The regulation, function and expression of Δ40p53 in breast cancer

Brianna Catherine Morten
BBiomedSci(Hons) ***(Newcastle)***

Doctor of Philosophy (Medical Genetics)
University of Newcastle
Submitted for examination August 2016

Declarations

Statement of Originality:

*This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository**, subject to the provisions of the Copyright Act 1968.*

***Unless an Embargo has been approved for a determined period.*

24/08/16

……. ……

Date **Brianna Morten**

Statement of Collaboration:

I hereby certify that the work embodied in this thesis has been done in collaboration with other researchers. I have included as part of the thesis a statement clearly outlining the extent of collaboration, with whom and under what auspices.

Statement of Authorship:

I hereby certify that the work embodied in this thesis contains a published papers/scholarly work of which I am a joint author. I have included as part of the thesis a written statement, endorsed by my supervisor, attesting to my contribution to the joint publications/scholarly work.

Thesis by Publication:

I hereby certify that this thesis is in the form of a series of published papers of which I am a joint author. I have included as part of the thesis a written statement from each co- author, endorsed by the Faculty Assistant Dean (Research Training), attesting to contribution to the joint publications.

24/08/16

…… ……

Date **Brianna Morten**

Acknowledgements:

This thesis would not have been completed without the help and support of a number of people. I would like to thank all the many people who made this possible, and let them know I am forever grateful for this opportunity.

Firstly, I would sincerely like to thank my supervisors, Professor Rodney Scott and Dr Kelly Avery-Kiejda, for giving me the opportunity to complete my PhD under their supervision. Rodney, thank you for providing a wonderful laboratory and guidance throughout my candidature. Kelly, thank you for being a great mentor, friend and supervisor who always provided me with all the help I required, but also gave me the space I needed to mature into an independent researcher. I really do appreciate all the red pen feedback from my writing, and the time you put in to helping me succeed, especially during those times on maternity leave.

To all the team at CIBM, past and present, thank you all for your friendship, and help in so many ways throughout my many years here. You all made it an enjoyable workplace to come to each and every day. In particular, I would like to thank Tiff, Andrea, Katherine, Bente, Kelly and Summer for the many tea breaks, cake days, lunches and other times that we shared together throughout my candidature. I am certain I would not have made it this far without your amazing support, your kind words and advice. I feel very honoured to have made so many lifelong friends during my time here.

To my family, Mum, Dad and Matt, I am forever thankful for having the most supportive, loving and caring family who always support me in whatever journey in life I choose to follow. Mum and Dad, it is because of you that I have the determination, the persistence and the ability to chase my dreams. You have always been there for me, you have listened patiently to all my problems, providing me with endless amounts of support, guidance, and love which I could always rely on, so I knew I was never on this journey alone. I cannot thank you enough for all you have done for me. Matt, you are the big brother that I have always looked up to, perhaps the reason I ended up in medical research, and you are one of my biggest supporters as well. Spending these years here at HMRI, completing our PhD's together, are memories that I will cherish for the rest of my life.

To my partner, Ben, thank you for coming into my life when I needed you most. You have been my rock throughout this crazy, rollercoaster of a journey that is a PhD. Without your support, and shoulder to cry on occasionally, I would not have made it to the end. Thank you for your

love, your understanding and for always knowing how to make me smile. To my friends, thanks for putting up with my stress and for always giving me an outlet to get away from the PhD and for helping to keep the balance in my life.

Finally, I would like to thank the University of Newcastle for providing me with a fantastic opportunity to study a PhD and providing me with my APA scholarship. I would like to acknowledge the Hunter Medical Research Institute, for allowing me to perform my research in state of the art facilities, and in a welcoming and friendly environment. I would also like to acknowledge the Sawyer family and thank them for their ongoing support throughout my candidature. To be a MM Sawyer scholarship holder was an honour, and I thank them wholeheartedly for giving me a wonderful opportunity to contribute to the field of medical research.

This thesis is dedicated to the most loving, caring and supportive team of people who pushed me to chase my dreams and believe that I could succeed. Mum, Dad, Matt and Ben, without you I could not have accomplished this milestone.

Thank you.

List of publications included as part of this thesis:

1. **Morten, B.C**, Wong-Brown, M.W, Scott, R.J and Avery-Kiejda, K.A, 'The presence of the intron 3 16bp duplication polymorphism of p53 (rs17878362) in breast cancer is associated with a low Δ40p53:p53 ratio and better outcome.' *Carcinogenesis*. 2016 Jan;37(1):81-6. doi: 10.1093/carcin/bgv164. Epub 2015 Nov 19.

2. **Morten, B.C**, Scott, R.J and Avery-Kiejda, K.A, 'Comparison of three different methods for determining cell proliferation in breast cancer cell lines.' J. Vis. Exp. (In press), e54350, doi:10.3791/54350 (2016).

3. **Morten, B.C**, Scott, R.J and Avery-Kiejda, K.A, 'Comparison of the QuantiGene 2.0 Assay and real-time RT-PCR in the detection of p53 isoform mRNA expression in formalin-fixed paraffin-embedded tissues.' *Submitted to PloS One (August 2016).*

Contributions by others to the thesis:

There were a number of collaborators who have made important contributions to the unpublished work presented in this thesis as follows-

- Dr Hamish Campbell and Professor Antony Braithwaite generated the MCF-7-LeGO and MCF-7-Δ40p53 cell lines used in Chapter 3 and Chapter 4.
- Dr Trisha Al-Mazi provided technical support for the MRM-MS studies in Chapter 4, and aided in the analysis of the data generated.

List of additional publications:

Publications:

1. Avery-Kiejda, K.A, **Morten, B**, Wong-Brown, M.W, Mathe, A, and Scott, R.J, 'The relative mRNA expression of p53 isoforms in breast cancer is associated with clinical features and outcome.' *Carcinogenesis*, 2014; 35 (3):586-596.

3. Mathe, A, Wong-Brown, M., **Morten, B.**, Forbes, J.F., Braye, S.G., Avery-Kiejda, K.A., and Scott, R.J., 'Novel genes associated with lymph node metastasis in triple negative breast cancer.' *Scientific Reports*, **5**, 15832 (2015).

List of oral conference presentations:

1. **Morten, B,** Scott, R.J and Avery-Kiejda, K.A, The p53 isoforms in breast cancer. *Confirmation presentation at HMRI*, 6th May 2013, Newcastle, NSW, Australia.

2. **Morten, B**, Scott, R.J, and Avery-Kiejda, K.A, Δ40p53 can alter breast cancer cell growth by mediating the estrogen response. *Hunter Cancer Research Alliance Annual Symposium,* 21st November 2014, Newcastle, NSW, Australia. Abstract published in: *Asia-Pac J Clin Oncol* 2014;10 (Suppl. 10): 1–7.

3. **Morten, B**, Campbell, H, Braithwaite, A, Scott, R and Avery-Kiejda, K, Δ40p53 can modulate ERα expression in breast cancer cell lines, *HEAPs Seminar Series*, 29th April 2015, Newcastle, NSW, Australia.

4. **Morten, B**, Campbell, H, Braithwaite, A, Scott, R and Avery-Kiejda, K, The Δ40p53 isoform in breast cancer- friend or foe? *The 2nd International p53 Isoforms Workshop*, 20-23 September 2015, Aix-en-Provence, France.

5. **Morten, B**, Scott, R and Avery-Kiejda, K, Identifying new targets for the treatment and prevention of breast cancer, *Lake Macquarie U3A Meeting*, 28th October 2015, Toronto, NSW, Australia.

6. **Morten, B,** Wong-Brown, M, Mathe, A, Scott, R and Avery-Kiejda, K, The expression of p53 isoforms in breast cancer, *Sungkyunkwan University and University of Newcastle Joint Symposium*, 26th November 2015, Newcastle, NSW, Australia.

Table of contents:

Abstract:

Breast cancer is the most common malignancy in women and it is the second highest cause of cancer-related death. The tumour suppressor gene *p53* is crucial for the prevention of cancer through its role in the maintenance of cellular growth and differentiation. Inactivation of *p53* through mutations is the most common event in cancer. However, in breast cancer, *p53* is mutated in only ~24% of cases and this suggests that other mechanisms are responsible for the loss of p53 function. Ascertaining the mechanisms responsible for its inactivation may lead to the identification of novel treatment targets or prognostic tools. The p53 isoforms were discovered over a decade ago and they may represent one mechanism that regulates p53 functionality. Currently, over 14 p53 isoforms have been discovered and their expression is deregulated in many human cancers. One such isoform, Δ40p53, has been found to inhibit p53, or support its tumour suppressive functions, depending on its ratio to full-length p53 (FLp53). Previous studies from our laboratory have shown that Δ40p53 is the most highly expressed p53 isoform in breast cancer, but its function and the clinical implications of its expression are yet to be elucidated. Furthermore, current methods of detecting Δ40p53 at the mRNA and protein level lack the sensitivity and specificity to analyse the endogenous expression of this isoform in clinical specimens. The aims described in this thesis were to elucidate the regulation and function of this isoform in breast cancer. Further studies aimed to design and evaluate novel methods for the detection of Δ40p53 mRNA and protein expression to aid in the clinical implementation of Δ40p53 as a breast cancer biomarker.

Δ40p53 can be produced by alternative translation or alternative splicing, but our previous studies have indicated that alternative splicing is likely to be a major route of Δ40p53 production in breast cancer. G-quadruplex structures within intron 3 of p53 are important for the production of FLp53 mRNA and disruption of these structures by a polymorphism in intron 3 of *p53* (PIN3) has been shown to lead to increased levels of Δ40p53 mRNA *in vitro*. The first part of this thesis reports on whether PIN3 was associated with the expression level of Δ40p53 in breast tumour tissues. PIN3 was shown to be associated with a low Δ40p53:FLp53 ratio, and that this was correlated with improved disease-free survival. This suggests that the Δ40p53:FLp53 ratio is modified by PIN3 in breast cancer and that the Δ40p53:FLp53 ratio and PIN3 may be potential prognostic indicators for breast cancer outcome.

Following this, the function of Δ40p53 was investigated in estrogen receptor (ER)-positive breast cancer. p53 can interact with and regulate the expression of another important transcription factor in breast cancer, ER, and this interaction may be important in breast

cancer development and progression. However, the role that Δ40p53 may play in this interaction is unclear. In this thesis, knockdown of Δ40p53 caused a reduction in the expression of ER and its target genes PR and pS2 in the presence of estrogen, and this was supported by overexpression studies examining Δ40p53. Furthermore, a high Δ40p53:FLp53 ratio was shown to be an indicator of worse disease-free survival in ER-positive breast cancers, suggesting that the level of Δ40p53 expression is related to treatment outcomes.

After determining a role for Δ40p53 in mediating the expression and function of ER in breast cancer, the next chapter evaluated two new methods of detecting Δ40p53 expression in tumour tissues. Firstly, a method using branched DNA probes (QuantiGene 2.0 assay) was examined to determine if it was superior to real-time PCR for the detection of Δ40p53 mRNA in formalin-fixed paraffin-embedded (FFPE) tissues. The results were also compared in matched fresh frozen (FF) tissues. Δ40p53 was unable to be detected by either real-time PCR or the QuantiGene 2.0 assay in FFPE tissues, but FLp53 was detected. However, the expression of FLp53 in FFPE tissues was not correlated between the two different methods. This study confirmed the difficulties in quantitating mRNA from these archival specimens. Finally, the use of targeted mass spectrometry in the quantification of Δ40p53 protein expression was investigated in a small pilot study. While only preliminary, the results suggested that Δ40p53 endogenous expression was distinguishable from FLp53 and was accurately quantitated in breast cancer cells. This method provided a novel technique for determining the abundance of Δ40p53 protein compared to FLp53 protein.

Taken together, the work portrayed in this thesis has demonstrated that Δ40p53 expression is associated with PIN3 in breast cancer. A novel functional role for Δ40p53 has also been identified, the regulation of ER expression in breast cancer which is associated with clinical outcomes, and a novel method for the quantification of Δ40p53 protein expression has been developed that may be useful for the analysis of clinical samples. These findings highlight the importance of high Δ40p53 expression in breast cancer, which has been previously reported on by our laboratory, and suggests that Δ40p53 may be an important prognostic marker in breast cancer.

List of Figures:

List of Tables:

List of abbreviations:

Abbreviation	Expanded term
AML	acute myeloid leukemia
ANOVA	analysis of variance
ATM	ataxia telangiectasia mutated
ATR	ATR serine/threonine kinase, a protein coding gene
BMI	body mass index
BRCA1/2	breast cancer 1/2
Chk2	checkpoint kinase 2, a serine/threonine kinase
COSMIC	catalogue of somatic mutations in cancer
CV	coefficient of variation
DBD	DNA binding domain
DCIS	ductal carcinoma *in situ*
DMEM	dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
dPCR	digital PCR
ER	estrogen receptor
ERE	estrogen response element
ESR1	estrogen receptor 1
FCS	fetal calf serum
FF	fresh frozen
FFPE	formalin-fixed paraffin-embedded
FLp53	full-length p53
G4	g-quadruplex structure
GATA3	GATA binding protein 3, a transcription factor
HCD	higher-energy collisional dissociation
HER2	human epidermal growth factor receptor 2
IDC	invasive ductal carcinoma
IGF-1	insulin-like growth factor 1
IRES	internal ribosome entry site
kDa	kilodalton
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MAPK	mitogen-activated protein kinase
MDM2	mouse double minute 2
mp53	mutant p53
MRM-MS	multiple reaction monitoring-mass spectrometry
mRNA	messenger RNA
mTOR	mechanistic target of rapamycin, a serine/threonine kinase
NANOG	Nanog homeobox, a protein coding gene
NLS	nuclear localisation signal
NTC	non-targeting control siRNA

p53RE	p53 response element
PCR	polymerase chain reaction
PIK3CA1	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha, a protein coding gene
PIN3	polymorphism in intron 3 of p53
PR	progesterone receptor
pS2	protein PS2, trefoil factor 1
PTEN	phosphatase and tensin homolog, a protein coding gene
PUMA	p53 upregulated modulator of apoptosis
RNA	ribonucleic acid
SFRS1	serine/arginine-rich splicing factor-1
siRNA	small interfering RNA
SNP	single nucleotide polymorphism
SRM	selected reaction monitoring
SRSF3	serine and arginine rich splicing factor-3
TAD	transactivation domain
TNBC	triple negative breast cancer
wtp53	wild-type p53