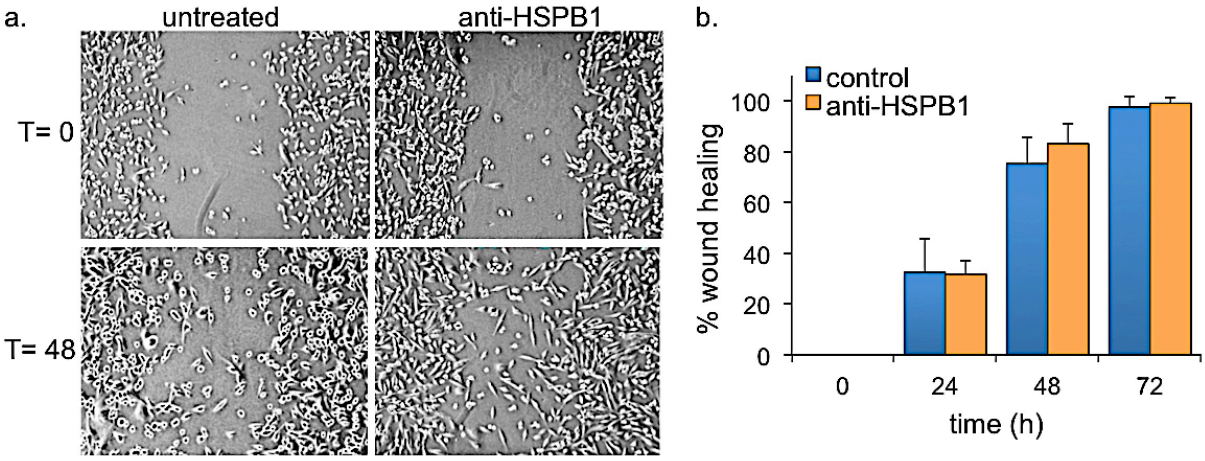


Figure 5.8: eHSPB1 have no impact on cell migration

MDA-MB-231 cells were cultured in 6-well plates till they became confluent at 48 h, and streak was done in triplicates. Conditioned media was added on day of streak and images were taken at 24 h, 48 h and 72 h post streaking. a. Images of MDA-MB-231 scratch assay at 0 and 48 h (T = 0 and T = 48). B. Scratch width was measured on day 0 and at 24 h, 48 h and 72 h post scratch. Percentage wound healing was calculated relative to measurement at day 0. Results show results from 3 repeat experiments ± standard error.

5.2.9. HSPB1 and HSPA1 expression in BC patients

Analysis of patient BC databases to investigate the significance of HSPB1 and HSPA1 in the development and prognosis of TNBC showed no impact of HSPB1 or HSPA1 in TNBC. Several studies have reported that HSP expression is increased in cancer (Calderwood and Gong, 2012, Vargas-Roig et al., 1997). However, analysis of patient databases downloaded from NCBI Gene Expression Omnibus (GEO) (GSE19615, GSE12276, GSE6532, GPL570, GSE9195, GSE12093, GSE11121, GSE1378, GSE1379, GSE9893, GSE2034, GSE1456-GPL96, GSE7378, E-TABM-158, GSE3494-GPL96, GSE4922, GPL96, GSE2990 and GSE7390) shows that HSPB1 expression is lowest in TNBC and highest in Luminal B BC with p(Wallis) < 0.0001 (Figure 5.9a); moreover, HSPA1 expression is slightly higher in Luminal B and comparable among all BC subtypes (p(Wallis)

< 0.0001) (Figure 5.9b). Kruskal-Wallis analysis is used for comparing two or more independent samples of equal or different sample sizes and a significant Kruskal-Wallis test indicates that at least one sample stochastically dominates one other sample. Moreover, Kaplan Meier survival graphs show no significant difference in adverse-event (AE) free survival among patient samples with high vs low HSPA1 or HSPB1 expression levels (Figure 5.9c). Moreover, Table 5.1 shows expression of HSPA1 and HSPB1 relative to mean expression in BC subtypes. HSPA1 and HSPB1 expression associate with Luminal B subtype, where 75% of Luminal B samples express high HSPB1 and 61% express high HSPA1; however, higher percentage of other subtypes express low HSPA1 and HSPB1.

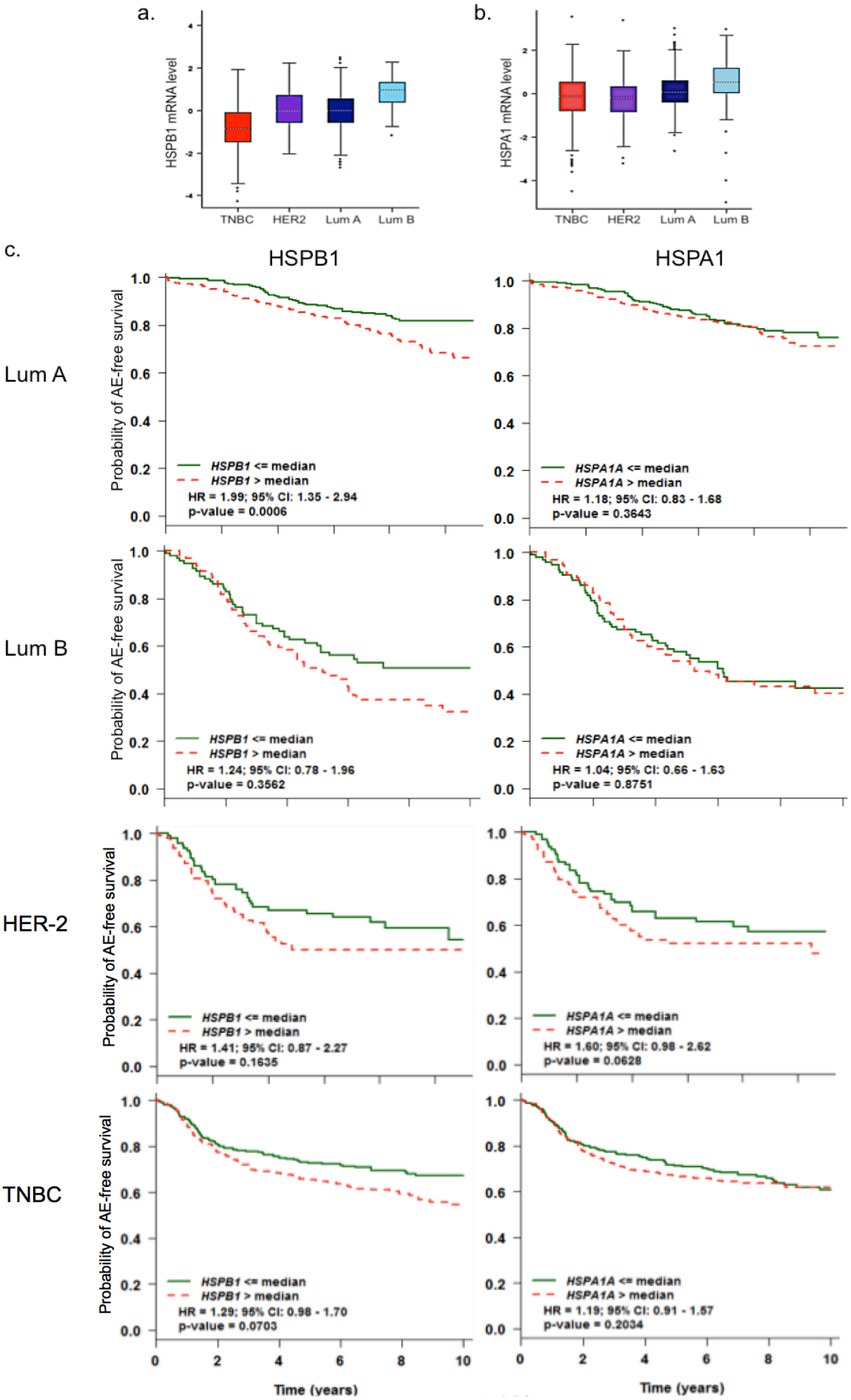

a.
HSPB1 mRNA level
TNBC
HER2
Lum A
Lum B
b.
HSPA1 mRNA level
TNBC
HER2
Lum A
Lum B
c.
HSPB1
HSPA1
Lum A
Lum B
HER-2
TNBC
Probability of AE-free survival
HSPB1 <= median
HSPB1 > median
HR = 1.99; 95% CI: 1.35 - 2.94
p-value = 0.0006
HSPA1A <= median
HSPA1A > median
HR = 1.18; 95% CI: 0.83 - 1.68
p-value = 0.3643
HR = 1.24; 95% CI: 0.78 - 1.96
p-value = 0.3562
HR = 1.04; 95% CI: 0.66 - 1.63
p-value = 0.8751
HR = 1.41; 95% CI: 0.87 - 2.27
p-value = 0.1635
HR = 1.60; 95% CI: 0.98 - 2.62
p-value = 0.0628
HR = 1.29; 95% CI: 0.98 - 1.70
p-value = 0.0703
HR = 1.19; 95% CI: 0.91 - 1.57
p-value = 0.2034
Time (years)
Time (years)

Figure 5.9: mRNA expression of HSPB1 and HSPA1 in BC patient samples.

a. Patient online GEO NCBI databases were analyzed for expression of HSPB1 mRNA among the different cancer subtypes. Box plot graph representing the relative HSPB1 mRNA level. Dots indicate outliers; b. Patient online GEO NCBI databases were analyzed for expression of HSPA1 mRNA among the different cancer subtypes. Box plot graph representing the relative HSPA1 mRNA level. Dots indicate outliers; c. Kaplan Meier graphs showing AE free survival of patients with BC based on HSPB1 and HSPA1 mRNA expression and subtype. All data were analyzed using SPSS statistical software.

Table 5.1: Percentage patients expressing high vs low HSPB1 and HSPA1 mRNA levels in different BC patient samples.

molecular subtype	*Basal-like*		*HER-2*		*Luminal A*		*Luminal B*	
relative expression	*Low*	*High*	*Low*	*High*	*Low*	*High*	*Low*	*High*
HSPB1	*91%*	*9%***	*65%*	*35%*	*69%*	*31%*	*25%*	*75%***
HSPA1	*70%*	*30%*	*76%*	*24%*	*63%*	*36%*	*40%*	*61%**

*Relative mRNA expression was stratified based on mean mRNA, mRNA expression less than mRNA mean expression was referred to as "low" and mRNA expression more than mRNA mean expression was referred to as "high". Significance difference between samples was calculated using Kruskal Wallis analysis and represented by * indicates $p < 0.05$ and ** indicates $p < 0.01$.*

5.3. Discussion

This chapter investigates the role of the HSR and its interaction with the UPR in mediating survival in TNBC. This study shows that the HSPB1 and HSPA1 expression in TNBC is important for cell survival and inhibiting HSF1 can sensitize MDA-MB-231 cells to drug induced cell death; however, interaction between the HSR and the IRE1 or PERK pathways of the UPR was not observed. Moreover, HSPB1 release to the extracellular space was identified in MDA-MB-231 and MDA-MB-453 TNBC cell lines, but not MCF-7.

The relevance of HSPA1 and HSPB1 in cancer development has been well reported in the literature. HSPs, including HSPA1 and HSPB1, are overexpressed in wide range of cancer and display an important role in cancer progression and resistance (Ciocca and Calderwood, 2005). Knockdown of HSPB1 in MDA-MB-231 cells via siRNA caused a significant increase in apoptotic cell death. However, this observation cannot be attributed only to HSPB1 knockdown since a decrease in HSPA1 expression was also observed in HSPB1 siRNA transfected cells. HSPB1 and HSPA1 are reported to act synergistically but no impact on regulation of HSPA1 expression by HSPB1 is known.

Stress induced transcription of HSPs requires activation of HSF1, the master regulator of HSP genes and supports the ability of cancer cells to accommodate stress, and has been implicated as a target in cancer therapy (Whitesell and Lindquist, 2009). Inhibiting HSF1 in MDA-MB-231 cells using KNK437, affected HSPB1 and HSPA1 expression and induced cell death. Moreover, HSF1 inhibition sensitized MDA-MB-231 cells to taxol and doxorubicin treatments. Hansen et al demonstrated that HSPB1 overexpression confers resistance to the chemotherapeutic agent doxorubicin in MDA-MB-231 BC cells (Hansen et al., 1999). Moreover, in melanoma cells overexpression of HSF1 confers resistance to doxorubicin and paclitaxel (Vydra et al., 2013). Other mechanism of HSP enhanced protection involves inhibition of apoptosis through direct interactions of HSPA1, HSPB1 and HSP90 with apoptotic molecules (Garrido et al., 2006). Regardless of the known protective function of HSPs, their role in the effectiveness of chemotherapy is contradictory. Several reports show that overexpression of HSPA1, HSPB1 or HSP90 increases cell resistance to cisplatin or doxorubicin (Yang et al., 2012, Liu et al., 2011, Lee et al., 2007, Fortin et al., 2000). In addition, HSPA1 mediates heat-induced carboplatin resistance in p53-dependent hepatoma cells (Sharma et al., 2010). However, several reports indicate that HSP expression does not enhance survival in patients with different types of neoplasia (Hettinga et al., 1996, Richards et al., 1996, Kimura and Howell, 1993). Recently, it was reported that despite the importance of HSPs in tumour initiation (Meng et al., 2011), HSF1 regulates a network of genes in malignant cells different from the heat shock induced transcription

(Mendillo et al., 2012).Therefore, cancer response to treatment does not depend solely upon HSF1 induction of HSP genes, but also on HSF1 mediated regulation of other genes or pathways such as glycolysis and energy metabolism, cell cycle signaling, DNA repair, apoptosis, cell adhesion, extracellular matrix formation, and translation (Mendillo et al., 2012, Dai et al., 2007, Zhao et al., 2009).

The role of HSPB1 and HSPA1 in ER stress induced cell death is not yet fully described. ER stress-mediated cell death is well known to depend on intrinsic apoptosis and regulated by the Bcl-2 protein family (Hetz, 2012). HSPB1 has been shown to inhibit several stages of the apoptotic pathway enhancing survival and resistance (Concannon et al., 2003). Moreover, studies in the lab show that HSPB1 can act upstream of MOMP and prevent the release of cytochrome-c protecting cells against ER stress induced caspase activation (Kennedy et al., 2014). Therefore, the effect of ER stress pathways inhibition or induction of HSPB1 and HSPA1 were investigated. This was carried out by observing the changes in expression of HSPB1, p-HSPB1 and HSPA1 in BC cell lines upon treatment with UPR inhibitors, MKC8866 and GSK797800 that inhibit the IRE1 and PERK arms of the UPR pathway respectively; and treatment with ER stress drugs, Tg and Tm. Although it is reported in the literature that the two pathways are linked (Weindling and Bar-Nun, 2015, Hou and Taubert, 2014), TNBC MDA-MB-231 cells did not show any substantial change in HSPB1 or HSPA1 expression upon either PERK or IRE1 inhibition. A very slight decrease in HSPB1 levels was observed at 48 and 72 h MKC8866 treatment, but was neither reproducible nor significant. No substantial change in HSPA1 or HSPB1 expression was observed upon inhibition of RNAse activity of IRE1. Similarly, PERK inhibition caused no significant change in expression of these proteins nor had an effect on the phosphorylation of HSPB1. Elevated HSPB1 and HSPA1 levels are associated with doxorubicin resistance in BC cells (Ciocca et al., 1992). However, HSPB1 and HSPA1 expression was not induced upon treatment of cells with ER stress drugs (Figure 5.4). Ciocca et al also reported that multidrug resistance is not associated with elevated HSP levels, and that specific HSPs, HSPA1 and HSPB1, are associated with doxorubicin resistance in certain human BC cells in a mechanism independent of the multidrug resistance system (Ciocca et al., 1992).

Moreover, IRE1 or PERK inhibition and ER stress did not result in any alteration of phospho-HSPB1 levels in TNBC cells. HSPB1 phosphorylation is characteristically observed in tumour cells and associated with LN metastasis of BC (Kaigorodova et al., 2015) and may be involved in the mediation of the NF-κB and JNK pathways (Ruan et al., 2015). Moreover, the inhibition of HSPB1 phosphorylation results in the increased sensitivity of colorectal cancer cells to chemotherapy (5-FU) (Matsunaga et al., 2014). On the other hand, p-HSPB1 reportedly suppresses cell growth of hepatocellular carcinomas via inhibition of extracellular

signal-regulated kinase (Matsushima-Nishiwaki et al., 2008, Yasuda et al., 2005). A similar role for pHSPB1 might be relevant in BC since basal-like expression of HSBP1 in BC cells lines, except MDA-MB-468, is very low in comparison to breast control cell line (MCF10A); moreover, no induction of HSPB1 phosphorylation was observed upon Tg or Tm drug treatment or upon inhibition of IRE1 or PERK in TNBC. However, the exact role of phosphorylated HSPB1 in the various types of cancer is contradictory and not fully understood.

In addition to HSPA1 and HSPB1 role in TNBC HSR to stress, HSPB1, but not HSPA1, release from MDA-MB-231 and MDA-MB-453 TNBC into extracellular space was also determined. No HSPB1 release was detected in MCF-7 cells. HSPB1 and HSPA1 are well reported to be released into the extracellular space; however, the release mechanism is unknown, but Graner et al. 2007 did show that exosomes are shed into the media of brain tumour cell lines contain HSBP1 and HSPA1 (Graner et al., 2007). Extracellular release of HSPA1 by glial cells was as a defense mechanism supplying neurons by stress response proteins to protect them from injury (Guzhova et al., 2001). Moreover, in stress induced prostate cancer cells, HSPA1 are secreted by non-classical lysosomal pathway and take part in paracrine or autocrine interactions with adjacent cell surfaces, and bind to the cell surface prostate cancer cells (Mambula and Calderwood, 2006). Therefore, the role of HSPB1 secreted in TNBC cells was investigated to determine whether released HSPB1 has an autocrine effect and induces cell proliferation and/or resistance. Targeting released HSPB1 with anti-HSPB1 antibody, no impact was observed on cell count, cell viability or resistance to taxol or ceramide treatment. Moreover, no significant effect on cell migration was observed by scratch assay. Role of extracellular HSPB1 detected in the culture media of MDA-MB-231 should be investigated further at it might have immunological implications or angiogenesis induction as described in HMEC cultures (Thuringer et al., 2013).

HSPB1 and HSPA1 are both reported to be over expressed in BC and play a role in the response of cells to therapy and have been attributed with worse patient prognosis (Vargas-Roig et al., 1998, Ciocca and Calderwood, 2005, Ciocca et al., 2010). However, analyses of patient databases described above showed an association of high HSPA1 and HSPB1 mRNA expression with Luminal B BC patients, and significantly low HSPB1 expression was observed in basal-like BC patients. Moreover, high HSPB1 levels in Luminal A BC were associated with worse prognosis as determined by decreased probability of AE-free survival ($p < 0.01$). In the literature, the impact of HSPB1 expression on BC is mostly reported in HER-2 and ER positive subtypes (Awada et al., 2012, Lee et al., 2005).

Despite the fact that patient data reveal that high mRNA expression of HSPB1 or HSPA1 have no significant effect on adverse-event free survival of basal-like BC patients, the role of HSPB1 and HSPA1 in TNBC can be further investigated in response to various stressors, and further identify if there any significant impact of phosphorylated HSPB1 in TNBC on cell survival or resistance, since HSP interaction and/or function can impact other cellular pathways (Zhang et al., 2015, Cayado-Gutierrez et al., 2013). Moreover, the role and mechanism of HSPB1 release in TNBC is an interesting feature to be further investigated since it is observed in TNBC MDA-MB-231 and MDA-MB-453 but not in luminal BC subtype MCF-7, and not as a result of treatment or stress. In addition, high levels of HSPB1 were also found in sera of BC patients (Rui et al., 2003); therefore HSBP1 extracellular release could be a potential diagnostic marker of basal-like BC.

Chapter 6

General discussion

According to statistics, almost one in eight women would be diagnosed with BC, while less than 1% of breast carcinomas occur in men. BC is highly heterogeneous and varies based on origin, type and underlying causes. Therapeutic response to treatment regimens can also differ greatly, for example, according to Cancer Research UK, patients diagnosed with BC stage 0 or 1 have 100% 5-year survival rate, while patients presenting with BC stage IV have only 22% 5-year survival rate. BC stage is defined at first diagnosis and evaluated according to size of tumour, association with LN, invasiveness and metastatic potential, in addition to histological markers to determine its subtype (Duffy et al., 2005). Basal-like BC subtype usually presents with worse prognosis and shortest recurrence free survival compared to HER-2 and luminal subtypes (Makki, 2015). Moreover, the majority basal-like BC are triple-negative lacking the ER, PR and HER-2 proteins; therefore, these two terms are used interchangeably (Badve et al., 2011).

Genetic predisposition and hereditary factors are recognized as predisposing factors causing BC. They comprise mutations in BC genes (BRCA1 and BRCA2) that account for around 10% of BC incidence (Larsen et al., 2014). Non-hereditary BC, similar to other cancers, often carry mutations in main regulatory genes, and despite the heterogeneity, different cancers may share common features at the cellular and tissue levels, such as uncontrolled cell proliferation, angiogenesis, evasion of apoptosis and metastasis (Makki, 2015). Such observed mechanisms are often due to up-regulation or down-regulation of critical genes and pathways, leading to disruption of cellular homeostasis and resistance to therapy (Hanahan and Weinberg, 2011). Current conventional treatments for BCs include mastectomy, radiation, cytotoxic or genotoxic drugs and combination therapies in addition to adjuvants such as tamoxifen, specific to ER positive tumours, and herceptin a HER-2 antagonist (Lord et al., 2004). However, basal-like or TNBC are resistant to hormonal targeted treatments since they lack surface receptors and markers (Bayraktar and Gluck, 2013). Current, research is directed towards developing personalized treatment approach which is based on tumour profiling allowing the identification of patient specific markers and factors that can be targeted (Alers, 2016). This approach has lead to development of diagnostic methods that can identify activity of certain genes in early-stage BC, and can help in treatment decisions based on the cancer's risk of recurrence within 10 years after diagnosis, such as the MamaPrint, Mammostrat and Oncotype DX (Drukker et al., 2013). These tests are potential diagnostic methods and most are developed for hormone receptor positive subtypes, therefore more research is needed to validate their efficacy and identify new diagnostic targets (Drukker et al., 2013). The main aim of this thesis is to identify specific pro-survival pathways and genes that contribute to the development of basal-like or TNBC, phenotype and resistance to conventional treatment regimens.

In order to identify specific markers that might enhance basal-like BC aggressive profile, BC patient databases were analyzed. This analysis was based on genetic approach comparing gene mutation and mRNA expression of genes implicated in pro-survival pathways in different patient BC subtypes, and the correlation of these genes to tumour grade, size and LN involvement, in addition to its impact on adverse-event free survival. Secondly, being able to identify the NF-κB and the HSRas possible pathways up-regulated in basal-like BC, MDA-MB-231 cell line was used as a model to understand their relative pro-survival mechanism.

Successive mutations of oncogenes and tumour suppressor genes are common acknowledged causes of cancer (Budillon, 1995). For example, mutations that inactivate Rb and p53 reduce death of BC cells in mice, and further inactivation of BRCA1 would exert an additive effect on tumour growth and predisposes breast epithelium to metastatic adenocarcinomas (Kumar et al., 2012). Tumours caused by such mutations lack proper differentiation and share basal-like and claudin-low TNBC BC features (Yadav et al., 2015). Therefore, identifying mutations and genetic variations common to basal-like or TNBC subtype might improve the understanding of this BC complexity. Unfortunately, the databases used did not give enough information about mutation of genes known to be responsible for progression of cancer such as *BRCA*, *Ras* oncogene, *PTEN, BRAF* or *RB*, while *TP53* tumour suppressor gene mutation status was identified in some databases. Almost 50% of cancers display mutations in *TP53,* and it is also observed in up to 30% of BC (Borresen-Dale, 2003). In patient samples analyzed, *TP53* positive mutational status was associated with basal-like BC. In addition, looking at BC cell lines mutation information, 17 out of 18 basal-like or TNBC panels developed by ATCC have mutated *TP53*, while no other gene mutation was common within the same panel (Lehmann et al., 2011). Hence, *TP53* mutational status might be responsible for basal-like BC tumourigenesis.

While genetic factors act as main drivers of cancer initiation and development remain a main theory in the field of cancer research, the “Warburg’s theory” postulates that insufficient cellular respiration caused by mitochondrial damage might be the driver of tumourigenesis, since cells would switch to non-aerobic respiration shutting down the mitochondria (Warburg et al., 1965). This probably explains in what way cells escape mitochondria mediated cell death (Elmore, 2007). Cells survival and tumourigenesis are maintained by different mechanisms particularly apoptosis evasion and proliferation (Hanahan and Weinberg, 2011). Therefore, BC patient databases were further analyzed to investigate gene expression of main survival pathways that are known to enhance cell proliferation by activating pathways that drive changes in cellular metabolism or escape cell cycle checkpoints, or down-regulate apoptosis by inhibiting cell death effectors (Hanahan

and Weinberg, 2011). The pathways investigated included the PI3K-Akt, mTOR, JAK/STAT, HSR, UPR and NF-κB. Expression of main genes involved in these pathways was compared between the BC subtypes; in addition, their impact on patient prognosis defined by tumour size, grade and LN involvement in addition to patient survival was analyzed.

Among the pro-survival pathways analyzed, the NF-κB and to a lesser extent the HSR pathway related genes were observed to be associated with the basal-like BC phenotype, while the AKT and mTOR pathway related genes expression were more associated with the HER-2 BC subtype, displaying worse prognosis. The PI3/AKT pathway enhances anti-apoptotic signals and is activated by insulin like growth factor and is responsible for aromatase resistance in ER+ BC, and to endocrine therapy, HER-2-directed therapy and cytotoxic therapy in BC (Ma et al., 2015, Nahta and O'Regan, 2010). However, Moestue et al suggest that PI3K inhibitors might be an effective therapy in basal-like BC since they demonstrate higher PI3K activity as shown by high pAKT levels (Moestue et al., 2013). Moreover, mutations in *PIK3CA*, *PTEN* loss and high pAkt levels are often detected in basal-like BC. Several studies have failed to identify a correlation between PIK3CA mutations and response to PI3K inhibition; however, HER-2 BCs may actually benefit more from drugs that inhibit the PI3K/AKT molecular pathway (Moestue et al., 2013). Similarly, no effect of mRNA expression of JAK/STAT pathway genes was determined in basal-like BC data. Clinical trials of selective JAK1/2 inhibitors in BC patients showed that JAK inhibition is not able to conquer basal-like BC progression (Quintas-Cardama and Verstovsek, 2013, Barbie et al., 2014).

On the other hand, enhanced NF-κB activity is reported to be associated with basal-like BC pathogenesis (Barbie et al., 2014, Yadav et al., 2015, Khan et al., 2013). In this study, patient database analyses showed a significant association of NF-κB related genes mRNA expression with basal-like BC, and a negative impact on prognosis and adverse-event free survival. In addition, NF-κB showed a significant positive correlation with *MKI-67* expression, which suggests that NF-κB enhances the proliferation rate in basal-like BC. Besides, positive correlation between mRNA expression of NF-κB genes and *TP53* was obtained. This positive correlation might be due to mutated *TP53* expression, since mutated p53 is stable and has been reported to accumulate in cells (Alsner et al., 2008). In addition to their distinct roles in cancer, NF-κB and p53 repress each other's activities, since p53 is a tumour suppressor while NF-κB is a tumour activator (Webster and Perkins, 1999). So, combined treatment against both pathways would be promising in BC. In fact, positive cooperation between p53 and NF-κB has been recently reported in the context of the responses of an epithelial cancer cell to standard chemotherapy, and in the presence of active signaling by a pleiotropic inflammatory cytokine, such as TNFα (Bisio et al., 2014).

Additionally, in macrophages activated p53 and NF-κB co-regulate several pro-inflammatory cytokines and chemokines, gene expression (Lowe et al., 2014). Therefore, p53 and NF-κB crosstalk could have a significant role in intensifying basal-like BC associated immunity.

For additional understanding of NF-κB mechanism and identify its new activators in basal-like BC, RIP2 function was investigated. RIP2 is required for NOD signaling, NF-κB induction and is a main mediator of inflammatory and immune response (Hasegawa et al., 2008). Besides, it is involved in regulation of cellular proliferation, differentiation and death (McCarthy et al., 1998, Adams et al., 2010, Munz et al., 2002, Khursigara et al., 2001). Compared to other BC subtypes, basal-like BC samples express higher RIP2 mRNA, which is associated with weak prognosis and reduced AE-free survival. High RIP2 mRNA expression was also positively correlated with increased *TP53* and *MKI-67* mRNA expression. While RIP2 is associated with p53 expression in basal-like BC, and is known to be an activator of NF-κB, it would be interesting to investigate whether it is involved in p53 and NF-κB crosstalk discussed earlier.

Additionally, the role and mechanism of action of RIP2 in basal-like subtype was explored *in vitro*. In MDA-MB-231 cell line model, RIP2 overexpression increased cell number and survival, while RIP2 knockdown and CARD-only RIP2 overexpression, significantly decreased survival of MDA-MB-231 cells in comparison to plasmid controls. Also, NF-κB activity was up to 1.8 fold higher in RIP2 overexpressing cells; however, it was significantly reduced with CARD-only RIP2 and RIP2 knockdown. Moreover, RIP2-K209R mutant construct having a non-ubiquitinated site, showed a decreased survival and NF-κB activity relative to RIP2 wild-type, which indicates an important role of ubiquitination in RIP2 function. RIP2 gets K63-polyubiquitinated on lysine (K) 209 and is fundamental for NOD dependent NF-κB induction (Hasegawa et al., 2008). BC cell-lines having basal-like characteristics show higher endogenous RIP2 expression compared to luminal and HER-2 subtypes. In Luminal and HER-2 BC cell lines (MCF-7, T47D and SKBR3), overexpression and knockdown of RIP2 induced cell death; therefore, RIP2 may need to be maintained at certain levels in these cells to control cell survival. Supporting this hypothesis, RIP2 was shown to be a key modulator of myoblast proliferation and differentiation and its down-regulation is crucial for myogenic differentiation (Munz et al., 2002). In rhabdomyosarcoma cells, overexpressing RIP2 highly reduced differentiation, and is responsible for deficient or incomplete differentiation (Ehlers et al., 2008, Munz et al., 2002). Therefore, RIP2 displays an important role in regulating proliferation and differentiation of cells and probably demonstrate a similar role in mammary tissue.

RIP2 has an auto-phosphorylation activity essential for its stability (Nembrini et al., 2009). RIP2 kinase activity was inhibited using PP2, what caused a decrease in RIP2 expression and consequent reduction in cell viability. Yet, RIP2 is not the only PP2 target, since it also inhibits the family of Src kinases activity. Recently, WEHI-345 was developed and confirmed to be a specific inhibitor of RIP2 kinase function. WEHI-345 is an ATP analogue and acts through binding to RIP2-ATP binding site with high specificity delaying and RIP2 ubiquitylation and subsequent NF-κB induction (Nachbur et al., 2015). Using this compound in TNBC cultures would help further confirm the role of RIP2 kinase activity and further understand its mechanism of action. Moreover, RIP2 auto-phosphorylation site was identified as Serine-176 and mutating it to alanine, prevents auto-phosphorylation and highly reduces its kinase function (Dorsch et al., 2006). Therefore, it would be important to specifically target the S176-RIP2 to inhibit autophosphorylation and its impact on RIP2 kinase activity, and monitor the activation state of RIP2 in basal-like BC patient samples via immunofluorescence targeting S176 to better understand and verify its role. This can be achieved by using anti-phospho-RIP2 antibodies, which are available commercially from Cell Signall (CST) and Thermofisher, and described as suitable for immunostaining, however, no publications confirming their validity are available yet.

Furthermore, RIP2 enhancement of BC resistance to drug induced cell death was studied. MDA-MB-231 cells overexpressing RIP2 has increased cell survival against ceramide and taxol, and decreased viability in response to these drugs was observed with RIP2 mutants or knockdown. In MDA-MB-231 cells RIP2, through induction of NF-κB, increased the expression of anti-apoptotic proteins including Bcl-2, Bcl-xL and IAPs, which might be the mechanism by which these cells evade apoptotic cell death. Ceramide is important in regulating Bcl-x alternative splicing, hence decreasing Bcl-xL levels and mediating Bak, Bax and Bcl-2 function in apoptosis and alters Bcl-xL expression levels in colon and BC *in vitro* models (Chalfant et al., 2002, Paschall et al., 2014). It also targets IAPs in colon and BC metastasis (Paschall et al., 2014). Moreover, NF-κB decreases response to chemotherapy by preventing apoptosis in solid cancers, and is associated with mammary carcinogenesis by regulating of Bcl-x_L, cFLIP and cIAP (Antoon et al., 2014). Therefore, in basal-like BC, RIP2 shows a significant role in mediating resistance to drug induced cell death via enhancing the expression of anti-apoptotic proteins. *In-vitro* data were further confirmed by patient data analyses, where high RIP2 expression was correlated with anti-apoptotic proteins expression. This is the initial research project describing RIP2 role in NF-κB activation leading to basal-like BC drug resistance.

In addition to genomic factors and pro-survival genes up-regulation, the tumour microenvironment enhances neoplasm growth and has an essential impact on

tumourigenesis (Natrajan et al., 2016, Jiang and Shapiro, 2014, Hanahan and Weinberg, 2011, Fritz and Gommerman, 2011). NF-κB has a contradictory effect on cancer. It is a part of the immune defense mechanism by which transformed cells are targeted and eliminated, and complemented by the activity of cytotoxic T-cells against cancer (Del Prete et al., 2011). However, it can also enhance tumourigenesis and survival through modulating factors released to the tumour microenvironment, a process by which cancer cells could evade immunity (Khan et al., 2013, Rayet and Gelinas, 1999). As shown here, RIP2 has a potential role in modulating microenvironmental factors. MDA-MB-231 cells overexpressing RIP2 have induced cytokines and growth factors release into the culture media. Specifically, in supernatant collected from cells overexpressing RIP2, a significant increase in IL-8, GROα, GM-CSF and VEGF concentration was detected. All of the factors detected in the media are NF-κB transcription targets, what validates that RIP2 role is mediated through enhancing NF-κB. RIP2 modification of the tumour microenvironment has been likewise described. Andersson *et al* reported that RIP2 alters induction of VEGF pathway and elasticity of vessels in myocardial ischaemia (Andersson et al., 2015). Interestingly, Adams et al reported that RIP2 expression is induced upon wound initiation in epithelial cells and after the stimulation of cell differentiation (Adams et al., 2010). Besides, they showed that RIP2 can be induced by growth factors and cytokines, and p38 MAPK dependent TNF-alpha-dependent induction (Adams et al., 2010). Moreover, it was demonstrated that inflammatory cytokines such as TNF-alpha, IL-1 beta, and IFN-gamma activated RIP2 transcription and translation in the endothelium. This induction was inhibited upon inhibiting NF-κB signaling indicating that NF-κB signaling was involved in cytokine-induced transcriptional activation of RIP2 gene expression (Yin et al., 2010). Coherent with published research, this study showed a decrease in RIP2 expression once NF-κB activity was inhibited. This data supports a feed-forward mechanism involving NF-κB induction of RIP2 transcription and a subsequent increase in RIP2 protein levels, which further induces NF-κB. Therefore, investigating the induction of RIP2 expression via cytokines and growth factors present in the tumour microenvironment might explain the higher expression of RIP2 in TNBC and basal-like BC patients, and suggest an autocrine activation loop responsible for increased proliferation, resistance and advanced BC phenotype. Additionally, identifying RIP2 as a distinctive marker of basal-like or TNBC is promising target for diagnostic and therapeutic options.

Finally, the heat shock and the unfolded protein pathways were also described to enhance tumourigenesis and studied here (Ciocca and Calderwood, 2005, Jager et al., 2012). Database analysis show that in basal-like BC increased HSPB1 and HSF1 mRNA expression and low XBP1 expression are responsible for worse prognosis and decreased

AE-free survival. Moreover, HSF1, HSPA1A and HSPB1 expression showed a positive correlation with *MKI-67* mRNA expression in basal-like BC only, and HSF1 and HSPA1A showed a significant increase in percentage of patients with tumour grade 3, which indicates a potential impact of these genes on cells proliferation. HSPB1 overexpression in tumours was previously described (Shin et al., 2005). As shown here, inhibiting HSPB1 expression using siRNA induced death of MDA-MB-231 cells; however, the expression of HSPA1 was likewise decreased. It was previously shown that upon stress, HSPB1 act synergistically with the stress-induced HSPA1 in order to maintain cellular tolerance, saving cells from apoptosis (Huang et al., 2007). Therefore, investigating the link between HSPB1 and HSPA1, and their impact on cell death under ER stress and hypoxia conditions might help further understand the role of HSR in BC and its interaction, if any, with the UPR pathway.

HSPB1 becomes phosphorylated as a response to ER stress, and hence exerts protection against cell death (Ito et al., 2005). However, in this study, pHSPB1 level was not induced upon thapsigargin and tunicamycin drug treatments in TNBC. Besides, BC cell-lines studied have high HSPB1 but low pHSPB1 levels. However, Kaigorodova et al reported that pHSPB1 in biopsies from BC patients showing LN metastasis, are significantly higher in the nucleus and cytoplasm of cells in comparison to biopsies with no LN metastasis (Kaigorodova et al., 2015). Therefore, HSPB1 phosphorylation induction and role could be further investigated in BC cell lines to understand physiological function of pHSPB1.

Through mRNA profiling, this study was able to identify high RIP2 expression as a novel marker and potential therapeutic target in TNBC. Moreover it proposes a feed-forward mechanism of NF-κB activation via RIP2, which leads to increased transcription of anti-apoptotic proteins and cytokines release in TNBC. However, gene expression analysis may not always provide sufficient information about cancer associated factors, since the driving forces for cancer initiation, progression and metastasis may be affected by other factors such as epigenetics, miRNAs and the microenvironment (Hanahan and Weinberg, 2011). Lately, miRNAs have been recognized as basic molecular components important for tumour growth, invasion and angiogenesis (Hayes et al., 2014). Furthermore, some proteins might have higher activity or regulatory role in cancer development and not be over-expressed.

In-vitro cell culture was another approach to explain RIP2 mediated NF-κB tumourigenesis. However, it is important to point out that cancer is the result of complex interactions between cells and their microenvironment. Therefore, it is important to further verify the RIP2 activation and downstream signaling described here, in other cancer types and *in-vivo* model. Additionally, the effectiveness of the new RIP2 inhibitor, WEHI-345, in the inhibition of basal-like or TNBC *in-vitro* or *in-vivo* xenografts can be investigated. Only with

comprehensive approach, which combines genetic profiling and well-established *in-vivo* and *in-vitro* experiments, it is possible to find and identify potential and effective therapeutic targets in cancer.

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