The functional role of PPP2R2A in luminal breast cancer

Abdul Mannan

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STATEMENT OF ORIGINALITY

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TABLE OF CONTENTS

Contents

ABSTRACT

Breast cancer is a leading cause of morbidity and mortality worldwide, and despite advancements made in early diagnosis and new treatments over the past few decades, disease progression and therapy resistance remains an unmet challenge. A better understanding of the mechanisms involved in disease progression and resistance to therapies is essential in order to develop improved treatment strategies.

Reversible phosphorylation is controlled by the balanced activities of protein kinases and phosphatases, and is a central regulator of the signal transduction pathways required for cell proliferation, differentiation and survival. As such, dysregulation of this balance can result in loss of cellular differentiation and sustained proliferation and survival – key characteristics of cell transformation. While the role of protein kinases in tumourigenesis has been extensively studied, the role protein phosphatases is less well understood.

Protein phosphatase 2A (PP2A), is a family of serine/threonine phosphatases that is inactivated in many cancers, including breast cancer, and as such is considered a tumour suppressor. PP2A controls over 50% of serine/threonine phosphatase activities in cells, and regulates numerous growth and survival signalling pathways including the PI3K-AKT and MAPK pathways. PP2A is a trimeric protein complex consisting of a structural subunit (PP2A-A), a catalytic subunit (PP2Ac) and a regulatory subunit (PP2A-B), of which there are at least 4 families each with multiple isoforms. While the dimeric complex of PP2A-AC can dephosphorylate multiple proteins, it is the PP2A-B regulatory subunit that provides substrate specificity and subcellular localization of PP2A. Recent large scale genomic analyses have identified recurrent loss of heterozygosity (LOH) at the *PPP2R2A* gene locus, which encodes the PP2A-B55α regulatory subunit. *PPP2R2A* LOH was most common in estrogen receptor positive (ER^+) luminal breast tumours, and in particular in the aggressive Luminal B subtype. However, the functional role of *PPP2R2A* loss in breast cancer is not known. Therefore, the overall goal of this thesis was to characterise the gene and protein expression of PP2A subunits, including *PPP2R2A* (PP2A-B55α), in human breast tumours, and to identify the specific functional role of reduced PP2A-B55α in luminal breast cancer.

The first aim of this thesis was to use *in silico* analysis of publically available gene expression databases, and immunohistochemistry (IHC) of human breast tumours, to compare the gene and protein expression of PP2A subunits and associating proteins between normal breast

tissues and breast tumours, and to examine the association of PP2A subunit expression with breast tumour subtypes and disease outcome. This analysis, presented in chapter 3, revealed that low gene and protein expression of the PP2A-B55α and PP2Ac subunits was associated with high grade tumours, and with Luminal B, Human epidermal growth factor positive ($HER2^+$), and triple negative breast (ER^-, progesterone receptor $(PR)^-$, $HER2^-$; TNBC) molecular subtypes, which are more aggressive than the Luminal A breast tumour subtype. Thus, low PP2A-B55α and PP2Ac protein expression is associated with aggressive breast tumours, and this is likely regulated at the gene expression level. Low *PPP2R2A* (PP2A-B55α) was further shown to predict for poor relapse-free and overall survival in breast cancer patients, most notably for patients with luminal (ER^+) tumours. Reduced gene expression of the PP2A regulators SETBP1 and alpha-4, was also associated with aggressive subtypes and worse outcome, suggesting that they may function as tumour suppressors. In contrast, high expression of a number of PP2A-B56 family subunits, and the PP2A inhibitors SET and CIP2A, were associated with more aggressive breast tumours, and thus may play an oncogenic role and be targets for breast cancer therapy.

To determine if the reduced *PPP2R2A* expression observed in aggressive poor outcome tumours, is functionally important, I next investigated the effects of molecular inhibition of *PPP2R2A* in the human luminal breast cancer cell lines, ZR751, MCF7 and BT474. The data in chapter 4 shows that short-hairpin RNA (shRNA) mediated inhibition of *PPP2R2A*, resulting in reduced PP2A-B55α protein expression, increased breast cancer cell proliferation, migration and invasion, and increased tumour growth in an orthotopic xenograft mouse model. This was associated with increased activation of ER and AKT signalling, and evidence of an epithelial-to-mesenchymal transition (EMT) phenotype. These results suggest that functional inactivation of PP2A-B55α complexes is important for breast cancer progression, and supports a tumour suppressive role for *PPP2R2A*.

The introduction of anti-estrogen therapies, such as Tamoxifen, to ER^+ breast cancer patients has led to remarkable improvements in survival. However, many patients are either intrinsically resistant to therapy, or develop resistance and later relapse with therapy-resistant disease. Given that low *PPP2R2A* expression was associated with poor outcome in ER^+ patients, in chapter 5 I explored whether inhibition of PP2A-B55α mediated tamoxifen resistance. Analysis of publically available datasets showed that the loss of PPP2R2A was a strong predictor of earlier relapse and distant metastasis in tamoxifen treated breast cancer patients. Furthermore,

molecular knockdown of PP2A-B55α induced resistance to tamoxifen in ER^+ breast cancer cells. In contrast, ER^+ breast cancer cells selected for resistance to tamoxifen, expressed reduced PP2A-B55α compared to parental drug sensitive cells, demonstrating a functional role for PP2A-B55α in ER signalling and therapy resistance. In addition, PP2A-B55α knockdown in the $HER2^+$ breast cancer cell line, BT474, induced resistance to the anti-HER2 therapeutics, Trastuzumab and Lapatinib. Therefore, the poor outcome observed in patients with low *PPP2R2A* expression may be mediated by intrinsic resistance to standard therapies.

Importantly however, breast cancer cells with low PP2A-B55α were highly sensitive to pharmacological activators of PP2A. Clonogenic and cytotoxicity assays showed that PP2A-B55α knockdown cells were just as sensitive, and in some cases were more sensitive, than control cells, to sphingolipid PP2A agonists (FTY720 and derivatives) and small molecule activators of PP2A (SMAPs). This suggests that PP2A activities can still be enhanced in tumours with low PPP2R2A, and thus is a potential therapeutic strategy for poor outcome breast cancer patients.

In chapter 6 I further showed that treating breast cancer cells with PP2A activating drugs can increase the sensitivity of breast cancer cells to targeted therapies, including tamoxifen, Lapatinib and the CDK4/6 inhibitor, Palbociclib. Importantly, the addition of a PP2A activating drug sensitized tamoxifen-resistant breast cancer cells to tamoxifen, providing a strong rationale to combine PP2A activating drugs with standard therapies for the treatment of therapy sensitive and resistant breast tumours. Finally, given that sphingolipid PP2A activators and SMAPs are distinct classes of drugs with different mechanisms of PP2A activation, I examined the effects of combining these two classes of drugs. Intriguingly, I found that the combination displayed highly synergistic cytotoxicity in breast cancer cells with or without PP2A-B55α knockdown.

Collectively, the body of work presented in this thesis enhances our understanding of the function of PP2A-B55α in breast cancer signalling and therapy resistance, and suggests that PP2A-B55α expression may be a useful biomarker for predicting disease outcome in luminal breast cancer. Furthermore, these data support the clinical testing of PP2A activating drugs alone and/or in combination, in relapsed/resistant ER^+ breast cancer patients, with the ultimate goal of improving the survival of breast cancer patients..

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ABBREVIATIONS

53BP1	p53 binding protein 1
CIP2A	Cancerous inhibitor of PP2A
3D	Three dimensional
4OH-tam	4 hydroxytamoxifen
5-FU	Fluorouracil
ADH	Atypical ductal hyperplasia
AI	Aromatase inhibitor
ATM	Ataxia telangiectasia mutated
B55α	PP2A regulatory subunit B55 isoform α
BME	Basement membrane extract
BRCA	Breast cancer associated
BSA	Bovine serum albumin
CHK	Checkpoint kinase
CI	Combination index
CtIP	C-terminal Binding Protein Interacting Protein
ERK	Extracellular signal-regulated kinase
DCIS	Ductal carcinoma in situ
DMEM	Dulbecco's modified Eagle's medium
DMFS	Distant metastasis free survival
DMSO	Dimethyl sulphoxide
DNA-PKcs	DNA-dependent protein kinase
DSB	Double strand DNA break
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
ER	Estrogen receptor
ERE	Estrogen response element
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate

g	gram
g	gravity
GOBO	Gene expression-based Outcome for Breast cancer Online
GSK-3β	Glycogen synthase kinase-3β
GWL	Greatwall kinase
HEAT (repeat)	Huntington-elongation-PP2A-A subunit-TOR
HER2	Human epidermal growth factor receptor
HR	Homologous recombination
HR	Hazard ratio
HRP	Horse radish peroxidase
ID50	concentration of drug that inhibits cell viability by 50%
JAK	Janus kinases
kDa	Kilodalton
KSR1	Kinase suppressor of Ras
LCMT-1	Leucine Carboxyl Methyltransferase
LOH	Loss of heterozygosity
Luc	Luciferase
MAPK	Mitogen activated protein kinase
Mdm-2	Mdouble minute homologue 2
MEFs	Mouse embryonic fibroblasts
mg	Milligram
ml	Millilitre
mM	Millimolar
mRNA	messenger RNA
mTOR	Mammalian target of rapamycin
NHEJ	Non-homologous end-joining
nM	Nanomolar
OS	Overall survival
PARP	Poly ADP ribose polymerase
PBS	phosphate buffered saline
PDK1	3-phosphoinositide-dependent protein kinase 1

PH	Plecstrin homology (domain)
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol (4,5)P2
PIP3	Phosphatidylinositol (3,4,5) phosphate
PKA	Protein kinase A
PME-1	Phosphatase methylesterase (specific for PP2A)
PP2A	Protein Phosphatase 2A
PP2A-A	Structural subunit of PP2A
PP2A-C	Catalytic subunit of PP2A
PPP2R2A	PP2A B55α regulatory subunit
PTEN	Phosphatase and tensin homologue
Rb	Retinoblastoma
RFS	Relapse free survival
RIPA	Radio-immunoprecipitation assay
RPMI	Roswell park memorial institute media
RTK	Receptor tyrosine kinase
SBDS	Shwachman-bodian-diamond syndrome
SDS	sodium dodecyl sulphate
SEM	Standard error of the mean
Ser	Serine
SERM	Selective estrogen receptor modulator
shB55α	Short hairpin targeting mRNA encoding for PP2A-B55α
shCont	short hairpin control
shRNA	Short hairpin RNA
SMAP	Small molecule activator of PP2A
STAT	Signal transducer and activator of transcription
TamR	Tamoxifen resistant
TBST	Tris buffered saline with 0.1% Tween 20
TCGA	The cancer genome atlas
Thr	Threonine

TNBC	Triple negative breast cancer
Tyr	Tyrosine
Wnt	wingless
WT	Wild-type
µg	Microgram
µl	Microlitre
µM	Micromolar