Genotypic phenotypic correlation in male breast cancer

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Thesis abstract

Male breast cancers (MBCs) are rare cancers, comprising less than 1% of all breast cancers and less than 1% of all cancers in men. As a result, these cancers have not been well characterized with almost all management extrapolated from the treatment of female breast cancers. More recent studies, however, have demonstrated differences from female breast cancers.

This thesis has examined genotypic and phenotypic correlation in a group of 61 familial MBCs (kConFab) and 225 mixed familial and sporadic MBCs (Lund University, Sweden) with robust clinical and pathological data. The hypothesis is that: 1) male and female breast cancer (FBC) is different, 2) familial male and familial FBC is different and 3) familial and sporadic MBCs are different with possible differences within familial MBC subgroups.

The penetrance of familial MBCs is different to that of familial FBC, showing an increased proportion of *BRCA2* male carriers and underrepresentation of *BRCA1* male tumours. Histopathology showed a paucity of lobular and medullary male breast cancers, with less frequent HER2 and Basal phenotypes. An association between *BRCA2* mutation carrier status and invasive micropapillary subtype was seen. A *BRCA1* associated medullary phenotype was not demonstrated and accordingly TP53 somatic mutations and associated hypoxic drive was not seen in these tumours, highly suggestive of a redundant role for *BRCA1* in MBC. The somatic mutation profile in familial MBCs was similar to that seen in luminal A FBCs, with the rare E547K *PIK3CA* seen several times in MBCs suggesting a possible gender correlation.

Methylation of the ERβ/eNOS complex associated tumour suppressor gene, *GSTP1*, was frequently seen in MBCs, more so in familial than sporadic MBCs. The clinical significance of this may be useful in screening for MBCs in these high-risk populations and may also be a risk modifier as high levels of *GSTP1* methylation have also been seen in *BRCA2*-associated prostate cancers. Similar to familial FBCs, there was observed clustering of tumors by methylation patterns into different *BRCA* subgroups, albeit with small numbers.

Compared to sporadic MBCs, familial MBCs overall showed an earlier median and mean age of onset, with more frequent multifocality or bilateral disease, Familial MBCs also showed a higher proportion of high grade tumours and invasive papillary carcinomas. Familial MBCs also showed a higher amount of gene losses and higher levels of candidate gene methylation.

The study demonstrated several differences between male and female breast cancers and sporadic and familial MBCs.

(Total words – 397).

Declaration

This is to certify that:

1. the thesis comprises only my original work towards the Doctor of Philosophy except where indicated in the Preface,

2. due acknowledgement has been made in the text to all other material used,

3. the thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

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Preface

Work carried out in collaboration indicating the proportion of contribution of others is as follows:

1. Familial male breast cancers procured from kConFab. I wish to thank Heather Thorne, Eveline Niedermayr, all the kConFab research nurses and staff, the heads and staff of the Family Cancer Clinics, and the Clinical Follow Up Study (funded 2001-2009 by NHMRC and currently by the National Breast Cancer Foundation and Cancer Australia #628333) for their contributions to this resource, and the many families who contribute to kConFab. kConFab is supported by grants from the National Breast Cancer Foundation, the NHMRC and by the Queensland Cancer Fund, the Cancer Councils of New South Wales, Victoria, Tasmania and South Australia, and the Cancer Foundation of Western Australia.

2. Male breast cancers from Melbourne Pathology were identified and collected from within their archives by Dr Leonie Constable.

3. The collection of male breast cancers from Sweden used in parts of this study was assembled by Ida Johansson, Ingrid Hedenfalk, Cecilia Nilsson, and Marie-Louise Fjällskog, including clinicopathological data linked to these cases.

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Publications arising from this thesis

1. **Genotypic and phenotypic analysis of familial male breast cancer shows under representation of the HER2 and basal subtypes in *BRCA*-associated carcinomas.** Siddhartha Deb, Nicholas Jene, kConFab Investigators, Stephen B Fox. *BMC Cancer. 2012 Nov 9;12:510. doi: 10.1186/1471-2407-12-510.*

   (38 citations).

2. ***PIK3CA* mutations are frequently observed in BRCAX but not *BRCA2* – associated male breast cancer.** Siddhartha Deb, David Byrne, Nicolas Jene, kConFab Investigators, Alexander Dobrovic, Stephen B Fox. *Breast Cancer Research. 2013 August 23; 15(4): R69.*

   (16 citations).

3. **Nuclear HIF1A expression is strongly prognostic in sporadic but not familial male breast cancer.** Siddhartha Deb, Ida Johansson, David Byrne, Cecilia Nilsson, kConFab Investigators, Leonie Constable, Marie-Louise Fjällskog, Alexander Dobrovic, Ingrid Hedenfalk, Stephen B. Fox. *Mod Pathol. 2014 Sep;27(9):1223-30. doi: 10.1038/modpathol.2013.231. Epub 2014 Jan 24.*

   (12 citations).

4. **Mutational profiling of familial male breast cancers reveals similarities with luminal A female breast cancer with rare *TP53* mutations.**

Siddhartha Deb, Stephen Q Wong, Jason Li, Hongdo Do, Jonathan Weiss, David Byrne, Anannya Chakrabarti, Trent Bosma, kConFab Investigators, Andrew Fellowes, Alexander Dobrovic, Stephen B Fox. *Br J Cancer. 2014 Dec 9;111(12):2351-60. doi: 10.1038/bjc.2014.511*

(12 citations).

5. **The cancer genetics and pathology of male breast cancer.** Siddhartha Deb. Sunil R. Lakhani. Laura Ottini. Stephen B Fox. *Histopathology. 2016 Jan;68(1):110-8. doi: 10.1111/his.12862. Review.*

(15 citations).

6. *BRCA2* **carriers with male breast cancer show elevated tumour methylation.** Siddhartha Deb, Kylie L Gorringe, Jai-Min B Pang, David J Byrne, Elena A Takano, kConFab Investigators, Alexander Dobrovic. Stephen B Fox. *BMC Cancer. 2017 Sep 11;17(1):641. doi: 10.1186/s12885-017-3632-7.*

Table of contents

Abbreviations

4EBP1	-	4E-binding protein 1
ABLI	-	Abelson Murine Leukemia Viral Oncogene Homolog 1.
AI	-	Aromatase Inhibitor
AJCC	-	American Joint Committee on Cancer.
Apo D	-	Apolipoprotein D
AR	-	Androgen Receptor.
ATM	-	Ataxia-Telangiectasia Mutated
ATP	-	Adenosine triphosphate.
BCoR-L1	-	BCL6 corepressor-like 1
BMI	-	Body Mass Index
BPH	-	Benign Prostatic Hyperplasia
BRCA1	-	Breast Cancer 1
BRCA2	-	Breast Cancer 2
BRCAX	-	Breast Cancer X
BRE	-	Bloom Richardson Ellis
BRIP1	-	BRCA1-interacting protein-terminal helicase 1
CA9	-	Carbonic Anhydrase 9
CASP8	-	Caspase 8
CCND1	-	Cyclin D1
CDH1	-	Cadherin 1
CDH13	-	Cadherin 13
CGH	-	Comparative Genomic Hybridization
CHEK	-	Checkpoint Kinase

CI	-	Confidence Interval
CK5	-	Cytokeratin 5.
CMI	-	Cumulative Methylation Index
CMF	-	Cyclophosphamide Methotrexate Fluorouracil
CONEXIC	-	Copy Number and EXpression In Cancer
CPD	-	Carboxypeptidase D
CT	-	Computed Tomography.
CTGF	-	Connective tissue growth factor
CYP17	-	17α-hydroxy/17,20-lyase
DCIS	-	Ductal Carcinoma In Situ.
DMSO	-	Dimethyl sulfoxide
EGFR	-	Epidermal Growth Factor Receptor.
eIF	-	Eukaryotic initiation factor
EMBRACE	-	Epidemiological Study of Familial Breast Cancers
ER	-	Oestrogen Receptor
ERBB2	-	Erythroblastosis oncogene B 2
ESR1	-	Estrogen Receptor 1
EZH2	-	Enhancer of Zeste Homologue 2
FBC	-	female breast cancer
FGFR	-	Fibroblast Growth Factor Receptors
FISH	-	Fluorescence in Situ Hybridization.
FOXA1	-	Forkhead box protein A1
FOXM1	-	Forkhead box protein M1
GAB2	-	GRB2-associated-binding protein 2
GADD45	-	Growth Arrest and DNA Damage-inducible 45

GATA5	-	GATA-binding factor 5
GLUT	-	Glucose Transporter
GnRH	-	Gonadotrophin-Releasing Hormone
GWAS	-	Genome-Wide Association Study
HEBON	-	Hereditary Breast and Ovarian Cancer Research Group Netherlands
HER2	-	Human Epidermal Growth Factor Receptor 2
HIF1	-	Hypoxia-Inducible Factor 1
HIF1a	-	Hypoxia-Inducible Factor 1 alpha
HOX	-	Homeobox
HR	-	Hazard Ratio
HRM	-	High Resolution Melting.
ICNST	-	Invasive Carcinoma of No Special Type
ISH	-	In Situ Hybridization
kB	-	kilobase
kConFab	-	Kathleen Cuningham Foundation Consortium for Research into Familial Breast Cancer
LHFP	-	Lipoma HMGIC fusion partner
LHRH	-	Luteinizing Hormone-Releasing Hormone
LKB1	-	Liver kinase B1
LOH	-	Loss of Heterozygosity
MAP	-	Microtubule-Associated Proteins
MAP3K1	-	Mitogen-activated protein kinase 3
MAP2K4	-	Mitogen-Activated Protein Kinase 4,
MBC	-	Male breast cancer

MGMT	-	O^6-methylguanine DNA methyltransferase
MHC	-	Major Histocompatibility Complex.
MLPA	-	Multiplex Ligation-dependent Probe Amplification
MMTV-PyVT	-	MMTV-polyomavirus middle T antigen
MRI	-	Magnetic Resonance Imaging.
MSH6	-	mutS homolog 6
MS-HRM	-	Methylation-Sensitive High-Resolution Melting (MS-HRM)
MS-MLPA	-	Methylation-Specific Multiplex Ligation-dependent Probe Amplification
mTOR	-	Mammalian Target of Rapamycin
NAT1	-	N-acetyltransferase-1
NHMRC	-	National Health and Medical Research Council
NRL	-	Neu-related lipocalin
OCCR	-	Ovarian Cancer Cluster Region
OR	-	Odds Ratio
OVCA	-	Ovarian Cancer Gene
OXPHOS	-	Oxidative Phosphorylation
PALB2	-	Partner and Localizer of the BRCA2
PARP	-	Poly [ADP-ribose] polymerase
PAX	-	Paired box
PET	-	Positron Emission Tomography.
PgR	-	Progesterone Receptor
PIK3CA	-	Phosphatidylinositol-4,5-bisphosphate 3-Kinase Catalytic Subunit Alpha
PNI	-	Perineural invasion.

PRC2	-	Polycomb Repressive Complex 2
PRL	-	Prolactin
PTEN	-	Phosphotase and Tensin homolog
QMSPCR	-	Quantitative Methylation-Specific Polymerase Chain Reaction
qRT-PCR	-	Real-Time Quantitative Reverse Transcription PCR
RARB	-	Retinoic Acid Receptor Beta
RASSF1A	-	Ras Association Domain Family Member 1
RR	-	Risk Ratio
RSF1	-	Remodeling and spacing factor 1
RUNX3	-	Runt-Related Transcription Factor 3
SEER	-	Surveillance, Epidemiology, and End Results
SHOX	-	Short-stature Homeobox-Containing Gene on Chromosome X
SMARCB1	-	SWI/SNF Related, Matrix Associated, Actin Dependent Regulator of Chromatin, Subfamily B, Member 1
SNP	-	Single Nucleotide Polymorphism
SSCP	-	Single Strand Conformation Polymorphism
STK11	-	Serine/threonine kinase 11
SWH	-	Salvador/Warts/Hippo
TAF4	-	TATA-binding protein associated factor 4
TAZ	-	Tafazzin
TCGA	-	The Cancer Genome Atlas
TGF-a	-	Transforming Growth Factor alpha
THY1	-	Thymus cell antigen 1
TMA	-	Tissue Microarray
TOX	-	Thymocyte selection-associated high mobility group box

TP53	-	Tumor Protein p53
TRAM	-	Transverse Rectus Abdominis
TTP	-	Time to progression.
UDG	-	Uracil-DNA glycosylase
VHL	-	Von Hippel-Lindau
WHO	-	World Health Organization.
WIF1A	-	Wnt Inhibitory Factor-1
WT1	-	Wilm's Tumour Gene
YAP	-	Yes Associated Protein
ZBRK1	-	Zinc Finger and BRCA1 interacting protein with a KRAB domain
ZNF217	-	Zinc Finger Protein 217
ZNF282	-	Zinc Finger Protein 282
ZNF350	-	Zinc Finger Protein 350

List of Figures and Tables

LITERATURE REVIEW

1.1 Breast cancer.

Breast cancer is a common cancer in Australia and worldwide, contributing to the second most common cause of cancer-related death in women(1). While the incidence has increased from 81.1 to 113.5 cases per 100,000 (age-standardized incidence rate) from 1982 to 2009, the age-standardized mortality has decreased by approximately 30% from 30.8 to 21.6 deaths per 100,000 women(1), possibly due to treatment advances, improved screening, early detection and increased awareness. Despite this progress, up to one third of women will develop metastasis with 5-year overall survival rates of 23%(1) for advanced disease.

Extensive research has been undertaken into understanding the biology of breast cancers mainly focusing on the most common epithelial tumors. As a result, and more so than most other cancer types, a molecular portrait of breast cancer has emerged with distinct molecular subtypes with good genophenotypic and clinical correlation and important implications for cancer treatment. Breast cancer has also been somewhat of an archetype for the study of familial cancers and inherited cancer risk with the characterization of several breast cancer predisposition genes.

Perhaps as a byproduct of this molecular revolution, there has been a reinvigoration of the study of male breast cancers. This is an uncommon cancer accounting for less than 1% of cancers in males(2). The current paradigm has been that MBCs are most similar to FBC arising in perimenopausal women, with management largely extrapolated from FBC studies.

Critically, as the future of breast cancer management shifts towards personalized medicine, a multidisciplinary approach will focus on; identifying high risk patients for preventative management, improvement of treatment of advanced breast cancers, greater utility of combination and next generation targeted therapies and possible introduction of immunotherapies. Greater characterization and the understanding of MBC biology is therefore imperative in ensuring the most optimal management of these tumors with development of male breast cancer specific guidelines.

1.2 Embryogenesis and puberty.

In utero development of the male and female breast is the same(3-5). Both have similar ectoderm and mesoderm derived epithelial (luminal and myoepithelial cells) and stromal (intralobular and interlobular fibrovascular tissue) components with the major difference between sexes being greater numbers of lobules in the female breast. As fetal breast tissue is extensively responsive to maternal hormones, variable glandular complexity is seen and may range from simple to branching structures with morphological variations of the epithelium, including secretory changes, also common in both male and female newborns. Changes after birth included formation of a nipple. At birth the ductal system opens onto the surface through the breast pit on the skin surface. This depression in the skin then forms a nipple and areolar as the skin surrounding the nipple proliferates. The breast then remains the same with little change in both sexes until the onset of puberty.

During puberty, the three-fold increase in circulating estrogens causes the ductal and periductal mesenchymal breast tissue of males to undergo proliferation(6). There is

subsequent involution of these structures due to later rising testicular androgen levels that increase up to adult levels which are up to 30 times higher than baseline pre-pubertal concentrations(7). Thus, during puberty almost two-thirds of adolescent boys will develop gynecomastia secondary to this proliferation, sometimes also aided by increased peripheral conversion of estradiol's by fatty tissue in more overweight adolescents. Almost 95% of cases will regress within 6-24 months of onset(7).

1.3 Male breast cancer.

1.3.1 Epidemiology.

Almost 1% of all breast cancers will occur in males, where incidence is approximately 1-1.1 per 100,000 males(8-11). Like FBC, there is regional and ethnic variation, with the highest rates noted in African and Jewish populations and the lowest rates seen in Asians(6, 8-13). Over time, the rates of MBC appear to have increased from approximately 0.86 to 1.06 per 100,000 males over a 26-year period in large population based analysis(14). Unlike, the bimodal peak of FBC due to cancers of early onset, MBCs shows a gradual cumulative increase in incidence with age and occur 5-10 years later with the peak incidence in the 6th decade (Figure 1.1)(15).

Similar to FBC, there appears to be racial/ethnic variation in MBC incidence. The lowest rates are seen in Asian men with intermediate and higher rates reported generally in Caucasian and West Africa/African Americans respectively(6, 11, 12). Notably, the highest incidence are reported in Jewish men(11).

1.3.2 Risk factors.

1.3.2.1 Hormonal risk factors.

Like FBC, excess estrogen, and in particular higher estrogen relative to androgen levels are associated with increased risk of MBC(9-11, 16-21), and a higher risk of MBCs is seen in Klinefelter's syndrome, androgen/testosterone deficiencies and testicular abnormalities such as undescended testis, orchitis and testicular injury.
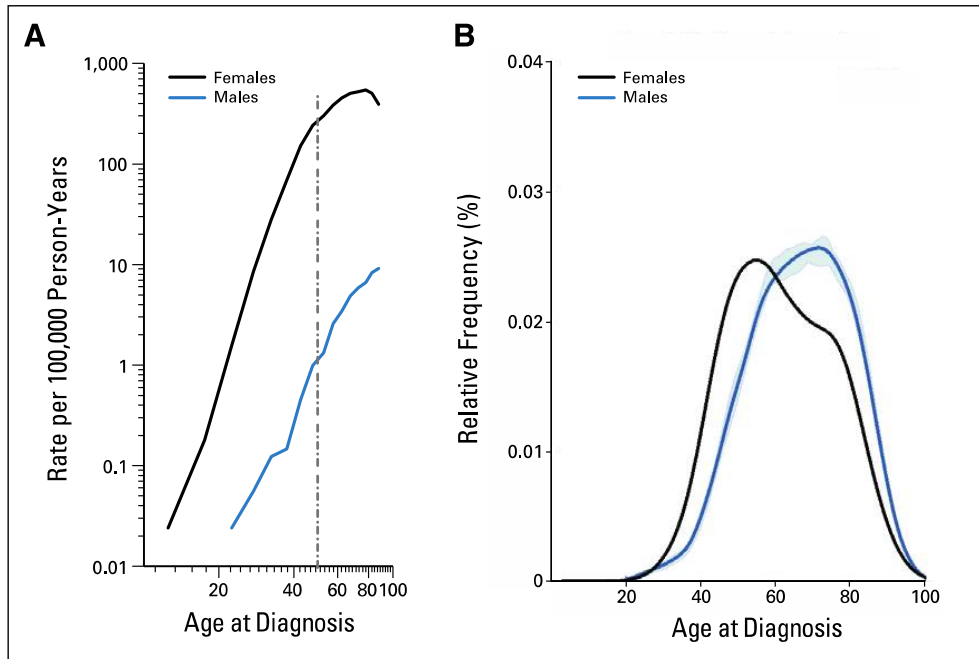
Figure 1.1 Comparison of age at diagnosis for male and female breast cancer. Surveillance, Epidemiology and End Results registry. 1973 to 2005. (A) Age-specific incidence rates. (B) Age distribution at diagnosis. From Korde LA *et al*.(2010)(15).

Evidence of the sensitivity of the male breast to hyperestrogenism is more commonly seen with the development of gynecomastia(22). This is most commonly seen in pubescent boys and while generally reversible can also persist into adulthood. While MBCs and gynecomastia often coexist (up to 38% of MBCs may have associated gynecomastia(23)), it appears that the presence of gynecomastia is not a risk factor for MBC. Nonetheless, there is evidence to suggest that conditions leading to hyperestrogenism do increase the risk of MBC. Common causes of hypoestrogenism

include chronic liver diseases affecting hepatic function and obesity(9, 11, 16) with peripheral aromatization of estrogen almost doubling the risk of male breast cancer occurrence. In particular, higher BMI is shown to be associated with higher MBC risk: $HR = 2.01(95\%CI:1.14\text{-}3.55, p = 0.015)$ in overweight $(25.0 \leq BMI < 30.0 kg/m^2)$ adolescents; and $HR = 4.97(95\%CI\ 2.14\text{-}11.53, p = 0.0002)$ in obese $(BMI \geq 30.0 kg/m^2)$ adolescents(24).

Interestingly, there are implications that *in utero* exposure may also be consequential, as a 1.71 times higher risk of MBCs in first-born males was seen in one study, when compared to younger male siblings, thought to occur due to higher levels of intrauterine estrogens in earlier pregnancies(25). Instances where exogenous estrogens or anti-androgen therapy has been given, such as in transsexuals(26) and in prostate cancer patients have also been suggested to increase breast cancer risk. Hence, while an association between MBC and BPH has also been noted, it is unclear whether the hormonal milieu contributes concurrently to both conditions or the treatment of BPH with finasteride increases MBC risk(27).

1.3.2.2 Occupational and lifestyle risk factors.

Several occupational associations have been described to increase MBC risk. Men with exposure to hot environments such as from blast furnaces, within steel works and rolling mills, are at a higher risk of MBC due to testicular dysfunction(28). An increased risk (RR-1.3) is also seen in males exposed to high electromagnetic fields, and with alcohol consumption, where a 6-fold increase in OR is seen in males consuming more than 90g/day(29). Polycyclic aromatic hydrocarbons, pollutants and

exhaust emissions may also increase MBC risk(30, 31), however, evidence is as yet still inconclusive due to difficulties measuring exposure.

RISK LEVEL	ENVIRONMENTAL RISK FACTOR	GENETIC RISK FACTOR
HIGH RISK	Hormonal imbalance	BRCA2
	Testicular or liver damage	Klinefelter's syndrome
	High oestrogen intake	Breast cancer family history – BRCAX/polygenetic.
	Radiation exposure	
MODERATE/LOW RISK	Occupational exposure - Heat	BRCA1
	Obesity	CHEK2
		Cowden Syndrome
SUSPECTED RISK	Occupational exposure - Exhaust emissions - Magnetic fields	Androgen Receptor
	Higher alcohol intake	CYP17

Table 1.1: Risk factors for Male Breast Cancer. From L. Ottini *et al*.(2010) 141-155(32).

Ionizing radiation is also a probable risk factor for MBC with a single case controlled multi-institutional study showing a modest trend of increasing risk with increased frequency of chest X-rays and an increase in risk in men with three or more total radiographic examinations(33). Risk was only seen from 20-35 years after the initial exposure. Grundy A *et al* reviewed the lifetime job histories of 115 cases of MBCs and 570 controls and found that those exposed to occupational magnetic fields for at least 30 years had a nearly threefold increase in risk of breast cancer (OR-2.77, 95%CI=0.98-7.82) when compared to those with background levels of exposure(33).

An association with smoking is also suggested by a cancer registry study in Florida(34). A total of 1573 cases were reviewed and showed those with exposure to

≥1 packs/day had worse survival (OR-2.48;CI:1.59-3.87) than lifetime non-smokers with a significant dose-response (p for linear trend <0.001). However, this association was not seen by in the Male Breast Cancer Pooling Project consortium(35), that included 2,378 cases and 51,959 controls for analysis from 10 case-control and 10 cohort studies. Cigarette smoking status, smoking pack-years, duration, intensity, and age at initiation were not associated with male breast cancer risk.

1.3.2.3 Inherited risk factors.

1.3.2.3.1 Germline predisposition.

Population based studies suggest up to 33% of MBCs may arise within a background of familial breast and ovarian cancer, suggesting germline susceptibility appears to be a significant contributor to the pathogenesis of MBCs(32, 36-38). Furthermore, 66-86% of familial MBC arise in BRCAX families with unknown underlying predisposing genetic mechanisms. Interestingly, genophenotypic correlation of familial MBCs also appears different to FBCs suggesting further hormonal modification of gene effects exists between genders.

1.3.2.3.2 *BRCA2*.

Perhaps the best characterized and studied predisposition gene is *BRCA2* in MBCs. With known associations with familial FBCs and ovarian cancer(39-41), it is also the strongest risk factor for MBC with incidence rates of up to 10% in *BRCA2* male carriers(9, 36, 42). Interestingly, the association of *BRCA2* and MBCs is further supported by frequent somatic alterations of *BRCA2* noted in MBCs outlined later in this review. Notwithstanding, a low threshold for germline testing should be present for any MBCs with multiple first-degree relatives affected, as evidenced by the

highest mutation frequencies noted (50-100%) in males with 3 or more breast/ovarian cancers in 1st degree relatives. Further evidence of *BRCA2* effect is seen in a single Italian study of the most common non-synonymous polymorphism in *BRCA2*, the N372H variant, that shows a significantly increased risk of MBC in HH homozygous male carriers younger than 60 years of age (OR – 5.6)(43).

Some bias in *BRCA2* mutation location and cancer type is seen with the ovarian cancer cluster region (OCCR) within exon 11 associated with a greater risk of ovarian cancers and a lower risk of female breast cancers(44). As such, similar MBC specific *BRCA2* mutations are not clearly seen, aside from proportionally more truncating *BRCA2* mutation in familial MBCs(45, 46) than in familial FBC. Possible genotypic gender bias may also be suggested by a study of 154 Finnish MBCs that showed predominance of the 9346(-2) A>G *BRCA2* founder mutation in MBCs but of the 999 del5 *BRCA2* mutation in FBCs(47).

Unlike FBC, phenotypic features have been noted particular to MBCs arising in *BRCA2* mutation carriers. These tumors appear to harbor a more aggressive phenotype with a higher proliferative index and higher grade(36, 48). Most are invasive carcinomas of no special type, but with overrepresentation of invasive micropapillary carcinomas. Immunophenotypically, there is also an association with increased frequency of the HER2 intrinsic phenotype(49). The onset or outcome of these tumors is otherwise similar to other familial and sporadic MBCs(36).

1.3.2.3.3 *BRCA1.*

The genophenotypic landscape of *BRCA1* is considerably different between males and females(50-55). In general, male carriers have a significantly decreased lifetime incidence of MBCs (1-2%)(36) when compared to females (cumulative risk of 70% by 70 years of age), with some studies demonstrating higher penetrance with specific mutations or within specific populations as outlined by Papi *et al*. showing MBC in 3 of 11 families harboring the *BRCA1* c.3228_3229delAG founder mutation(56). Similarly, while the incidence of *BRCA1* mutation carriers is relatively infrequent in most MBC populations (incidence <2%), a higher incidence is seen in MBCs arising with a family history of breast/ovarian cancer (up to 6.3%) and in founder populations, as demonstrated by an incidence of 10.5% (8/76) in a study of Ashkenazi Jews(57).

BRCA1 loss, either through methylation, pathway deregulation, or LOH, with phenotypic correlation (basal cell phenotype) is also seen in many sporadic FBCs(58), especially those of early onset. Both the lack of geno-phenotypic correlation in *BRCA1* MBCs, which are predominantly ER-positive invasive carcinomas of no special type (IC-NST), and the relatively paucity of the basal phenotype in MBC suggest a lack of *BRCA1* loss effect in MBCs(36). The still higher incidence of MBCs in *BRCA1* mutation carriers above the general population and variable penetrance of *BRCA1* in some MBC populations, however, suggests that smaller groups of mutation carriers may have some predisposition to MBCs. As yet, strong modifiers of MBC risk in *BRCA1* carriers have not been identified.

1.3.2.3.4 *PALB2.*

PALB2 is critical for the localization of *BRCA2* to sites of DNA damage to initiate repair and to mediate interaction of *BRCA1* and *BRCA2* in DNA-damage response(59,

60). Thus, the loss of *PALB2*, most frequently due to truncating mutation(59, 60), subsequently shares some functional (diminished homologous recombination and intra-S-phase checkpoint defects) and clinical features with *BRCA2* loss. Cancer phenotype is characterized by an overall increased risk of FBC (RR 2-6x) and probable earlier onset FBCs (<50 years of age), familial pancreatic cancer but not ovarian cancers(45). Generally seen in 1% (range 0.5-2.7%) of BRCAX families, *PALB2* mutations have been seen in families with FBC and MBC(59, 60). Due to the low frequency of mutation carriers, direct screening of unselected MBC populations has been inconclusive as to whether or not there is increased MBC risk. However, a targeted study of 1,144 BRCAX female patients showed a four-fold higher rate of MBC within families with *PALB2* heterozygous mutations, thus suggesting a role of *PALB2* in MBC predisposition(61).

1.3.2.3.5 *CHEK2*.

Cell cycle checkpoint kinase 2 (*CHEK2*) is a tumor suppressor gene encoding for the *CHEK2* kinase that regulates cell proliferation and initiates DNA repair in response to DNA double strand breakage(62). The main germline mutation is the *CHEK2**1100delC deletion mainly found within northern and eastern European families where carrier frequency may be up to 1.3-1.6% of the population(63, 64). In females, an increased risk of FBC in heterozygous carriers is seen (OR: 2.7, 95% CI: 2.1-3.4) with a cumulative lifetime risk of 37% (95% CI:26-56%) at 70 years of age(65). The risk effect of germline *CHEK2* mutation in MBC is not so clear. While in certain populations there is a 4-10- fold increase of MBC risk and enrichment of the mutation in BRCAX families with MBC (13.5% incidence(66)), other large MBC studies have failed to detect variants above the baseline population. As yet, it is

unclear what modifiers may affect *CHEK2* penetrance in MBC to account for such large difference between populations.

1.3.2.3.6 *RAD51.*

The *RAD51* family of genes, composed of *RAD51* and *RAD51* paralogs, encodes proteins involved in DNA damage repair mainly through homologous recombination(67). Germline mutations within these tumor suppressor genes results in Fanconi anemia–like disorders and contribute to familial breast and ovarian cancers(68). A large Genome-Wide Association Study (GWAS) genotyping 823 MBC patients and 2,795 controls identified SNPs increasing MBC risk(69). Of these, two were validated in a series of 438 MBCs and 474 controls of which one was the rs1314913 SNP located in intron 7 of the *RAD51*B gene at 14q24.1 (OR-1.57). The SNP has an allelic frequency of approximately 20% and is not reported to be associated with female breast cancer.

1.3.2.3.7 *BRCA1*-associated proteins.

Several *BRCA1*-associated proteins have been recently identified as female breast cancer predisposition genes. The *BRCA1*-interacting protein-terminal helicase 1 (*BRIP1*) gene was originally identified using a C-terminal fragment of *BRCA1* as bait(70). Mutations within the helicase domains of the *BRIP1* protein interfere with double-strand DNA break repair in a *BRCA1*-binding dependent manner(70). Germline mutations of *BRIP1* are associated with Fanconi anemia, early onset breast cancer and ovarian cancer(70). Within BRCAX families, the incidence of germline *BRIP1* mutations is 0.7-2% and mutation confers a 2-fold higher risk of breast cancer in heterozygous females(71-74). Only one study to date, performed on 97 unselected

MBCs with *BRCA1*, *BRAC2*, *CHEK2* and *PALB2* wild type showed a total of 5 germline alterations previously described in FBC of which two were non-coding(75). Of the three coding mutations, one was a silent variant (E879E), one likely to be pathogenic (R264W) but without *BRIP1* loss of heterozygosity (LOH) within the tumor, and one a common missense variant (P919S) frequently seen in FBC but not statistically enriched in this series of MBC(75). To date, the evidence is limited but suggests pathogenic loss of *BRIP1* may not be significant(75).

A probable tumor suppressor protein, BCL6 corepressor-like 1 (BCoR-L1)(76) is a newly described *BRCA1* interacting protein with considerable homology with DNA damage repair proteins, with transcriptional regulation properties and in particular transcriptional co-expression(76). Decreased expression has been seen in *BRCA1*/2 mutation carriers but also in sporadic FBCs(76). Located on the X chromosome (Xq26.1), gene inactivation is thought to be either due to LOH or by complete or skewed X chromosome inactivation reported in both early onset female breast cancers and ovarian cancers(76). Given that males carry only a single gene, Lose *et al*. hypothesize this gene may be susceptible to loss in MBC(76). Thus, twenty-one MBC families were tested showing little variation or LOH in the coding region but highly variable qRT-PCR BCoR-L1 expression in cancer free subjects and high risk cancer patients, thereby suggesting expression likely does not play a role in MBC predisposition in familial breast cancer(76).

Other genes examined in MBCs include *ZNF350/ZBRK1*(77), which complexes with *GADD45* to represses transcription in a *BRCA1*dependent manner. A single study included 21 breast and ovarian cancer families, some with MBCs and showed that

while some variants were identified, it appears unlikely that mutations in these genes account for a significant fraction of inherited breast cancer(77). MBC specific analysis or a statement on MBC outcome was not present in the paper.

1.3.2.3.8 *PTEN.*

PTEN is a phosphatase that negatively regulates the Akt/*PIK3CA* signaling pathway, and is thus a regulator of cell cycle, apoptosis and cell metabolism(78). While somatic mutations of *PTEN* in multiple cancers, including FBC, are relatively frequent, germline mutations of the *PTEN* tumor suppressor gene are very rare and result in Cowden Syndrome, a highly variable autosomal dominant syndrome(79). With an estimated incidence of 1:200,000 - 1:250,000, patients characteristically present with a constellation of non-cancerous skin lesions including mucocutaneous trichilemmomas, acral and palmoplantar keratoses, and papillomatous papules, and with an increased susceptibility to thyroid cancers and breast cancers in females(79). To date only three reports of possible male breast cancer arising with a background *PTEN* germline mutation have been documented in the literature(80, 81). One family with 2 males affected by a G129E mutation had a reported breast adenocarcinoma in one if not two brothers(81). The other two cases reported were in a 4-year-old male with a c.802delG *PTEN* germline mutation who subsequently died of the disease 2 years later, and a 43-year-old male with a *PTEN* c.347-351delACAAT germline mutation who died 14 years later due to hepatic metastases(80). All patients had preceding skin lesions and all reported cancers were infiltrating ductal carcinomas. As both Cowden syndrome and MBC are rare, it is difficult to ascertain from these limited case series as to whether germline *PTEN* mutation increases the relative risk of MBC. However, the observations suggest Cowden Syndrome may account for

some breast cancers especially those of early onset (<45 years of age) with a history of skin lesions, and screening for *PTEN* germline mutation may be warranted in these patients.

1.3.2.3.9 17α-hydroxy/17,20-lyase (*CYP17*).

Polymorphism of the *CYP17* gene has been implicated in the pathogenesis of male breast cancer. Encoding for the cytochrome P450c17a enzyme, a key component in the steroidogenic pathway involved in the synthesis of estrogens as well as progestins and androgens(82), a polymorphic T to C substitution in the 5' untranslated region of the gene 34bp upstream from the start codon creates an additional Sp-1-type promoter motif (CCACC) therefore affecting transcription(83). Allele frequency varies across populations with proportions of carriers ranging from 46% in the UK to 79% in Japan(84). The resultant increased transcriptional activity is hypothesized to increase steroid production, however, studies in females with this polymorphism have only shown inconsistent association with increased oestradiol in premenopausal women, suggesting the increase may be negligible in most women and thus an increased risk for female breast cancer is not seen per se(85).

The possibility that increases in circulating oestradiol in males harboring the CYP17 polymorphism may be clinically relevant in the development of MBC is suggested by a study of 64 MBCs and 81 controls that showed presence of the polymorphism (heterozygous or homozygous) was significantly more frequent in MBC (73.4% vs 58.8%, OR:2.1(95%CI:1.04-4.27,p=0.038))(86). A second study evaluated the potential role of the polymorphism in Icelandic carriers of the 999del5 founder *BRCA2* mutation(87); 309 controls and 39 MBCs with 15 *BRCA2* MBCs were tested.

Presence of the polymorphism was not significantly increased in frequency amongst MBC cases (69.2% vs 66.6%), however the frequency of the CC genotype was higher among carriers of the *BRCA2* 999del5 mutation (33.3%) than non-carriers (16.7%) (did not reach a statistical significance). This association was not observed in female *BRCA2* carriers. Thus, it appears that *CYP17* gene polymorphisms may increase MBC risk in some populations but may also modify *BRCA2* risk for MBC, however, further large studies in well-defined populations are warranted.

1.3.2.3.10 Male breast cancer associated low risk alleles.

A recent GWAS study(69) of 823 MBCs germline DNA and 2,795 controls identified one novel SNP within *RAD51*B as being associated with increased MBC risk (mentioned above) and a second SNP, rs3803662 (allele frequency 49.6%), localized to the *TOX3* gene (mapping to 16q12.1) associated with increased MBC risk (OR-1.5). Belonging to the TOX subfamily of transcription factors which function to modify chromatin structures, the rs3803662 variant is associated with increased female breast cancer susceptibility (OR-1.2) and triple negative breast cancers(88). Furthermore, within primary breast cancers, *TOX3* LOH(33.9%) and somatic mutations(4.5%) have also been noted(88), however, to date somatic changes of *TOX3* in MBCs have not been studied.

A second cohort of 433 MBCs and 1569 controls(69) also revealed 5 SNPs showing an association with cancer susceptibility: rs13387042(2q35)(OR-1.30), rs10941679(5p12)(OR-1.26), rs9383938(6q25.1)(OR-1.39), rs2981579(*FGFR2*)(OR-1.18), and rs3803662(*TOX3*)(OR-1.48). While these low risk alleles are also seen in FBC, rs3803662(*TOX3*) and rs13387042 (2q35) showed excess relative risk of male

breast cancer more than double that observed in FBC, suggesting altered risk between genders. Ottini *et al*.(89) also genotyped 413 MBCs and 745 age-matched male controls in the Italian population, focusing on 9 SNPs from known breast cancer susceptibility loci: (rs13387042/2q35, rs1045485(*CASP8*), rs10941679/5p12, rs889312(*MAP3K1*), rs2046210(*ESR1*), rs2981582(*FGFR2*), rs3817198(*LSP1*), rs3803662/*TOX3*, and rs2363956/19p13). Of these, three loci were associated with MBC risk: rs2046210(*ESR*)(6q25.1)(OR-1.71) (which has a role in estrogen metabolism), rs2981582(*FGFR2*)(10q26.13)(OR-1.26) and rs3803662(TOX3) (16q12.1) (OR 1.59).

1.3.2.3.11 Klinefelter's Syndrome.

Klinefelter syndrome is the most common sex chromosome disorder in males affecting approximately 150 per 100,000(90). Pathogenesis is due to germline mutation due to presence of an extra X chromosome resulting in a 47XXY karyotype. The syndrome is clinically characterized by a combination of hypergonadotropic hypogonadism, infertility, gynecomastia and learning difficulties(90). The super-numerical X-chromosome may be inherited from either parent and undergoes variable silencing. Considerable phenotypic difference are seen, with the *SHOX* gene and CAG repeat numbers in the androgen receptor appearing to influence phenotype(91). Patients with Klinefelter syndrome are at a higher risk of some cancers including MBCs (RR 30-50) due to increased circulating estrogen. Interestingly, the presence of a mosaic 47XXY/46XY karyotype correlates more strongly with breast cancer mortality than pure 47XXY genotype for unestablished reasons. The clinicopathological features of male breast cancers in Klinefelter's otherwise appears similar to other MBCs with a median age of onset reported as 58 years(90).

1.3.2.3.12 Androgen Receptor.

Data relating to germline variations in the androgen receptor gene suggest a possible role in some breast cancers. Located on chromosome Xq11-12, a region within exon 1 of the gene is highly pleomorphic with a variable number of CAG repeats(92). This has biological significance as *in vitro* studies show relatively short CAG repeat sequences increase levels of transactivation of the androgen receptor. Conversely, abnormally long repeats are associated with X-linked spinal and bulbar muscular atrophy (Kennedy's disease) of which gynecomastia and infertility are noted as phenotypic outcomes of androgen insensitivity(93). While genotyping has suggested short AR CAG lengths to be associated with higher levels of testosterone(94, 95), several studies have examined a corresponding association of long CAG lengths with MBCs. Results show that while the majority of AR CAG lengths (most commonly 20-25 repeats long) are of comparable length between controls and men with MBC, long CAG repeats (i.e. > 28 repeats) are rarely seen in control males but noted in males with MBC (alleles with 29, 30 and 49 repeats noted) suggesting that relatively long CAG repeat sequence within the androgen receptor gene may be implicated in a few cases of male breast cancer(96). Interestingly, comparison of *BRCA2* mutation carriers and non-mutation carriers with MBCs showed a difference in median length, with shorter repeats seen in the *BRCA2* group (21 vs 24 repeats), suggesting hormonal influence may be diminished in *BRCA2* carriers(97). Other studies have examined AR germline mutation, with two cases of MBCs with AR gene mutations (R607Q and R608K)(98) reported and two series that consist of; 117 MBCs that were negative for the AR Arg726Leu germ-line mutation (99)(thought to confer a growth advantage to prostate cancer(100)), and no exon 2 or 3 mutations detected in 37 Polish MBCs(101).

1.3.3 Clinicopathologic features.

1.3.3.1 Clinical presentation.

Currently, widespread awareness and screening programs for male breast cancer are not present in clinical practice. As such, almost all male breast cancers are symptomatic at initial presentation, and often with more advanced disease than FBC at presentation(102). Due to reduced breast tissue, men also often present with nipple (retraction, oozing, bleeding) and skin involvement(ulceration, retraction) (103). Some may also present with axillary lumpiness due to nodal spread(104, 105). As a significant number of males (up to 40%) present with advanced stage disease (AJCC 7th edition stage III/IV), presentation may also initially be due to metastatic disease, where bone and lung are the most common sites of distant spread(106-108).

The signs of breast cancer are otherwise similar between men and women with the main difference being more central retroareolar tumor masses and skin and nipple changes seen in males. Like female breast cancer, adjunct mammographic and/or ultrasound examination is recommended. At the time of imaging, fine needle aspiration (FNA) or core biopsy have been both shown to be valid methods of attaining a tissue diagnosis(109-112).

1.3.3.2 Radiological features.

The most common radiological findings reported appear to be: an ill-defined mass, a spiculate mass or as a well-defined hyper-intense mass mammographically or on ultrasound, where most solid cancers are hypoechoic(113-115). MBC may be

distinguished from gynecomastia, which is relatively common, by increased intensity on imaging, calcification and asymmetric shape and position in the breast(116-118).

As complex cysts are rare in men, the presence of these lesions on imaging appears to be pathognomonic for either papillary carcinoma in situ or of invasive papillary carcinoma(116-118). Other features of a malignant papillary lesion include the presence of an eccentric mass, ill-defined edge of lesions, a spiculate mass and calcification(116-118).

Like FBCs, malignant calcifications can also be seen in MBC. However, some patterns of calcification seen in some MBC cases were not overtly atypical and would be labeled benign in females(119)(120), suggesting radiological assessment of malignant calcification may be different between MBCs and FBCs. Interestingly, a proportion of male breast cancers have also been detected on advanced imaging techniques such as CT(121), MRI(122) and PET(123-125).

1.3.3.3 Pathological features.

The gross features of male breast cancer are similar to that seen in female breast cancer. Histologically, classification of breast cancers is as per the WHO 2012 criteria with no guidelines or differences suggested for male and female breast cancer. The majority (approximately 90%) of cancers arising in males are invasive carcinomas of no special type. This histological type, along with invasive papillary carcinoma and invasive micropapillary carcinomas are proportionately more frequently seen in males(36). Lobular differentiation is rare in male breast cancers.(14, 36, 49). Invasive carcinomas of basal cell phenotype are also underrepresented in males(14, 49). Other
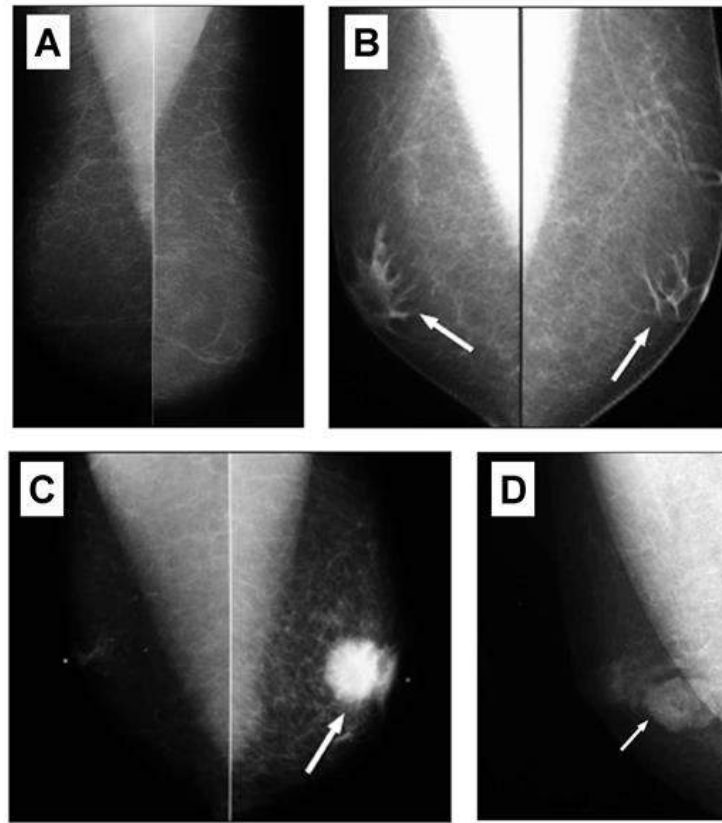
Figure 1.2 Radiographic appearances of benign and malignant male breast disease: a) normal - mediolateral oblique mammograms of both breasts in a male b) gynecomastia - showing linear or flame-shaped projections (arrows) that radiate out into the fatty tissue, c) invasive ductal carcinoma - showing round high-density mass (arrow) with irregular margins and subtle spiculation in the retroareolar region, d) invasive papillary carcinoma - showing a circumscribed oval mass (arrow) with lobulated margins and associated microcalcifications with gynecomastia also present in the retroareolar region. Adapted from Yitta S et al.(115).

cancer types (Table 1.2) have been rarely reported in MBCs. Compared to FBCs, MBCs express ER and PgR more frequently (ER >90%, PgR >75%)(36, 126). Data on HER2 expression is a little less clear with many studies showing a frequency of

HER2 amplification half that seen in FBC(36, 42, 49, 126, 127). An association between HER2 amplification and positive *BRCA2* mutation carrier status has been shown in one study(49) but not validated further. Of the intrinsic phenotypes described by Nilsson *et al*., MBCs have proportionately more luminal cancers and almost half the proportion of HER2, Basal and Null subtypes seen in FBC(36).

Benign and pre-malignant conditions
Gynaecomastia
Pseudoangiomatous stromal hyperplasia
Mastitis
Granular cell tumour
Lipoma
Fibromatosis
Fibroadenoma
Nodular Fasciitis
Myofibroblastoma
Schwannoma
Haemangioma
Ductal Carcinoma In Situ
Malignant
Invasive carcinoma - no special type
IC-NST with micropapillary carcinoma
Invasive carcinoma with basal cell phenotype
Secretory carcinoma
Invasive papillary carcinoma
Invasive lobular carcinoma
Invasive pleomorphic lobular carcinoma
Liposarcoma
Dermatofibrosarcoma Protuberans
Pleomorphic hyalinizing angiectatic tumour
Basal cell carcinoma
Haematopoietic malignancies
Melanoma
Secondary metastasis

Table 1.2 Benign, pre-malignant and malignant lesions reported in the male breast. Adapted from Breast Pathology, 2nd edition, D.J. Dabbs (2016)(128).

1.3.4 Prognosis.

There is still some contention as to whether MBC has a worse overall prognosis when compared to FBC(129-134). The largest study to date of MBC examined outcome in 13,000 MBC and 1,440,000 FBC from the national cancer database in the US between 1998 and 2007(132) and showed that compared to FBC, overall survival was worse in MBC with stage I/II disease but comparable for stage III/IV disease. Conversely, the second largest study evaluating over 2,665 MBCs and 450,000 FBCs from various registries diagnosed between 1970-2007 showed worse DSS in men compared to women(133). However, when patients were matched for age, year of diagnosis, stage, follow up time, treatment and region, there was a slightly better outcome in males compared to females. Overall, the approximate 5 and 10-year survival rates are close to 60% and 40% respectively(129-134).

1.3.5 Histological reporting and prognostic and predictive factors.

Currently, the histological reporting, grading and staging of MBCs is performed in a similar format to women. Different guidelines for MBC do not exist and all published reports have used the same categories devised from FBC studies and used for FBC reporting. Potential areas that may be different between MBC and FBC could include tumor staging as per the guidelines set by the AJCC. Compared to FBCs, MBCs tend to present with a higher nodal stage. It is uncertain how T and N stage correlate between MBC and FBC and whether MBCs metastasize at an early T-stage when compared to FBCs due to the smaller size of the male breast.
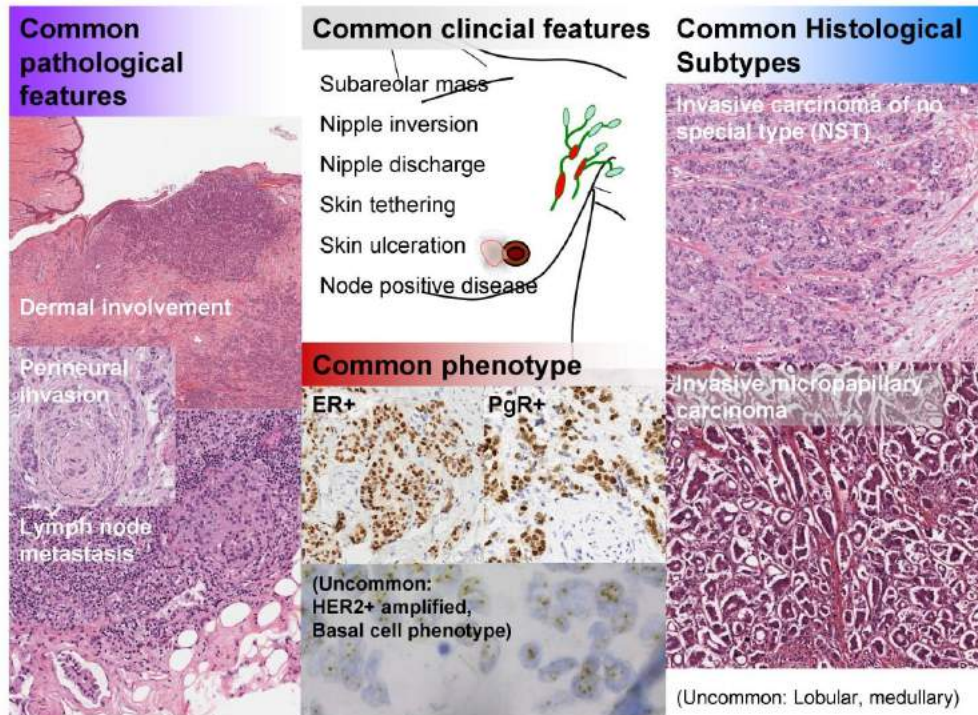
Figure 1.3 Clinical and pathological characteristics of male breast cancer. From Deb et al. (2016) (135).

Nonetheless, several prognostic and predictive factors have emerged in MBC. Like female breast cancer, the Nottingham Bloom Richardson Ellis (BRE) grade is used as a marker of tumor differentiation based on the percentage of tubule formation, mitotic rate and nuclear characteristics(129, 136, 137).

The AJCC staging of MBCs has consistently shown that increased tumor size and nodal involvement are associated with worse prognosis in MBC both on univariate and multivariate analysis(138). Age at diagnosis also appears to be consistently prognostic, with increased age associated with worse overall and disease-specific prognosis(36, 139). As yet, no predictive factors for treatment response have been demonstrated in male breast cancer.

1.3.6 Somatic molecular alteration in male breast cancer.

In comparison to the study of germline predisposition in MBCs, the characterization of somatic changes in MBCs is relatively understudied. Much of this partly due to the haphazard presentation and collection of MBCs making assembly of large MBC cohorts difficult. The studies to date, however, demonstrate several differences from female breast cancers suggesting alternate molecular pathways may exist in MBCs. Furthermore, several novel prognostic biomarkers and MBC subsets have also been identified.

1.3.6.1 Gene mutations.

To date, only eleven studies(140-150) have investigated somatic mutations within a combined sample size of just over 300 MBCs. Most studies have focused on one to two genes with only three studies, including the TCGA study, examining a larger panel of genes. Even within these limited studies, the findings are somewhat mixed. The most commonly mutated genes appear to be *PIK3CA*(140, 142, 143, 146, 147), at a frequency slightly lower than FBCs (11-33% vs 16-40%). The most common hotspots for activating mutations are found in exons 9 and 20.

Of the other mutations studied, conflicting results have emerged as to the frequency of *TP53* and *KRAS* somatic mutations. Early studies by Dawson *et al.*(141) showed *KRAS* and *TP53* mutations in respectively 12% and 25% of MBCs tested. The more recent MBC studies utilizing high throughput sequencing platforms have, however, failed to detect *KRAS* mutations in a further 66 MBCs and found only 2 TP53 mutations in 57 MBCs which occurred in two *BRCA2* mutation carriers(143). A study of *EGFR* and *ESR1* mutations by direct sequencing by Single Strand Conformation

Polymorphism (SSCP) in 103 MBCs by Rizzolo *et al.*(147) demonstrated no pathogenic mutations.

Other MBC studies have shown no somatic mutations in the androgen receptor(144) but a moderately high frequency (21%) of somatic *BRCA2* loss, further highlighting the potential importance of this gene in MBC pathogenesis(48). Over 300 somatic mutations were also noted in the 9 MBCs sequenced by the TCGA, however, the only gene mutated in multiple samples was *PIK3CA*(150).

1.3.6.2 Chromosomal changes.

Several studies have examined somatic chromosomal and copy number changes either by aCGH, MLPA, ISH(151-156) or sequencing(143, 157). These studies indicate there are both similarities and differences noted between MBC and FBCs, with potential identification of specific MBC subsets. Most of the studies used different cut-offs of frequency to define common gains or losses. For comparison, where possible any regions present in >25% of samples have been included. Of the six major studies (Figure 1.4), three demonstrate more regions with gains and less with losses in MBCs when compared to FBCs. Interestingly, changes that were common across at least 4 of the studies (gains of loci incorporating 8q11-q24 and 17q12-q25 and loss of loci incorporating 13q13-q21) were all commonly found in FBC. Of all the loci altered in multiple studies, only two; the gain of 7q36.1 and 11q13.2, was not commonly found in FBC(150). This region contains several partial oncogenes including *ZNF282*, *PAK1*, *RSF1* and *GAB2*. Of the changes commonly seen in FBCs, those not seen in any of the MBC studies included frequent loss at 13q14, 15q11, 17p13-11 and 19p13. These amplicons include *RB1*, *TP53*, *OVCA1*, *OVCA2* and

LKB1/*STK11*. MBCs, however, demonstrate more frequent gains of *EGFR* and *CCND1*, and more frequent losses of *BRCA2*, *PALB2*, *CHEK1*, *CHEK2*, *EMSY* and *CPD* than FBCs and notably, loss of *TP53* is almost half as frequently seen(135, 143).

Due to the infrequency of *HER2* amplification in MBC, Lacle M *et al*(158) used MLPA and FISH to detect and characterize copy number changes on chromosome 17 in a cohort of 139 MBC. The study found similar patterns to FBC but with less complex rearrangements and fewer copy number changes. Frequent gains of 17q, encompassing two distinct amplicons, and losses of 17p were observed with a true amplification rate of *HER2* of 5.8%.

Clinicopathological and genotypic correlation with specific gains and losses is also seen in MBC. Interestingly, almost all studies have noted two distinct groups of MBCs, a smaller subset with few alterations, designated "MBC simple" by Johansson *et al*.(157, 159), and a larger set of cases with more extensive changes designated "MBC complex". The MBC complex group appears to align best with the luminal B female breast cancers, but with significantly more whole chromosomal arm gains. The MBC complex cancers also appear to be associated with a greater propensity to metastasize, but strength of this observation has been limited due to underpowering of the MBC complex group.

1.3.6.3 SNPs.

Silvestri *et al*.(160) examined 386 MBC including 50 *BRCA1*/2 carriers for 29 susceptibility SNPs using Sequenom iPLEX technology. By logistic regression models, they found a significant association with MBC risk for five SNPs: rs1562430
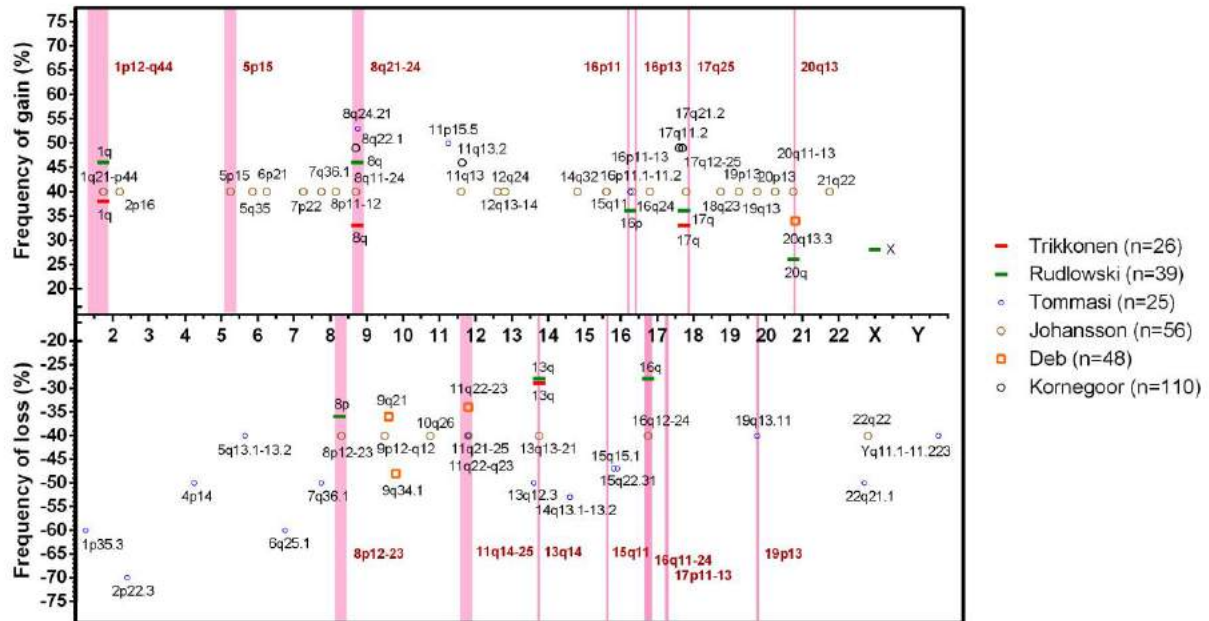
Figure 1.4. Common gains and losses in male breast cancer studies to date compared to gains and losses commonly reported in female breast cancer (shaded pink). Deb S *et al*. (2016)(135).

(p=0.002) and rs445114 (p=0.026) both within the 8q24.21 region; rs1011970/9p21.3 (p=0.011), rs614367/11q13.3 (p=0.016) and rs1314913/14q24.1 (p<0.0001). Association was seen between rs614367/11q13.3 and ER status (p=0.006), and of rs1011970/9p21.3 and HER2 status (p=0.002). Association of rs1011970/9p21.3 risk genotype with HER2+MBC was confirmed by a multivariate analysis. rs1314913/14q24.1 was associated with increased MBC risk in analyses restricted to male *BRCA1*/2 mutation carriers (p=0.041) suggestive of a possible risk modifier locus in male *BRCA1*/2 mutation carriers.

1.3.6.4 Expression profiling.

Only two studies(161, 162) have examined expression profiles in male breast cancer, demonstrating differences from female breast cancer and also identifying clinically distinct subsets. Gene expression analysis performed by Callari *et al.*(161) comparing 37 ER+ MBCs and 53 ER+ FBC showed extensive differential expression of almost 1000 genes, mapping to cell processes involved in metabolism, protein translation and synthesis, cell signaling, cell motility and immunological mechanisms. It suggested than compared to female breast cancer, in male breast cancer both aerobic glycolysis (Warburg effect) and anaerobic glycolysis pathways were not the preferential glucose metabolic pathway for most tumors, with upregulation instead of enzymes involved in different steps of mitochondrial oxidative phosphorylation (OXPHOS), mitochondrial ATP complex and peroxiredoxins (antioxidant enzymes that reduce hydrogen peroxide and indirectly affect cell proliferation).

Divergence of genes associated with cell motility is also seen between FBC and MBCs with upregulation of tubulin genes in MBC but downregulation of microtubule-associated proteins (MAPs)(161). Again, as these molecules are potentially important targets for anticancer therapy(163), this may be important as they differ between male and female breast cancers. The lower frequency of HER2 amplified cancers in MBCs was mirrored by a tenfold lower number of genes found to be correlated with *ERBB2*, thus reiterating a minor role for this gene in the pathogenesis and as a target in males. Conversely, there were more genes correlating with AR in MBCs than FBCs suggesting greater importance of this receptor in males. Interestingly, while in FBC AR expression is often observed in cancers with apocrine differentiation, this association is not demonstrated in MBC studies to date with only a handful of apocrine carcinomas described in males(164). Focusing on signaling

pathways, in MBC there was up-regulation of effectors of the PI3K/AKT/mTOR pathway and of *FGFR2* which may translate to promising future targets for therapy. Increased protein synthesis in MBCs is also implied with upregulation of many ribosomal proteins of both the 40S and 60S subunits and associated proteins, and of poly(A) binding proteins compared to FBCs. An increase in some chemokines and differential expression of major histocompatibility complex (MHC) II and immune receptors indicated generally a reduction of immune response in MBC.

Johansson *et al*(162) examined a subset of MBCs (n=53) where gene expression and array CGH data were present. The computational framework Copy Number and EXpression In Cancer (CONEXIC) was used to integrate the two data platforms in an attempt to identify candidate driver genes. The MBCs were compared with 359 FBCs with a mixture of intrinsic subtype. The results were highly divergent between male and FBC with only 2 common drivers (*TAF4* and *CD164*) identified amongst the 30 candidate drivers in MBCs and 67 identified in FBC, suggesting considerable difference between genders. Of the drivers in MBC, only three well known cancer genes were identified; *LHFP*, *ZNF217* and *MAP2K4*. The remaining genes were most commonly associated with molecular process involving cell differentiation, cell cycle, cell division and signal transduction. A follow up study(159) examined 66 male breast cancers and by unsupervised cluster analysis, two subgroups of male breast cancers (designated luminal M1 and luminal M2) were differentiated. Again, neither clustered well with any of the intrinsic female subgroups but showed correlation with chromosomal instability, with the M1 group harboring a more complex genome with greater numbers of losses and gains. Gene ontology indicated M1 tumors were more aggressive with up-regulation of genes associated with proliferation, cell migration,

cell adhesion, angiogenesis, cell cycle, cell division, HER2 and HOX (Homeobox) genes. Clinically, there was a trend towards worse outcome in M1 tumors. Using the independent dataset from Callari *et al*, unsupervised clustering was again able to demonstrate the M1 and M2 subgroups.

1.3.6.5 miRNA

Gene silencing by miRNA is now well recognized as a method of gene silencing both in normal physiological processes but also aberrantly within cancer. The target of miRNAs are post transcription gene mRNAs resulting in inhibition of, or degradation of, the mRNA(165). Notably, multiple miRNA may inhibit hundreds of mRNA and a single mRNA may similarly be inhibited by multiple miRNA. Thus, depending on which mRNA are targeted, aberrant miRNA may resemble tumor suppressor genes or oncogenes.

Only a handful of studies have investigated miRNA expression in MBCs. These have examined and shown difference between MBC and gynecomastia(166), further reiterating that the molecular pathogenesis of the two entities is different and that gynecomastia is not a likely precursor for MBC. Unfortunately, no studies have attempted to compare miRNA profiles between normal male breast epithelium and MBCs to better suggest the alterations that may take place from baseline normal tissues. Nonetheless, differences are seen between MBCs and FBCs as demonstrated by Pinto *et al*.(167), who analyzed a limited miRNA cancer panel (miR17, miR21, let-7a and miR124) on 27 familial MBCs, 29 familial FBC and 26 sporadic FBC. Lower miR17 (41% vs 66%, p=0.05) and let-7a (15% vs 45%, p =0.015) expression was seen in men as well as absence of a correlation between miR17 and let-7a

expression and estrogen receptor (decreased with increased miR expression) that was seen in FBCs. While this may be possibly due to inadequate power, it may also indicate that, aside from differential expression between MBCs and FBCs, miRs may also function differently between FBCs and MBCs with different gene networks affected.

1.3.6.6 Promoter methylation

The methylation of tumor suppressor genes is also a common mechanism of gene silencing during early stages of tumor development. Quantitative assessment of 25 genes by methylation specific MLPA was performed in 108 consecutive unselected MBCs(168). Compared to FBC, hypermethylation was less common in several genes: *ESR1*(p=0.005), *BRCA1*(p=0.010), *BRCA2*(p<0.001), CD44(p=0.05), *STK11*(p=0.04), *RARB*(p=0.03), *PTEN*(p=0.03) and *VHL*(p=0.03) and *ATM*(0.02). Interestingly, the most frequently methylated genes were *MSH6*(96% of cases), *WT1*(83%), *PAX5*(79%) and *CDH13*(77%), which was similar to FBC.

Clinicopathological correlation showed tumors with higher levels of methylation (calculated as a sum of the percentage of methylation for each gene), were associated with higher grade and mitotic count. Hypermethylation of only two genes, *ESR1* and *GSTP1*, was shown to be associated with higher grade and *MGMT* (O^6-methylguanine DNA methyltransferase) hypermethylation was also associated with larger tumor size. No other correlation was seen either with single genes or clusters of genes. While methylation of an individual gene was not seen to be prognostic, overall high methylation was an independent prognostic factor in the cohort (HR:2.5, p=0.048).

A second study by Pinto *et al.*(169) examined methylation patterns of two genes: *RASSF1A* and *RARB* in a cohort of 27 familial MBCs and 29 familial FBCs by QMS-PCR. *RASSF1A* methylation was observed more frequently men than in women (76% vs. 28%, respectively, p=0.0001). While slightly underpowered, there appeared to be a reversal in the association between *RASSF1A* and *RARB* methylation and ER and PgR expression. Whereas in males there appeared to be a correlation between gene hypermethylation and loss of hormonal receptor expression, the opposite was observed in FBC. This data from these initial two studies suggests that while certain groups of genes in MBC show similar methylation patterns and rates similar to FBC, there are also specific genes which differ considerably between the genders thus supporting the concept of different pathogenesis between some MBCs and FBCs. Furthermore, markedly different clinicopathological associations with hypermethylation of the same gene between the two sexes also implies that gene silencing by methylation may have a different effect or association within the tumor.

1.3.6.7 Molecular biomarkers of prognostic and predictive significance.

Of the studies to date in MBCs, several MBC molecular markers, including protein biomarkers, have been described.

Along with some unique drivers, the two studies by Johansson(170) also identified two novel prognostic biomarkers of MBC; THY1 and NAT1. Loss of THY-1(171, 172) may be linked to epithelial mesenchymal transition and increased invasive and metastatic capabilities in MBC, and clinically is indicative of worse metastasis-free survival (HR:3.6). NAT1 is a highly conserved cytosolic enzyme with possible links to folate homeostasis but has also been shown to have drug metabolizing

activity(173). Like THY-1, lower NAT1 expression correlated with worse clinical outcome (HR-2.5).

The influence of AR on prognosis has also been studied with mixed results. Immunohistochemical studies have varied with only Kwiatkowska *et al.*(174) showing shorter survival with AR expression (33% vs 74%, p = 0.03 for DFS and 57% vs 71%, p = 0.05 for OS) in 43 MBCs. Analysing 81 MBCs, Song *et al.*(175) also demonstrated the presence of long CAG repeats in the AR gene within MBCs correlated with worse 5 year DFS (39.3% vs 60.0%, p=0.04) and worse 5 year OS (49.2% vs 70.0%).

Similar markers to FBCs have also been described. Koornegoor *et al.*(127) identified CCND1 amplification as being associated with worse 5 year survival (HR: 3.0) with a follow up study also demonstrating that the hypoxic protein, HIF-1a was an independent prognostic factor for 5 year survival (HR-2.5)(176). A study of 54 MBCs by Dakin Hache *et al.*(177) using immunohistochemical analysis of intratumoral aromatase expression showed strong expression was associated with improved 5 year overall survival (92% vs 49%, p=0.04)(177). Similar to FBC, several studies demonstrate ERBB2 amplification and p53 accumulation appear to be associated with worse DFS and overall survival in MBCs(49, 127, 178). Immunohistochemical examination of Apolipoprotein D (Apo D), a component of the human plasma lipid transport system, by Serra Diaz *et al.*(179) in 57 MBCs showed association with worse relapse-free survival which is similar to observations in FBCs in patients >70 years of age but not in FBCs occurring at early onset or in premenopausal women.

Several transcription factors have also been studied in MBC. Already well described in FBC, Abdeljaoued *et al*(180) have shown on multivariate analyses of 130 MBCs that the transcription factor FOXM1 is an independent prognostic factor for overall survival (HR-0.69(CI:0.43-0.96,p<0.001)). Humphries *et al*. (181) have shown FOXA1 (HR-0.41(CI:0.22-0.77,p=0.005) to be a positive prognostic for DFS in a cohort of 446 cases, remaining upon multivariate analysis. Both factors appear to correlate inversely and directly with tumor ER expression and resistance respectively. FOXM1 was also significantly associated with tumor size, histological grade, lymph node spread, Ki-67 proliferation index and molecular subtype.

In a combined total of 697 MBC, Humphries *et al*(182), examined eIF4E and eIF5, described translation initiation factors shown to be significant drivers in several cancer subtypes(183), including breast cancer. In multivariate Cox regression analysis both showed worse overall survival (eIF4E HR-2.38(CI:1.18-4.8,p=0.016), eIF5 HR-2.55(CI:1.14-5.7,p=0.022); with coexpression being highly significant (HR-7.04(CI:2.22-22.26,p=0.001)). With mTOR inhibitors targeting this pathway now in the clinic, the data also showed reduced eIF4E and eIF5 expression post BEZ235/everolimus, correlated with extended survival, suggesting that these biomarkers may represent new targets for potential future therapeutic intervention.

Di Benedetto *et al*.(184, 185) have examined biomarkers of the Salvador/Warts/Hippo (SWH) pathway. Within 129 eligible patients, using immunohistochemistry for Hippo transducers TAZ/YAP and their target CTGF, multivariate analyses confirmed that TAZ+/CTGF+ and YAP+/CTGF+ phenotypes were independent predictors of

survival (HR-2.03,95%CI:1.06-3.90,p=0.033 and HR-2.00,95%CI:1.04-3.84,p=0.037 respectively).

To date, no correlation with survival has been seen with methylation of individual genes, however, Johansson *et al*.(170) (2015 *et al*) showed two distinct methylation patterns within a cohort of 47MBCs, with a correlation between one subgroup with hypermethylation of PRC2 target genes, high expression of EZH2, clustering with luminal B FBCs and a tendency toward inferior survival. Koornegoor *et al*.(168) observed a high overall methylation status of the tumor (calculated as the sum of the methylation percentage of all genes) correlated with poor survival (HR 2.5). Assessment of DNA ploidy by Pich *et al*.(186) on 34 primary MBCs also showed worse survival in patients with tumor aneuploidy (median survival 38 vs 77 months, p=0.03).

Several small studies have revealed predictive markers in MBCs for hormonal therapy. Within a cohort of 104 MBCs, Wenhui *et al*.(187) observed that for patients who received tamoxifen therapy, AR-negative patients (determined immunohistochemically) showed a higher clinical benefit rate than AR-positive patients (P=0.025). Additionally, the median TTP and OS were significantly different (P=0.02 for TTP; P=0.029 for OS). In 53 MBCs, Abreu *et al*.(188) showed that in men receiving adjuvant tamoxifen therapy, there was an association between CYP2D6*4 polymorphism and a probability of recurrence (p = 0.034).

1.3.7 Treatment

1.3.7.1 Locoregional treatment

To date, the majority of data generated regarding clinical management of male breast cancers have been retrospective, usually single institutional and often only single armed without comparison to relevant controls but rather comparing outcomes with actuarially based estimates. Unfortunately, many of these studies are biased by collection of data across large time spans, and often contain great treatment heterogeneity. More so, as the management of most of these patients has been based on practices from female breast cancer, and to date no concrete guidelines for specific management of MBC are present.

1.3.7.2 Surgery

The mainstay and the gold standard of primary treatment is also surgical in MBC. Relatively lower rates of conservative surgery(13.2%)(189) have been seen previously seen (SEER) with a growing shift towards more conservative therapy, with the rate of patients undergoing lumpectomy between 2007-2009 significantly higher than the corresponding rate between 1983-1986 (15.1% vs. 10.6%)(190). The use of more radical surgery appears to be utilized more frequently in older patients and in advanced (stage 4) disease(190). Interestingly, a single study of men showed self-image perception appeared to be an important driving factor for conservative surgical management(191).

Not surprisingly, conservative therapies appear to result in inferior local control, however despite this, OS (46.9% vs 46.4%) and DSS (82.8% vs 77.3%) appear comparable(189), possibly due to higher uptake rates of adjuvant therapy in patients undergoing conservative surgical management.

Compared to females, removal of MBCs has different technical challenges. As MBC are more frequently retroareolar, often involve the nipple and skin, and are relatively large when compared to background breast tissue(32, 192), modified partial mastectomies are often skin bearing and often leave comparatively larger defects than in the female breast. Similarly, problems with radical mastectomies also included large chest wall defects which may require significant reconstruction with the use of a transverse thoracoepigastric skin flap and TRAM flaps, the two proposed approaches in the surgical literature(192). A recent series has also reported increased rates of seroma among men when compared to women post-operatively (80.6% vs 59.4%), with equivalent levels of post-operative infection and necrosis(193).

Surgically axillary node sampling, either through axillary lymph node dissection or sentinel node biopsy is an important component of breast cancer management and staging. To date, limited studies of lymph node assessment have been performed in male breast cancer with the only large body of work comprising an analysis of the SEER database of male (n=712) and female (n=382,030) breast cancer patients undergoing lumpectomies from 1983-2009(190). The data shows strikingly lower levels of lymph node sampling in male patients (59.2% vs 81.6%, $p<0.0001$) compared to female cancers. The difference between the genders was not accounted for by variables such as year of diagnosis, patient age, race or cancer stage. Ironically, irrespective of sampling, the overall rates of lymph node positivity in this study, and also consistently within the wider male breast cancer literature, are higher in males than females (39.4% vs 20.1%, $p<0.0001$). The trend towards assessing nodal disease by examining the draining lymph node by sentinel node biopsy has been established

and well-studied in female breast cancer. Several studies in MBC suggest the method is an effective method of assessment in MBC(190, 194).

Subsequent disease control by prevention of recurrences, lymph node disease and metastasis are by a combination of hormonal therapy, chemotherapy, radiotherapies and potentially further surgery. Similar to FBC, the most common sites of metastasis are the bone (56.4%) and lung (23.1%)(106, 195).

1.3.7.3 Radiotherapy

A review by Cloyd *et al*.(190) of radiotherapy use in 5425 male breast cancers collected between 1983 and 2009 (SEER) showed generally lower use of this adjuvant therapy in males undergoing both partial (35.4%) and complete mastectomy (20.8%) than females (generally used in >50% of cases). Smaller studies are more inconsistent in the rates of uptake, with 85% of males receiving postoperative radiotherapy in one larger study(196-198) which showed relatively low rates of chest recurrence (1.8%) and nodal recurrence (5.3%) suggesting good efficacy. When radiotherapy is used, it appears local control rates are excellent at >92% and may be superior to surgery alone, as suggested by a retrospective study(196) of 690 patients from 20 French centers, that showed a significant difference in local relapse between irradiated and non-irradiated patients (7.3% vs 13%). Similarly, Macdonald *et al*.(199) compared radiotherapy use between 60 males and 4181 females showing gender was not a prognostic factor with similar clinical outcomes (locoregional recurrence, breast cancer specific survival and overall survival). Acknowledging that the male breast is anatomically smaller than females, that local male breast cancer recurrence occurs in a similar pattern to female breast cancer, and that nodal positivity is more frequently

seen in MBC, some authors(32) suggest that post mastectomy radiotherapy may be utilized in MBC where tumors are >10mm. This is in contrast to others who recommend the same indications be used as for female breast cancer(200).

1.3.7.4 Chemotherapy:

Due to the difficulties of recruiting large numbers, very few prospective trials on the use of chemotherapy in male breast cancer have been performed. Data pertaining to the use of chemotherapy has been collected retrospectively and consistently shows lower uptake (26.7% vs 40.6%) of chemotherapy and lower compliance compared to women(201). Historically, higher rates of use are seen if the breast cancer was diagnosed post 1980, was associated with positive lymph node disease, and if patients were younger(189).

Analysis of recurrence rates with the use of adjuvant cyclophosphamide, methotrexate and 5-FU (CMF) in 24 MBCs was performed by Bagley *et al*.(136) All patients had modified or radical mastectomy with positive nodal involvement without radiotherapy. Five-year survival was projected to be 80% (CI:74-100%) which is significantly improved compared to their historical controls (5-year DFS of 30%). While the study suggested a distinct benefit of conventional chemotherapeutics in the treatment of node-positive disease, the CMF regimen is not currently used as standard breast cancer treatment. Similarly, smaller retrospective studies have also suggested a benefit of adjuvant chemotherapy(202), however, due to small numbers of patients the studies have lacked sufficient power to reach significant conclusions.

As yet, there are no clear guidelines as to the use of chemotherapy in male breast cancers.

1.3.7.5 Adjuvant Hormonal therapy.

As most (>90%) MBCs are estrogen and progesterone receptor positive(14, 36, 42), endocrine therapies may be of potential use in MBC treatment. Biologically and clinically, it is still unclear as to what the role of estrogen may be in MBCs beyond an association as a risk factor for MBC, and secondly what the treatment efficacy and adverse effect profile of anti-estrogen therapy may be. Retrospective data(32, 202) suggests hormone therapy has been more commonly given in tumors of larger size or when there has been a positive family history. A prospective study(203) of tamoxifen effect on 39 operable stage II and III patients, who all received radiotherapy to the primary site, showed an actuarial 5-year breast cancer specific survival advantage of tamoxifen (61.5% vs 44%, p=0.006) when compared to historical controls. A DFS advantage was also seen (56% vs 28% at 5 years, p=0.005).

While in FBC there is a considerable body of evidence supporting the use of aromatase inhibitors (AIs) as either adjuvant treatment or in advanced disease, there is no supportive data in MBCs. Rather, a multicenter retrospective study(204) in which 257 MBC patients were treated with adjuvant endocrine therapy showed OS was significantly increased in the group treated with tamoxifen and that the treatment of the patients with AI was associated with increased mortality compared to the patients treated with tamoxifen (HR 1.55; 95 % CI: 1.13–2.13; $p = 0.007$). Hence, the most recent St Gallen guidelines on treatment of early stage breast cancer do not support the use of AIs, with or without Luteinizing Hormone-Releasing Hormone (LHRH), in

the adjuvant setting(205). The benefit of AIs in the metastatic setting is essentially unknown with only 5 ongoing studies to date with between 5 to 24 recruited patients.

1.3.7.6 Clinical trials

Currently, almost 5,000 clinical trials are listed worldwide recruiting male breast cancers into mixed gender studies. Historically, however, almost all studies fail to report specific male breast cancer participation or outcomes. More specific review, thus, only shows 3 active male breast cancer specific clinical studies and trials, of which one is a phase 3 trial of Tamoxifen +/- GnRH (Gonadotrophin-Releasing Hormone Analogue) vs Aromatase Inhibitor + GnRH Analogue in Male Breast Cancer Patients (MALE) (NCT01638247), a second is an observational study evaluating the potential risk of MBC development and finasteride exposure (MK-0906-162/2003.021), and the third is a prospective, randomized, multi-center phase II trial evaluating treatment with Tamoxifen +/- GnRH Analogue vs Aromatase Inhibitor + GnRH Analogue in MBC (NTC01101425).

1.3.8 Development of preclinical models

The development of preclinical models is a useful tool in testing hypotheses. Significant advantages are gained by generating accurate, reproducible and robust assays and animal modes, particularly for rare clinical conditions.   Several mouse-based studies have examined interventions in male breast cancer *in vitro*(206).

1.3.8.1 Animal models

Shishido *et al*(206) examined the effects of antineoplastic drugs in a male spontaneous mammary tumor model using the MMTV-PyVT (MMTV-polyomavirus

middle T antigen) transgenic mouse strain. Of four experimental groups, the data showed that while only Tamoxifen and paclitaxel treated animals changed hormone receptor expression levels, only treatment with cisplatin decreased tumor volume. Markers of apoptosis were subsequently examined for a mechanistic explanation for the effect of cisplatin. The data showed cisplatin was the only compound to induce a significant increase in caspase 3, a marker of apoptosis induction. There was a significant decrease in the expression of survivin (an inhibitor of apoptosis) after tamoxifen or cisplatin treatment, however, tamoxifen was shown to increase Bcl-2 expression, an inhibitor of apoptosis possibly negating the effect of survivin.

Nagasawa H *et al*(207) examined the effect of pituitary hormones on male breast cancer development using in house SHN/Mei and SLN/Mei (both containing mammary tumor virus locus Mtv4), and GR/AMei (which carries MMTV via an endogenous proviral gene) mouse strains within which spontaneous female, but not male, breast cancers develop. An untreated control group was compared with a group grafted with isologous anterior pituitaries placed under the kidney capsule. Results showed no tumors in any control mice or GR/A strains but in 7/13 SHN and SLN mice at 12 months of age.

Arendt *et al*.(208) also examined the effect of prolactin and also TGF−α on MBC development. Using a mammary-selective, estrogen-insensitive promoter neu-related lipocalin(NRL), two transgenic lines were created incorporating prolactin (NRL-PRL) and TGF-a (NRL-TGF-a) genes. All female strains developed tumors with only bi-transgenic males producing ERα-positive tumors with variable Androgen receptor expression.

1.3.8.2 Cell lines

Several cell lines have been characterized and utilized in the study of female breast cancers *in vitro*, allowing standardized examination of a variety of cellular biological process ranging from gene and protein function to drug effects *in vitro*. This method is particularly useful in examining rare gene variants or rare diseases where traditional descriptive studies may not show associations due to low power. As yet a male breast cancer cell line has not been described in the English literature. A single Japanese study by Maeda *et al*.(209) exists describing an unregistered human male breast cancer cell line, KBC-2.

1.4 Statement of problem, research approach and aim of study.

At the commencement of the doctorate, there was a paucity of study into male breast cancer. The assumption was that it was similar to post-menopausal female breast cancer, and with little clinical supporting data, treatment of these cancers was entirely based on guidelines for female breast cancers. Furthermore, more so than in female breast cancer, a larger proportion of male tumors arose in breast cancer families, a subset not well-defined to date.

This study proposed further characterization of male breast cancers in a group of up to 61 familial male breast cancers from kConFab and 225 male breast cancers from the Lund University in Sweden with robust clinical and histopathological data with evaluation of common cancer pathways known in female breast cancers.

The aims were to:

- Characterize familial and sporadic male breast cancers

- Describe genophenotypic associations and differences between subgroups;

    o female and male breast cancers,

    o familial female and familial male breast cancers

    o familial and sporadic male breast cancers.

(Word count – 9,861)

Chapter 2- Materials and Methods:

2.1 Summation of Materials and Methods.

The entire results and chapters are based around accepted peer-reviewed papers. The Material and Methods sections of these publications include are summaries below.

2.2 Paper 1 - Genotypic and phenotypic analysis of familial male breast cancer shows under representation of the HER2 and basal subtypes in BRCA-associated carcinomas. Siddhartha Deb, Nicholas Jene, kConFab Investigators, Stephen B Fox. *BMC Cancer. 2012 November 9;12:510.*

2.2.1 Study group

Males with breast cancer were obtained from the kConFab repository (http://www.kconfab.org). Criteria for admission to the kConFab study has been previously published [19] and patients were attained from within Australia and New Zealand between 1998 and 2009. The cases used in the analysis had a diagnosis of breast cancer between 1980 – 2009. Clinical parameters, including TNM staging, tumour recurrence, occurrence of non-breast primary tumours and death were obtained from referring clinical centres, kConFab questionnaires and state death registries. Information on pedigree, mutational status and testing were available from the kConFab central registry. All available slides from all cases were reviewed for relevant histopathological parameters. Histological classification was based on criteria set by the World Health Organisation. This work was carried out with approval from the Peter MacCallum Cancer Centre Ethics Committee (Project No: 11/61).

2.2.2 Germline mutation detection

Mutation test results were attained either on referral to a previously tested patient entering the kConFab database or following testing performed in the kConFab core research laboratory, where testing for BRCA1 and BRCA2 mutations was performed on DNA extracted from 18 ml sample of anticoagulated blood or mouthwash kit [20]. The blood processing protocol (kConFab Biospecimen Protocol. http://www.kconfab.org/epidemiology/biospecimen_protocol.html.) generated a nucleated cell product for DNA extraction. DNA was extracted as required (QIAamp DNA blood kit, Qiagen GmbH, Hilden, Germany). Testing of index cases in kConFab families was carried out by denaturing high performance liquid chromatography or multiplex ligation-dependent probe amplification(210). BRCA1 and BRCA2 variants were classified into the following categories with criteria: pathogenic, splice-site variant, variant of unknown significance and polymorphism. Once the family mutation had been identified, all pathogenic (including splice site) variants of BRCA1 and BRCA2 were genotyped by kConFab in all available family members' DNA.

BRCAX cases were defined by cases with a strong family history meeting kConFab eligibility criteria (http://www.kconfab.org/Collection/Eligibility.shtml), but with absent BRCA1 or BRCA2 mutations within family members.

2.2.3 Tissue microarrays (TMAs) and expression analysis by immunohistochemistry (IHC)

TMAs were created from archival paraffin material. Two 1mm cores were taken for each tumour. TMA sections were cut at 4 μm thick intervals, de-waxed and hydrated. Antigen retrieval was performed according to manufacturers' instructions and endogenous peroxidase activity blocked before incubating sections with desired antibodies. Tumours were separated into molecular phenotypes as per Nielsen et al(211). Expression of estrogen receptor-α (ER) (Ventana, clone SP1), progesterone receptor (PgR) (Ventana, clone 1E2), epidermal growth factor receptor (EGFR) (Zymed, clone 31G7) and cytokeratin (CK) 5 (Cell Marque, clone EP1601Y) was performed. HER2 amplification was assessed by silver in situ hybridisation (SISH) using the INFORM HER2 DNA probe (Ventana). Nuclear expression of ER and PgR was scored as per the Allred scoring system(212) (intensity + percentage of tumour cells staining, 0–8) and separated into absent (score 0/8), low (1-5/8) and high (6-8/8). HER2 gene status was reported as the average number of copies of the HER2 gene per cell in 30 tumour cells. Gene status was assessed as per the guidelines recommended by Wolff et al(213). EGFR was scored positive for any membranous staining of tumour cells. Expression of CK5 was defined as positive when cytoplasmic and/or membranous staining was observed in tumour cells. Tumours were assigned to the following subtypes; Luminal (ER positive, HER2 negative), HER2 (HER2 positive), Basal (ER PgR and HER2 negative, CK5 and/or EGFR positive), and Null/negative (ER, PgR, HER2, CK5/6 and EGFR negative).

2.2.4 Statistical analysis

Comparison of groups was made with using Mann–Whitney U for non-parametric continuous distributions and chi-square test for threshold data. Kaplan-Meier survival

curves were plotted using breast cancer related death as the endpoint and compared using a log rank test. Regression analyses as time to fail curves were plotted for age of diagnosis and occurrence of second non-breast primary tumours. Cox proportional hazard regression model was used to identify independent prognostic factors for disease specific survival (DSS). Analysis was performed with GraphPad Prism 5 software (Graph-Pad Prism version 5.04 for Windows, GraphPad Software, La Jolla California USA). A two-tailed P-value test was used in all analyses and a P-value or less than 0.05 was considered statistically significant.

2.2.5 Contribution to the paper

Pathology review of all cases, review and interpretation of all immunohistochemistry and HER2 ISH, statistical analysis and manuscript preparation.

2.3 Paper 2 - *PIK3CA* mutations are frequently observed in BRCAX but not *BRCA2* –associated male breast cancer. Siddhartha Deb, David Byrne, Nicolas Jene, kConFab Investigators, Alexander Dobrovic, Stephen B Fox. Breast Cancer Research. 2013 August 23; 15(4): R69.

2.3.1 Study group

Only primary breast cancers were examined in this study. Breast and Ovarian Analysis of Disease Incidence and Carrier Estimation Algorithm (BOADICEA) scores(214) were generated from family pedigree and stratified by BRCA1/2 mutation carrier status. The flow of patients through the study was according to the REMARK criteria. Of the 118 cases within the kConFab registry, 58 cases were excluded due to

unavailability of tissue. Of the 60 cases where tissue was available, 2 cases had insufficient tumour tissue for DNA extraction or for a core to be taken for assembly of a tissue microarray (TMA) and a further single case had an extremely low DNA yield and insufficient material for tissue microarray.

Fifty-seven cases had sufficient material at an appropriate DNA concentration for somatic mutation testing and one case did not have adequate tissue for TMA construction with all tissue committed to DNA extraction. Clinical parameters, including disease specific mortality were obtained from referring clinical centres, kConFab questionnaires and state death registries. Information on pedigree, mutational status and testing were available from the kConFab central registry. Histological classification was based on criteria set by the World Health Organization 2012(215). The tumours were stratified into intrinsic phenotypes based on Nielsen *et al.*(211).

2.3.2 Germline BRCA1/2 testing

Mutation testing for BRCA1 and BRCA2 mutations was performed as reported previously in section 2.2.2.

2.3.3 High-Resolution Melting (HRM) assay

Genomic DNA was extracted from formalin-fixed, paraffin embedded (FFPE) samples. A 3 μM haematoxylin and eosin (H&E) stained slide was cut from FFPE blocks and stained to identify tumour enriched areas. From the relevant area on the

FFPE block, a 2 mm punch biopsy core was taken. The cores were then dewaxed and hydrated through gradient alcohol. Genomic DNA was then extracted using the DNeasy Tissue kit (Qiagen, Valencia, CA, USA)) following proteinase K digestion at 56°C for three days.

The PIK3CA, AKT1, BRAF and KRAS primer sequences as shown in section 4.3.4 Additional file 3: Supplementary table 2. PIK3CA exon 9 and 20 primers produced amplicons with 104 base pairs (bp) and 102 bp, respectively. AKT1 exon 4, BRAF exon 15 and KRAS exon 4 primers produced 78 bp, 144 bp and 92 bp amplicons, respectively. PCR for HRM analysis was performed in 0.1 ml tubes on a Rotor-Gene Q (Qiagen) utilising the fluorescent DNA intercalating dye, SYTO 9 (Invitrogen, Carlsbad, CA, USA). A 20µL final reaction volume contained $1 \times$ PCR buffer, 0.5 to 2.0 mM MgCl2, 200 to 400 nM of forward and reverse primer, 200 µM of dNTPs, 5 µM of SYTO 9, 0.5 U of HotStarTaq polymerase (Qiagen), 5 ng of genomic DNA, Uracil-DNA glycosylase (UDG) (0.5 units/reaction), UDG buffer (New England BioLabs, Ipswich, MA, USA) and PCR grade water. The cycling and melting conditions are shown in in section 4.3.4 Additional file 3: Supplementary table 2. All reactions had initial UDG treatment for FFPE artefacts at 37°C for 30 minutes(216), followed by an incubation step at 95°C for 15 minutes, denaturation step at 95°C, annealing steps at the temperatures listed in section 4.3.4 Additional file 3: Supplementary table 2, and an elongation step at 72°C. A single cycle of 97°C for one minute preceded a melt phase run between temperatures listed in section 4.3.4 Additional file 3: Supplementary table 2, rising 0.2°C per step. Samples were run in duplicate. HRM analysis was performed on the Rotor-Gene Q Software (v1.7) (Qiagen, Valencia, CA, USA).

2.3.4 DNA sequencing

All samples with either or both duplicates showing abnormal melts were sequenced for detection of mutations. PIK3CA exon 9 and 20 HRM products were amplified using M13 tagged primers initially and then M13 primers for a second step for PIK3CA exon 9 (amplicon 185 bp) and a single step PCR reaction for PIK3CA exon 20 (amplicon 149 bp) using primers listed above. The composition of a total reaction mixture of 20 μL contained; 1 × PCR buffer, 2.5 mM MgCl2, 400 nM of each primer, 200 μM of dNTPs, 0.5 U of HotStarTaq polymerase (Qiagen), 5 ng of HRM DNA products and PCR grade water. The PCR conditions were as follows: an initial incubation at 95°C for 1 minute, followed by 35 cycles of 95°C for 10 seconds, 55°C for 10 seconds and 72°C for 4 minutes. The sequencing reaction was then performed using the Big Dye Terminator v3.1 chemistry according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA) using 6 μL of the PCR products that were purified with 2 μL of ExoSapIT (GE Healthcare, Little Chalfont, Buckinghamshire, UK). After ethanol precipitation, the sequencing products were run on a 3700 Genetic Analyser (Applied Biosystems). The sequencing data were then analysed using Sequencher 4.6 (Gene Codes Corporation, Ann Arbor, MI, USA). Each mutation was confirmed by sequencing a second independent PCR reaction.

2.3.5 Immunohistochemistry

Tumour-tissue microarrays (1-mm cores), with a twofold redundancy, were prepared from archival FFPE tissue blocks. TMA sections were cut from each block at 4 μm thick intervals, dewaxed, placed through graded alcohol and then into water. For

phosphorylated 4EBP1 (p4EBP1) and phosphorylated S6 (pS6), antigen retrieval was performed using high pH antigen retrieval buffer (DAKO, Glostrup, Denmark) in pressure cooker for three minutes at 125°C.

For phosphorylated AKT1 (pAKT), antigen retrieval was performed with CC1 high pH retrieval solution (Roche, Basel, Switzerland) at 100°C for 36 minutes. Staining for p4EBP1 (dilution 1:400, clone 2855, Cell Signalling Technology, Danvers, MA, USA) and pS6 (dilution 1:200, clone 2211, Cell Signalling Technology) was performed using a monoclonal and polyclonal rabbit antibodies respectively. Antigen-antibody complex was detected using the Envision FLEX system (EnVision FLEX/HRP and EnVision FLEX DAB + Chromogen, DAKO). Staining for pAKT1 (dilution 1:1,000, clone LP18, Novocastra, Newcastle upon Tyne, UK) was performed using a monoclonal mouse antibody with secondary detection using Ventana Ultraview detection reagents (Roche). Slides were then counterstained with haematoxylin, dehydrated, cleared and mounted for assessment. Phosphorylated 4EBP1 expression was assessed for both cytoplasmic and nuclear expression, nuclear expression for pAKT1 and cytoplasmic expression for pS6 (Figure 2a). A histoscore was generated by multiplying staining intensity (0, no staining; 1, weak; 2, moderate; 3, strong) by the percentage of positive tumour cells (0, 0; 1, < or = to 25%; 2, >25% to 50%; 3, >50% to 75%, 4, >75%). The histoscores ranged between 0 and 12. For subsequent analysis, histoscores were categorised into either absent (histoscore = 0) or present (1 to 12) or low (0 to 3) and high (4 to 12) to differentiate from baseline staining of adjacent normal breast epithelium.

A PIK3CA mutation phenotype was defined by either a tumour harbouring a somatic PIK3CA activating mutation or showing an absence of p4EBP1 expression and moderate to strong pS6 expression (histoscore 4-12/12) on immunohistochemistry.

2.3.6 Statistical analysis

Comparison of groups was made using Mann-Whitney U for non-parametric continuous distributions and chi-square test for threshold data. Kaplan-Meier survival curves were plotted using breast cancer related death as the endpoint and compared using a log rank test. Analysis was performed with GraphPad Prism 5 software (GraphPad Prism version 5.04 for Windows, GraphPad Software, La Jolla, CA, USA). A two-tailed P-value test was used in all analyses and a P-value of less than 0.05 was considered statistically significant.

2.3.7 Contribution to the paper

Conception and design of study, pathology review of cases, DNA extraction, HRM and Sanger Sequencing of samples, interpretation of pAKT, p4EBP1 and pS6 immunohistochemistry, statistical analysis and manuscript preparation.

2.4 – Paper 3 - Nuclear HIF1A expression is strongly prognostic in sporadic but not familial male breast cancer. Siddhartha Deb, Ida Johansson, David Byrne, Cecilia Nilsson, kConFab Investigators, Leonie Constable, Marie-Louise Fjällskog, Alexander Dobrovic, Ingrid Hedenfalk, Stephen B. Fox. Mod Pathol. 2014 Sep;27(9):1223-30. doi: 10.1038/modpathol.2013.231. Epub 2014 Jan 24.

2.4.1 Study group

An Australian based cohort of cases included familial male cases obtained from the KConFab resource (http://www.kconfab.org: criteria for admission to the kConFab study has been previously discussed in section 2.2.1) and sporadic male breast cancers obtained from the Peter MacCallum Cancer Centre and Melbourne Pathology. These cases were ascertained following a search of the relevant kConFab registry and pathology databases, and were diagnosed between 1980 and 2009 in Australia or New Zealand. Patients forming the Swedish cohort were identified through the Swedish National Cancer Registry(211). Males diagnosed between 1990 and 2007 within the Lund and Uppsala-Orebro regions that had available formalin-fixed paraffin-embedded tumor blocks, clinicopathological data and outcome data were included in the study. This work was carried out with approval from the Peter MacCallum Cancer Center Ethics Committee (Project No: 11/61) and the local ethics committee in Uppsala, Sweden (i2007/254), and the Lund University (2012/89). Clinical parameters, including the American Joint Committee on Cancer 7th ed TNM staging, tumor recurrence, occurrence of non-breast primary tumors, and death were obtained from referring clinical centers, kConFab questionnaires and state death registries when available. Information on pedigree, mutational status and testing were available from the kConFab central registry. All available slides from cases were reviewed by a pathologist for relevant histopathological parameters. Histological classification was based on criteria set by the World Health Organization (2012)(215).

2.4.2 Germline BRCA1/2 Testing

Any Australian and New Zealand cases of male breast cancer with a strong family pedigree were referred to kConFab preceding this study. Mutation testing for BRCA1

and BRCA2 mutations was performed as reported previously[1] on kConFab referred cases. Once the family mutation had been identified, all pathogenic (including splice site) variants of BRCA1 and BRCA2 were genotyped by kConFab in all available family member's DNA. In the Swedish cohort, only patients with a strong family history of breast and ovarian cancer had germline BRCA1/2 testing.

2.4.3 Tissue-Microarray Construction and Immunohistochemistry

Tumor tissue microarrays (1-mm cores), with a twofold redundancy, were prepared from archival formalin-fixed paraffin-embedded tissue blocks. Patient flow/use was as per the REMARK criterion(217). A total of 104 cases were excluded due to; blocks not being available, an absence of clinical and pathological information, or an absence of adequate material (i.e., core biopsy diagnosis of breast cancer) for tissue-microarray construction.

Tissue-microarray sections were cut from each block at 4 mm thick intervals, dewaxed, placed through graded alcohol, and then into water. Antigen retrieval was performed using high pH EnVision FLEX Target Retrieval Solution (Dako) for 4 min at 124°C for HIF1A and CA9. Staining for HIF1A (1:50 overnight incubation at 4°C, Novus Biologicals) and CA9 (1:4000, 30 min at room temperature, Novus Biologicals) was performed using rabbit polyclonal antibodies. Antigen–antibody complex was detected using the Envision FLEX system (EnVision FLEX/HRP and EnVision FLEX DABþChromogen, DAKO)

2.4.4 Scoring Criteria and Cut-offs

Scoring was performed according to a previously used semi-quantitative system.[6,16,22–24] Briefly, HIF1A was scored only according to the presence (1+) or absence (0) of nuclear expression. Only tumors showing a strong membranous staining in >10% cells were considered positive for CA9.[6]

2.4.5 Statistical Analysis

Comparison of groups was made with using Mann–Whitney U for non-parametric continuous distributions and χ^2- test for threshold data. Kaplan–Meier survival curves were plotted using breast cancer related death as the endpoint and compared using a log rank test. Analysis was performed with Graph-Pad Prism 5 software (GraphPad Prism version 5.04 for Windows, GraphPad Software, La Jolla CA, USA). A two-tailed P-value test was used in all analyses and a P-value of less than 0.05 was considered statistically significant.

2.4.6 Contribution to the paper

Preparation of kConFab cases for tissue microarrays, work up of antibodies used, interpretation and scoring of immunohistochemical stains, analysis and interpretation of data, statistical analysis, manuscript preparation.

2.5 Paper 4 - Mutational profiling of familial male breast cancers reveals similarities with luminal A female breast cancer with rare TP53 mutations. Siddhartha Deb, Stephen Q Wong, Jason Li, Hongdo Do, Jonathan Weiss, David Byrne, Anannya Chakrabarti, Trent Bosma, kConFab Investigators, Andrew Fellowes, Alexander Dobrovic, Stephen B Fox. Br J Cancer. 2014 Dec 9;111(12):2351-60. doi: 10.1038/bjc.2014.511

2.5.1 Study group.

Males with breast cancers were obtained from the kConFab repository and included cases from Australia and New Zealand diagnosed between 1980 and 2009. The flow of patients through the study, according to the REMARK criteria. Of the 118 cases within the kConFab registry, 58 cases were excluded because of the unavailability of tissue. Of the 60 cases where tissue was available, 12 cases had poor quality DNA or insufficient tumour tissue for DNA extraction. The patients were well annotated clinical, as outlined in section 2.2.1.

This work was carried out with approval from the Peter MacCallum Cancer Centre Ethics Committee (Project No: 11/61).

2.5.2. Germline BRCA1/2 testing.

Mutation testing for BRCA1 and BRCA2 mutations was performed as reported previously in section 2,2.2.

2.5.3 DNA extraction.

Genomic DNA was extracted from formalin fixed, paraffin embedded (FFPE) samples as reported previously in section 2.3.3.

2.5.4 UDG treatment.

The treatment of FFPE DNA with uracil-DNA glycosylase (New England Biolabs, Ipswich, MA, USA) was performed on the MyCycler instrument (Bio-Rad, Hercules, CA, USA). This has been demonstrated to significantly reduce sequence artefact induced by formalin fixation(216). One unit of UDG was added for each 20 ng of

FFPE DNA with 0.5_ of UDG buffer. The treatment conditions had two incubation steps: an initial activation at 37 1C for 2 h and an inactivation of UDG enzyme at 97 1C for 10 min.

2.5.5 Illumina TruSeq amplicon cancer panel.

The TruSeq Amplicon Cancer Panel comprises a total of 212 amplicons from 48 genes (Paper Supplementary Table 3)(143) and 6 amplicons from reporter sequences (RP5-1091E12.1, RP11-286H14.8, RP11-530I17.1, RP11-350N15.4, CTC-554D6.1, C11orf65) that are simultaneously amplified in a highly multiplexed and single-tube reaction. Five microlitres at a concentration of 25 ng ml_1 of each DNA sample was used for the experiment according to the manufacturer's instructions. The MiSeq system was used for paired end sequencing using a v1 150 bp kit (Illumina, San Diego, CA, USA). Forty-eight cases were able to examine gene mutation completely and 44 cases were able to assess copy number variation (CNV).

2.5.6 Sequencing validation.

Within all samples, hot spots on TP53 (exons 5–7) PIK3CA (exons 9 and 20), AKT1 (exon 1), BRAF (exon 15) and KRAS (exon 2) genes were analysed for mutation by high-resolution melting and Sanger sequencing. The PIK3CA, AKT1, BRAF and KRAS data using Sanger sequencing for these exons in these patients has been reported previously in section 2.3.4. Mutations of other cancer samples on the same runs were also validated by Sequenom MassARRAY platform (San Diego, CA, USA). Three MBC samples were also run at least two times across multiple sequence runs to examine for run-specific variation.

2.5.7 Bioinformatics.

Primer sequences prefixing the short reads were used to assign each read to an amplicon. Global alignment was then performed between the reads and the amplicon reference sequences to identify sequence variations. Positive variants (in the original biologic sample) were identified using VarScan2 (http://varscan.sourceforge.net). DNA CNV was estimated by comparing sequence read depth between the breast cancer samples and a pseudocontrol. The control was created by averaging the normalised read depth from 20 random human samples that were derived from the same protocols and location as the cancer samples. The averaging and normalisation of the control group was performed using the baseline creation workflow in CONTRA(218). Log ratios between a cancer sample and the control were then computed in 50 bp windows using CONTRA. Using 4600 inhouse samples, we estimated the null distribution of log ratios for each gene and each exon separately, and thereby making significant calls on genes/exons that lie at the extremes of the distributions (using a P-value cut-off of 0.05; Benjamini–Hochberg adjusted). Gains and losses were defined by a two-fold increase or decrease in reads, whereas amplification was determined by a fourfold increase. Deletions were not examined separate to losses. Comparison of groups was made using Mann–Whitney U-test for nonparametric continuous distributions and w2 test for threshold data. Kaplan–Meier survival curves were plotted using breast cancer-related death as the end point and compared using a log-rank test. A two-tailed P-value test was used in all analyses and a P-value or <0.05 was considered statistically significant.

2.5.8 Hierarchical clustering.

Unsupervised hierarchical cluster analysis of log 2 ratios of copy numbers for each gene was used to detect possible unique signatures. Analysis was performed using Cluster and Tree View software written by Michael Eisen (Stanford University, Stanford, CA, USA) as published previously(219, 220) and Elucidean metric distance was used.

2.5.9 Contribution to the paper

Conception and design of study, pathology review of cases, DNA extraction, UDG treatment, sequencing of samples, interpretation of sequencing data, statistical analysis and manuscript preparation.

2.6 – Paper 5 - *BRCA2* carriers with male breast cancer show elevated tumour methylation. Siddhartha Deb, Kylie L Gorringe, Jai-Min B Pang, David J Byrne, Elena A Takano, kConFab Investigators, Alexander Dobrovic. Stephen B Fox. BMC Cancer. 2017 Sep 11;17(1):641. doi: 10.1186/s12885-017-3632-7.

2.6.1 Study group

Primary male breast cancers examined in this study were obtained from the Kathleen Cunningham Foundation Consortium (kConFab) breast/ovarian familial cancer repository, describe previously in section 2.2.1.

The flow of patients through the study was according to the REMARK criteria. Of the 118 cases within the kConFab registry, 58 cases were excluded due to unavailability of tissue. Sixty cases had sufficient material at an appropriate DNA concentration for methylation testing as outlined below. These cases belonged to three groups: 3 MBCs

that arose in BRCA1 mutation carriers, 25 that arose in BRCA2 mutation carriers and 32 that occurred in males from BRCAX families.

Clinical parameters, including disease specific survival (DSS) were obtained from referring clinical centres, kConFab questionnaires and state death registries. Information on pedigrees, mutational status and testing were available from the kConFab central registry.

Histological classification was based on criteria set by the World Health Organisation 2012(215) and all slides and pathological records from all cases were reviewed centrally. Immunohistochemistry for estrogen receptor (ERα), progesterone receptor (PgR), basal markers (cytokeratin 5 (CK5), EGFR) and HER2 silver in-situ hybridisation (SISH) was performed as previously discussed in 2.2.3, with stratification in to intrinsic phenotypes was based on Nielsen et al(211).

Permission to access the kConFab samples and data was granted by the kConFab Executive Committee (Project #115/07–17). This work was carried out with approval from the Peter MacCallum Cancer Centre Ethics Committee (Project No: 11/61).

2.6.2 Germline BRCA1/2 testing

Mutation testing for BRCA1 and BRCA2 mutations was performed as previously reported in section 2.2.2.

2.6.3 DNA extraction

Genomic DNA was extracted from formalin-fixed, paraffin embedded (FFPE) samples as discussed previously in 2.3.3.

2.6.4 Bisulfite modification

Genomic DNA (600 ng) was bisulfite modified using the MethylEasy™ Xceed kit (Genetic Signatures, North Ryde, Australia) according to the manufacturer's instructions. The bisulfite modified DNA was eluted into 50 μL of EB buffer. CpGenome™ Universal Methylated DNA (Chemicon/Millipore, Billerica, MA) and whole-genome amplified DNA were used as the fully methylated and unmethylated controls, respectively. DNA methylation standards (10, 25 and 50%) were made by mixing the fully methylated control with the unmethylated DNA control.

2.6.5 Methylation-sensitive high-resolution melting (MS-HRM)

Methylation screening was performed using MS-HRM to quantitate methylation in bisulfite-modified samples according to the sequence-dependent thermostability in which the level and presence of homogenous and heterogeneous methylation can be detected. MS-HRM primers were specifically designed to generate short amplicons enabling use in formalin-fixed paraffin embedded (FFPE) samples and are summarised in **5.4.3 Additional file 2**. PCR amplification and HRM analysis were performed on the Rotor-Gene 6000 (Corbett, Sydney). Samples were run in duplicate. Conditions for each gene are described in **5.4.3 Additional file 2**. The reaction was performed using a final volume of 20 μL and the mixture consisted of 1 × PCR buffer (Qiagen, Hilden, Germany), 2.5–4.0 mmol/L of MgCl2, 200 μmol/L of each dNTP, forward and reverse primers, 5 μmol/L of SYTO9 intercalating dye (Invitrogen, Carlsbad, CA), 0.5 U of HotStarTaq DNA polymerase (Qiagen, Hilden, Germany)

and 10 ng of bisulfite modified DNA. The methylation level of each DNA sample was determined visually by comparing it against the standard curves. Heterogeneous DNA methylation was defined by melting profiles that did not directly conform to any of the methylation controls due to the formation of heteroduplexes between closely but not identically related single complementary DNA strands. Complexes that complete melting slightly after the unmethylated controls were indicative of low levels of DNA methylation. In contrast, complexes with a late melting profile typically contained more heavily methylated epialleles.

2.6.6 Methylation scoring

A cut-off of 10% methylation was used to primarily exclude low level methylation of uncertain biological significance. The remaining samples were further grouped into moderate methylation (10–50% fully methylated, or moderate heterogenous methylation) and high methylation (>50% fully methylated, or high-level heterogenous methylation). Positive methylation (hypermethylation) for each gene was thus considered when duplicate samples showed >10% or moderate to high heterogeneous methylation, the samples were also given a percentage methylation for each gene by comparing the methylation to the curves of the standard, which was then averaged across all the genes to give an average methylation index (AMI) scored between 0 and 100% for each tumour sample. The AMI measurement is based on the cumulative methylation index [24], which is the sum of the percentages of methylation of the individual genes, but corrects for the number of genes tested. Using the AMI scores, groups were dichotomised into low and high based on the median AMI as a cut-off point. This analysis does not make assumptions as to the effect of any particular level of methylation.

2.6.7 Cluster analysis

Unsupervised complete linkage clustering was performed with Euclidean metric distance. Unsupervised hierarchical cluster analysis of methylation at each gene was used to detect possible distinct molecular signatures. Analysis was performed using Cluster and Tree View software written by Michael Eisen (Stanford University) as previously published(219, 220).

2.6.8 Statistical analysis

Comparison of groups was made with using Mann-Whitney U for non-parametric continuous distributions and Fisher's exact test for threshold data. Kaplan-Meier survival curves were plotted using breast cancer related death as the endpoint and compared using a log rank test. Pearson's correlation coefficient was measured for the cluster analysis. Analysis was performed with Graph- Pad Prism 5 software (GraphPad Prism version 5.04 for Windows, GraphPad Software, La Jolla California USA). A two-tailed P-value test was used in all analyses and a p-value or less than 0.05 was considered statistically significant.

2.6.9 Contribution to the paper

Project conceptualization, DNA extraction and performing DNA methylation assays, data analysis, preparation of manuscript.

Chapter 3 - Genophenotypic correlation of familial male breast cancers:

This chapter is composed of one paper:

1) Genotypic and phenotypic analysis of familial male breast cancer shows under representation of the HER2 and basal subtypes in BRCA-associated carcinomas. Siddhartha Deb, Nicholas Jene, kConFab Investigators, Stephen B Fox. *BMC Cancer. 2012 November 9;12:510.*

Supplementary/additional figures and tables from the article are present at the end of each published article.

3.1 Aims and Rationale

Within male breast cancer, a considerable number of cases arise in female breast and ovarian cancer families. While only a handful of instances of multiple male breast cancers within the one family have been reported, the cancers arising in female breast/ovarian cancers families are commonly referred to as familial male breast cancer and those arising without such a familial history as sporadic male breast cancer.

As a large proportion of MBC studies describing pathological and molecular characteristics of the tumors do not separate cases based on whether these are sporadic or familial cancers, i.e. arising within a male within a family with a predisposition for breast cancers, there is a paucity of pathological detail pertaining to familial MBCs in general. What is known is that there are differences from FBC and probably between sporadic and familial MBCs.

The aim was therefore to characterize familial MBC, in particular, to characterize differences between familial MBC and familial FBC, and possibly sporadic MBCs and familial MBCs. As many previous studies have been multi-institutional with possible interpretive and testing inconsistencies, the aim was to look for genophenotypic correlation through thorough clinical and pathological review at a single referral center.

3.2 Summary

At the time of publication, this paper was the largest high-risk population-based study of male breast cancer. The risk of male breast cancer in breast and ovarian cancer families is higher than that of the general population. However, the penetrance of cancer amongst *BRCA1* and 2 carriers is different to female breast cancer with a much higher incidence in *BRCA2* than in *BRCA1* carriers. The majority of male breast cancers arose in BRCAX families with a lower penetrance overall when compared to *BRCA1* mutation carriers, but 4 to 5 times above the general population.

The majority of familial MBCs were grade 2 or 3, ER/PgR positive, and invasive carcinoma of no special type. These proportions within the MBC cohort were significantly higher than within familial FBCs from the same cohort. There were also less lobular and medullary carcinoma with lower rates of HER2 amplification than familial FBCs. These findings were mirrored by a later study by Silvestri V *et al*.(221) looking at a total of 419 MBCs from CIMBA (Consortium of Investigators of Modifiers of *BRCA1*/2) which showed the same associations.

Compared to other MBC studies(42, 48, 133, 195, 222-233), the cohort of familial MBC from this study showed the lowest percentage of grade I tumors (3.3%) and the highest proportion of invasive papillary carcinomas (6.7%). Notably, familial FBCs, and in particular *BRCA1*-associated tumors are associated with distinct histomorphological features that are not as discriminatory in male breast cancer.

Compared to sporadic and unselected MBC studies, the familial MBCs arise at a slightly earlier age, with a median age of diagnosis of 62.5 years. Unlike familial female breast cancers, however, very early onset cancers (at <40 years of age) were uncommon in this cohort of male breast cancer. Interestingly, there was also a high proportion of secondary cancers arising within the familial male breast cancer cohort. Within *BRCA2* male carriers, an increased incidence of prostate cancer is known, with a more aggressive clinical course. While the most common second cancer seen in the *BRCA2* carriers was also prostate acinar adenocarcinomas, there was also a similar incidence within BRCAX males with MBCs. Indeed, BRCA status did not affect the rate of secondary cancers between the *BRCA2* and BRCAX group or the interval between diagnosis of breast and second cancers. What is also notable is that even within *BRCA2* male mutation carriers, the risk of developing prostate cancer is higher in males who have developed MBC compared to non-MBC *BRCA2* mutation carriers, suggesting a higher propensity for cancer within these patients. The observation of an increased secondary cancer rate in MBC patients noted in multiple sporadic and familial MBC studies, including this study, is interesting and as yet unexplained. The association appears to be more prominent in familial MBCs and appears to also exist in BRCAX high-risk cancer families.

Most prognostic factors described in this study are similar to those described in other MBC studies with some novel associations noted in this cohort. More so than in FBC, due to the small volume of normal breast tissue, male breast cancers more frequently involve the nipple and skin. A relatively high rate of perineural invasion (PNI) was observed in the study that is higher than those reported in FBCs. The majority of invasive foci were noted in the dermis and subcutaneous tissue where neural bundles can often be found, and thus the increased rates of perineural invasion may be a byproduct of increased proximity for male breast cancers to overlying skin. Rather than a method of metastatic spread, the presence of PNI is likely to be an indicator of a tumor with greater metastatic potential and was shown to be prognostic.

Unlike FBC, a *BRCA1* phenotype is not clearly seen in MBC. A subset of *BRCA1* FBCs are associated with early onset (often <40 years of age), with distinct histological (well circumscribed, high mitotic count, necrosis, tumor infiltrating lymphocytes, syncytial growth pattern) and immunohistochemical (ER/PgR/HER2 negative, EGFR/Cytokeratin 5 positive) phenotype. More so, somatic *BRCA1* loss, either by methylation, LOH or mutation is not infrequently seen in FBC. When it occurs, the histological and phenotypic association with invasive carcinoma of basal cell phenotype is seen. This study was only able to access tissues from 3 *BRCA1* MBCs which is a reflection the rarity of *BRCA1* MBCs, even within large familial breast and ovarian cancers registries like this one. As a result, study and characterization of these tumors is particularly poor. However, to date, including this study and a subsequent paper by Silvestri R *et al*.(221) looking at 44 *BRCA1* MBC, there has not been a single *BRCA1*-associated medullary MBC described, suggesting this association is almost exclusively seen in females.

In contrast, and unlike what is seen in familial FBC, a *BRCA2* MBC phenotype is noted. Previously Ottini *et al.*(42) noted *BRCA2*-associated tumors were more proliferative and of a higher grade, particularly in *BRCA2* carriers with an earlier age of onset. There was also a predisposition for *BRCA2* associated tumors to contain areas of invasive micropapillary carcinoma. Interestingly, as both Ottini *et al. et al.*(49) and Kwiatkowska *et al.*(234) have also shown somatic LOH of *BRCA2* is frequently seen in MBC, it may be that the *BRCA2* phenotype may be diluted by comparison of MBCs arising in *BRCA2* mutation carriers against sporadic MBCs, of which a proportion may also harbor loss of *BRCA2* function. A large study comparing MBCs in *BRCA2* mutation carriers, sporadic MBCs with *BRCA2* loss and sporadic MBC with intact *BRCA2* may demonstrate this phenotype more clearly.

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Genotypic and phenotypic analysis of familial male breast cancer shows under representation of the HER2 and basal subtypes in BRCA-associated carcinomas

Siddhartha Deb[1,2,3*], Nicholas Jene[1], kConFab investigators[4] and Stephen B Fox[1,3,4]

Abstract

Background: Male breast cancer (MBC) is an uncommon and relatively uncharacterised disease accounting for <1% of all breast cancers. A significant proportion occurs in families with a history of breast cancer and in particular those carrying *BRCA2* mutations. Here we describe clinicopathological features and genomic *BRCA1* and *BRCA2* mutation status in a large cohort of familial MBCs.

Methods: Cases (n=60) included 3 *BRCA1* and 25 *BRCA2* mutation carries, and 32 non-*BRCA1/2* (BRCAX) carriers with strong family histories of breast cancer. The cohort was examined with respect to mutation status, clinicopathological parameters including TNM staging, grade, histological subtype and intrinsic phenotype.

Results: Compared to the general population, MBC incidence was higher in all subgroups. In contrast to female breast cancer (FBC) there was greater representation of *BRCA2* tumours (41.7% vs 8.3%, p=0.0008) and underrepresentation of *BRCA1* tumours (5.0% vs 14.4%, p=0.0001). There was no correlation between mutation status and age of onset, disease specific survival (DSS) or other clincopathological factors. Comparison with sporadic MBC studies showed similar clinicopathological features. Prognostic variables affecting DSS included primary tumour size (p=0.003, HR:4.26 95%CI 1.63-11.11), age (p=0.002, HR:4.09 95%CI 1.65-10.12), lymphovascular (p=0.019, HR:3.25 95%CI 1.21-8.74) and perineural invasion (p=0.027, HR:2.82 95%CI 1.13-7.06). Unlike familial FBC, the histological subtypes seen in familial MBC were more similar to those seen in sporadic MBC with 46 (76.7%) pure invasive ductal carcinoma of no special type (IDC-NST), 2 (3.3%) invasive lobular carcinomas and 4 (6.7%) invasive papillary carcinoma. A further 8 (13.3%) IDC-NST had foci of micropapillary differentiation, with a strong trend for co-occurrence in *BRCA2* carriers (p=0.058). Most tumours were of the luminal phenotype (89.7%), with infrequent HER2 (8.6%) and basal (1.7%) phenotype tumours seen.

Conclusion: MBC in *BRCA1/2* carriers and BRCAX families is different to females. Unlike FBC, a clear *BRCA1* phenotype is not seen but a possible *BRCA2* phenotype of micropapillary histological subtype is suggested. Comparison with sporadic MBCs shows this to be a high-risk population making further recruitment and investigation of this cohort of value in further understanding these uncommon tumours.

Keywords: Male breast cancer, BRCA1, BRCA2, BRCAX, Micropapillary, Familial

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Background

Male breast cancer (MBC) is an infrequent and poorly characterised disease. Limited data to date suggests it is epidemiologically and biologically different from female breast cancer (FBC) but it is unknown whether current paradigms and treatment of female disease can be extrapolated to the pathobiology and management of MBC and vice versa. Although some recent large MBC studies have been undertaken, these are population-based and this current report is the largest to describe the genotype, tumour phenotype, complete clinicopathological parameters and survival in MBC from high-risk families.

Accounting for less than 1% of all male cancers, and 0.65% of all breast tumours [1-3], the incidence of MBC has increased steadily from approximately 0.86 to 1.06 per 100,000 males over a 26 year period [4,5] There is controversy surrounding mortality with some suggestion that MBC disproportionately accounts for a higher number of deaths than breast cancer in women [4-7] while other studies suggest parity when comparing age and stage matched cases [8].

Inherited risk factors for MBC appears to be a more significant contributor than in women with estimates of 10% of all MBC cases arising with a family pedigree suggestive of a genetic predisposition [2,9-11]. Unlike women, BRCA2 germline mutation in men confers a significantly higher lifetime risk of developing breast cancer than BRCA1 [2,9-11]. Other genes also implicated in the development of MBC including PTEN [12], P53 [13] and CHEK2 1100delC [14]. Kleinfelter's syndrome (XXY) [15], environmental and hormonal states that alter the ratio of androgens to estrogens are also thought to contribute to MBC [16]. Recent meta-analysis has also shown an association between previous breast disease, in particular gynaecomastia, and occurrence of MBC [17]. It is still unclear, however, whether this is a; precursor lesion, a risk factor for MBC or whether the aetiology and pathogenesis is the same for both conditions.

Despite extensive knowledge about female BRCA1, BRCA2 and other inherited familial breast tumours at present, little is know of male tumours from high-risk families. Comparison of sporadic tumours in both sexes shows; a steady linear increase in incidence in men with age in contrast to the bimodal distribution seen in FBC [2,3,18], an older median age of diagnosis in men [6,8,18], more advanced stage-related tumour characteristics (tumour size >2cm, positive axillary nodes) [2,18] but with more favourable histopathological characteristics (lower tumour grade) and biology (hormone receptor positive tumours) [2,18]. Most MBC studies have been performed with cohorts predominantly composed of "sporadic" population based patients whereas this study is focused on one of the largest groups of MBCs arising in high-risk families evaluating both clinicopathological and genetic associations.

Table 1 Mutation carrier status and male breast cancer with the kConFab cohort

	BRCA1	BRCA2	Non-BRCA1/2
All males in kConFab registry	429	339	19137
Breast Cancers	5 (1.2%)	35 (10.3%)	78 (0.4%)
Pathology Available	3	25	32

Methods

Study group

Males with breast cancer were obtained from the kConFab repository (http://www.kconfab.org). Criteria for admission to the kConFab study has been previously published [19] (Additional file 1: Table S1) and patients were attained from within Australia and New Zealand between 1998 and 2009. The cases used in the analysis

Table 2 Characterisation of BRCA1 and 2 mutations of males included within this study

Gene	Mutation	Effect
BRCA1	BRCA1 del exons 21_24	LGR
	BRCA1 2798_2801 del GAAA (STOP 998)	P
	BRCA1 5382_5383 ins C (STOP 1829)	P
BRCA2	BRCA2 del exons 1_2	LGR
	BRCA2 del exons 14_16	LGR
	BRCA2 2988 del C (STOP 959)	P
	BRCA2 2988 del C (STOP 959)	P
	BRCA2 5873 C>A (S1882X)	P
	BRCA2 5950_5951 del CT (STOP 1909)	P
	BRCA2 5950_5951 del CT (STOP 1909)	P
	BRCA2 6024_6025 del TA (STOP 1943)	P
	BRCA2 6503_6504 del TT (STOP 2098)	P
	BRCA2 6714_6717 del ACAA (STOP 2166)	P
	BRCA2 6854_6855 del TA (STOP 2223)	P
	BRCA2 6971_6983 del ATGCCACACATTC (STOP 2275)	P
	BRCA2 698_702 del AGTCA (STOP 180)	P
	BRCA2 7708 C>T (R2494X)	P
	BRCA2 8168_8169 ins C (STOP 2661)	P
	BRCA2 9132 del C (STOP 2975).	P
	BRCA2 9161 C>A (S2978X)	P
	BRCA2 9610 C>T (R3128X)	P
	BRCA2 9610 C>T (R3128X)	P
	BRCA2 983_986 del ACAG (STOP 275)	P
	BRCA2 995 del C (STOP 276)	P
	BRCA2 del exons 1_27	P
	BRCA2 IVS 7-1 G>A	P
	P BRCA2 8525 del C (STOP 2776).	P
	BRCA2 8714 A>G (del exon 19)	UV

Classification of Variants: P = Pathogenic, LGR = Large Genomic Rearrangement, UV = Unclassified Variant.

Table 3 Clinicopathological features

	All patients (n=60)	BRCA1 (n=3)	BRCA2 (n=25)	BRCAX (n=32)	P-value
AGE AT DIAGNOSIS					
Median	62.5 (30.1 - 85.6)	65.6 (49.5-80.1)	61 (31.0 - 85.7)	63.2 (30.1 - 81.8)	
<60 yoa	26 (43.3%)	1 (33.3%)	11 (44.0%)	14 (43.8%)	
>60 yoa	34 (56.7%)	2 (66.6%)	14 (56.0%)	18 (56.3%)	NS
DISEASE SPECIFIC MORTALITY	35.0%	33.3%	40.0%	31.3%	
SIDE					
Right	36 (60.0%)	1 (33.3%)	17 (68.0%)	18 (56.2%)	
Left	24 (40.0%)	2 (66.7%)	8 (32.0%)	14 (43.8%)	NS
Unifocal	56 (93.3%)	3 (100%)	22 (88.0%)	31 (96.9%)	
Multifocal	2 (3.3%)	0	2 (8.0%)	0	
Bilateral	2 (3.3%)	0	1 (4.0%)	1 (3.1%)	NS
HISTOLOGICAL SUBTYPE					
Invasive Ductal Carcinoma - No special type	46 (76.7%)	2 (66.7%)	18 (72%)	28 (87.5%)	
IDC with Micropapillary component	8 (13.3%)	0	6 (24%)	2 (6.3%)	
Invasive Papillary Carcinoma	4 (6.7%)	1 (33.3%)	1 (4%)	2 (6.3%)	
Invasive Lobular Carcinoma	2 (3.3%)	0	0	2 (6.3%)	NS
BRE GRADE					
1	2 (3.3%)	0	1 (4%)	1 (3.1%)	
2	31 (51.7%)	0	12 (48%)	19 (59.4%)	
3	27 (45.0%)	3 (100%)	12 (48%)	12 (37.5%)	NS
ER STATUS (ALLRED 0-8)					
0	1 (1.7%)	0	0	1 (3.3%)	
1-5	5 (8.6%)	1 (33.3%)	2 (8.0%)	2 (6.7%)	
6-8	52 (89.7%)	2 (66.7%)	23 (92.0%)	27 (90.0%)	
NA	2	0	0	2	NS
PR STATUS (ALLRED 0-8)					
0	5 (8.8%)	0	1 (4%)	4 (13.8%)	
1-5	8 (14.0%)	0	5 (20%)	3 (10.3%)	
6-8	44 (77.2%)	3 (100%)	19 (76%)	22 (75.9%)	
NA	3	0	0	3	NS
HER2					
Amplification	5 (9.1%)	0	2 (8.3%)	3 (10.7%)	
Non-amplified	50 (90.9%)	3 (100%)	22 (91.7%)	25 (89.3%)	
NA	5	0	1	4	NS
PHENOTYPE					
Basal	1 (1.7%)	0	0	1 (3.3%)	
Luminal	52 (89.7%)	3 (100%)	23 (92.0%)	26 (86.7%)	
HER2	5 (8.6%)	0	2 (8.0%)	3 (10.0%)	
NA	2	0	0	2	NS
TUMOUR SIZE					
Median	17mm (2-50mm)	15mm (9-25mm)	17mm (6-40mm)	16 (2-50mm)	
TUMOUR STAGE					
T1a	1 (1.7%)	0	0	1 (3.1%)	
T1b	8 (13.3%)	1 (33.3%)	4 (16.0%)	3 (9.4%)	
T1c	31 (51.7%)	1 (33.3%)	10 (40.0%)	19 (59.4%)	

94

Table 3 Clinicopathological features *(Continued)*

T2	19 (31.7%)	1 (33.3%)	11 (44.0%)	7 (21.9%)	
T3	1 (1.7%)	0	0	1	NS
LYMPHOVASCULAR INVASION					
Absent	32 (57.1%)	2 (66.7%)	14 (60.9%)	16 (53.3%)	
Present	24 (42.9%)	1 (33.3%)	9 (39.1%)	14 (46.7%)	
NA	4	0	2	2	NS
PERINEURAL INVASION					
Absent	31 (56.4%)	3 (100%)	12 (50.0%)	16 (57.1%)	
Present	24 (43.6%)	0	12 (50.0%)	12 (42.9%)	
NA	5	0	1	4	NS
PAGET'S DISEASE OF NIPPLE					
Absent	44 (84.6%)	2 (100%)	19 (86.4%)	23 (82.1%)	
Present	8 (15.4%)	0	3 (13.6%)	5 (17.9%)	
NA	8	1	3	4	NS
NODAL STATUS					
Cases with nodes examined	46 (76.7%)	3 (100%)	20 (80.0%)	23 (71.9%)	
Cases with positive nodes	20 (43.4%)	2 (66.7%)	9 (45.0%)	9 (39.1%)	NS
Average numbers of nodes examined per case	12.9 (1-30)	16.3 (13-24)	15.9 (1-30)	10.1 (1-29)	
NODAL STAGE					
N0	26 (56.5%)	1 (33.3%)	11 (55.0%)	14 (60.1%)	
N1	18 (39.1%)	2 (66.7%)	8 (40.0%)	8 (34.8%)	
N2	2 (4.3%)	0	1 (5.0%)	1 (4.3%)	NS
Cases with extranodal extension	8 (17.4%)	0	5 (25.0%)	3 (13.0%)	NS
MARGINS					
Clear	29 (48.3%)	1 (33.3%)	12 (48.0%)	16 (50.0%)	
Involved	15 (25.0%)	0	6 (24.0%)	9 (28.1%)	
Not assessable	16 (26.7%)	2 (66.7%)	7 (28.0%)	7 (21.9%)	NS
DCIS					
Absent	14 (25.0%)	0	7 (29.2%)	7 (24.1%)	
NA	4	0	1	3	
Present	42 (75.0%)	3 (100%)	17 (70.8%)	22 (75.9%)	NS
Nuclear Grade					
Low	2 (4.8%)	0	2 (11.8%)	0	
Intermediate	26 (61.9%)	1 (33.3%)	10 (58.8%)	15 (68.0%)	
High	14 (33.3%)	2 (66.7%)	5 (29.4%)	7 (31.8%)	NS

NS – Not significant.

had a diagnosis of breast cancer between 1980 – 2009. Clinical parameters, including TNM staging, tumour recurrence, occurrence of non-breast primary tumours and death were obtained from referring clinical centres, kConFab questionnaires and state death registries. Information on pedigree, mutational status and testing were available from the kConFab central registry. All available slides from all cases were reviewed by a pathologist for relevant histopathological parameters. Histological classification was based on criteria set by the World Health Organisation. This work was carried out with approval from the Peter MacCallum Cancer Centre Ethics Committee (Project No: 11/61).

Mutation detection

Mutation test results were generated through two avenues. If a clinic had performed mutation screening, the clinic report was passed onto the kConFab central registry. If no clinic mutation testing had been performed, the kConFab core research laboratory performed mutation testing. Testing for *BRCA1* and *BRCA2* mutations was performed on DNA extracted from 18 ml sample of anticoagulated

95

blood or mouthwash kit [20]. The blood processing protocol [21] generated a nucleated cell product for DNA extraction. DNA was extracted as required (QIAamp DNA blood kit, Qiagen GmbH, Hilden, Germany). Testing of index cases in kConFab families was carried out by denaturing high performance liquid chromatography or multiplex ligation-dependent probe amplification [22]. *BRCA1* and *BRCA2* variants were classified into the following categories with criteria as posted on kConFab's website [23]: pathogenic, splice-site variant, variant of unknown significance and polymorphism. Once the family mutation had been identified, all pathogenic (including splice site) variants of *BRCA1* and *BRCA2* were genotyped by kConFab in all available family members' DNA.

Tissue microarrays (TMAs) and expression analysis by immunohistochemistry (IHC)

TMAs were created from archival paraffin material. Two 1mm cores were taken for each tumour. TMA sections were cut at 4 μm thick intervals, de-waxed and hydrated. Antigen retrieval was performed according to manufacturers' instructions and endogenous peroxidase activity blocked before incubating sections with desired antibodies. Tumours were separated into molecular phenotypes as per Nielsen *et al* [24]. Expression of estrogen receptor-α (ER) (Ventana, clone SP1), progesterone receptor (PgR) (Ventana, clone 1E2), epidermal growth factor receptor (EGFR) (Zymed, clone 31G7) and cytokeratin (CK) 5 (Cell Marque, clone EP1601Y) was performed. HER2 amplification was assessed by silver in situ hybridisation (SISH) using the INFORM HER2 DNA probe (Ventana). Nuclear expression of ER and PgR was scored as per the Allred scoring system [25] (intensity + percentage of tumour cells staining, 0–8) and separated into absent (score 0/8), low (1-5/8) and high (6-8/8). HER2 gene status was reported as the average number of copies of the HER2 gene per cell in 30 tumour cells. Gene status was assessed as per the guidelines recommended by Wolff *et al* [26]. EGFR was scored positive for any membranous staining of tumour cells. Expression of CK5 was defined as positive when cytoplasmic and/or membranous staining was observed in tumour cells. Tumours were assigned to the following subtypes; Luminal (ER positive, HER2 negative), HER2 (HER2 positive), Basal (ER PgR and HER2 negative, CK5 and/or EGFR positive), and Null/negative (ER, PgR, HER2, CK5/6 and EGFR negative).

Statistical analysis

Comparison of groups was made with using Mann–Whitney U for non-parametric continuous distributions and chi-square test for threshold data. Kaplan-Meier survival curves were plotted using breast cancer related death as the endpoint and compared using a log rank test. Regression analyses as time to fail curves were plotted for age of diagnosis and occurrence of second non breast primary tumours. Cox proportional hazard regression model was used to identify independent prognostic factors for disease specific survival (DSS). Analysis was performed with GraphPad Prism 5 software (GraphPad Prism version 5.04 for Windows, GraphPad Software, La Jolla California USA). A two-tailed P-value test was used in all analyses and a P-value or less than 0.05 was considered statistically significant.

Results

Mutation analysis

The prevalence of MBCs in the kConFab registry with known gene mutations is summarised in Table 1 and 2. There were 5 (1.2%) of 429 known *BRCA1* mutation carriers and 35 (10.3%) of 339 *BRCA2* carriers who developed breast cancer. Of these, 3 and 25 cases respectively had reports, slides and tissues available for examination and were included in the study. Of the 3 *BRCA1* cases, 2 had a pathogenic mutation with 1 large genomic rearrangement. Of the 25 *BRCA2* cases, 22 had a pathogenic mutation, 2 large genomic rearrangements and 1 an unclassified variant. Within non-*BRCA1/2* families, of a total of 19,137 males, 78 (0.4%) developed breast cancer with 32 cases available for use in the study.

Clinicopathological features

The clinicopathological features are summarised in Table 3. The overall median age of diagnosis was 62.5 years (range 30.1-85.6 years), and mean age of diagnosis 60.0 years. There was no significant difference in clinicopathological factors between *BRCA1*, *BRCA2* carriers and BRCAX males including age of onset (Figure 1). Surgical treatment was by wide local excision (33.3%, 20/60) and mastectomy (66.6%, 40/60). All tumours
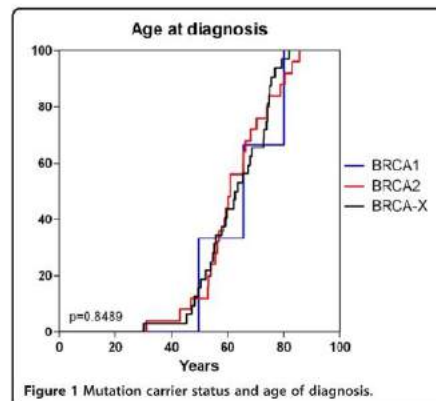
Figure 1 Mutation carrier status and age of diagnosis.

Figure 2 H&E histological subtypes in male breast cancer: a) invasive ductal carcinoma of no special type, b) & c) invasive lobular carcinoma, d) & e) invasive papillary carcinoma, f) invasive micropapillary carcinoma.
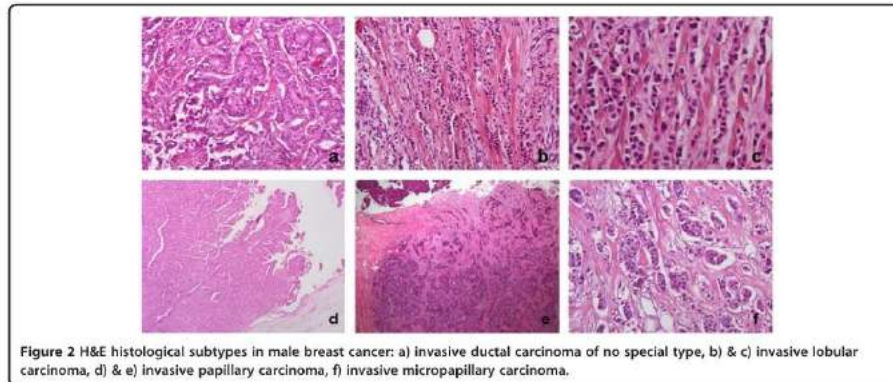
were present within 30mm of the subareolar region and the nipple. Four cases (6.6%) had multifocal disease with 2 cases of bilateral breast cancer, of which one was a metachronous BRCAX tumour with a 10 year interval and the other a *BRCA2* carrier with contralateral tumour occurring 12 years after the primary lesion.

Tumour size ranged from 2 mm to 50 mm (median 17 mm). The most common histological subtype was infiltrating ductal carcinoma of no special type (IDC-NST) (90%, 54/60) (Figure 2a) with 2 cases of invasive lobular carcinoma (3.3%) (Figure 2b and c) and 4 cases of invasive papillary carcinoma (6.7%) (Figure 2d and e). Of the

IDC-NST tumours, 8 had areas between 15 to 40% of invasive micropapillary carcinoma (Figure 2f).

Tumours were of mainly grade 2 (51.7%) and grade 3 (45.0%). Lymphovascular and perineural invasion (PNI) was identified in 42.9% (24/56) and 43.6% (24/55) of cases respectively when able to be assessed. Paget's involvement of the nipple was seen in 15.4% of cases (8/52) when assessable. Most tumours had a component of DCIS present (75%, 42/56). Normal breast tissue and gynaecomastia was observed in 65.1% (28/43) and 11.6% (5/43) of cases respectively. Forty six cases had lymph node sampling with 7 sentinel node biopsy only (15.2%) and

Figure 3 Immunohistochemical staining of male breast cancer for ER and PgR.

97
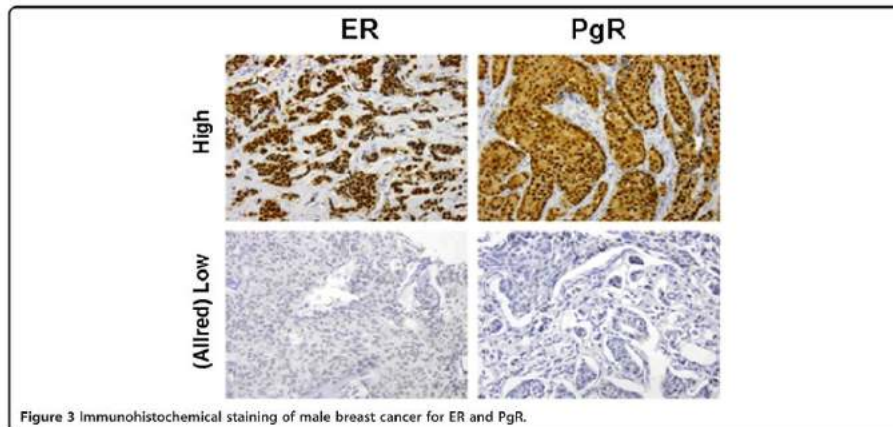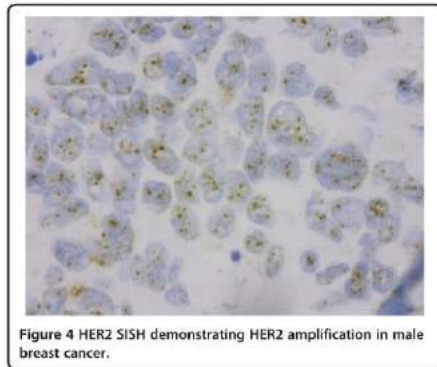
Figure 4 HER2 SISH demonstrating HER2 amplification in male breast cancer.

the remainder axillary dissection (84.7%). On average 1.6 sentinel nodes (median 1, range 1–3) were examined and an average of 15 nodes from axillary dissections (median 13, range 4–30). Of these, 1 (14.3%) sentinel node had metastatic disease and 19 axillary dissections had positive nodal disease (48.7%) with extranodal extension in 8 cases.

Most tumours were ER and PgR positive (Additional file 2: Figures S1 and Additional file 3: Figure S2), with

89.7% (52/58) and 77.2% (44/57) of cases respectively scored as high (Allred score 6-8/8) (Figure 3). HER2 amplification was seen in 9.1% (5/55) of cases (Figure 4). The range of HER2 amplification was 6.1-10.5 signals per nuclei in amplified cases. Two tumours were unable to be immunophenotyped completely. Based on analysis of the remainder, the most common intrinsic subtype was Luminal (89.7%, 52/58) followed by HER2 (8.6%, 5/58) and Basal (1.7%, 1/58). The Basal subtype (Figure 5) was a BRCAX tumour with prominent CK5 and EGFR staining but also low ER nuclear positivity. Morphology of this tumour was more consistent with a basal subtype rather than a luminal type tumour.

There was a trend towards *BRCA2* tumours having an invasive micropapillary component (24% 6/25, p=0.0574) and high Bloom Richardson Ellis (BRE) grade for *BRCA1* tumours (100% grade 3 3/3, p=0.0855), however these observations did not reach statistical significance. Overall, clinicopathological factors and intrinsic subtypes were not associated with *BRCA1* or *2* mutation carrier status and unlike in female breast cancer [27], there was no association between *BRCA1* mutational status and basal cell phenotype.

Characteristics are compared with other recent large MBC studies containing >50 patients and completed within the last 4 years [6-8,28-40] (Additional file 4: Table S2) and with the previous study of female breast cancers within the kConFab cohort [41].
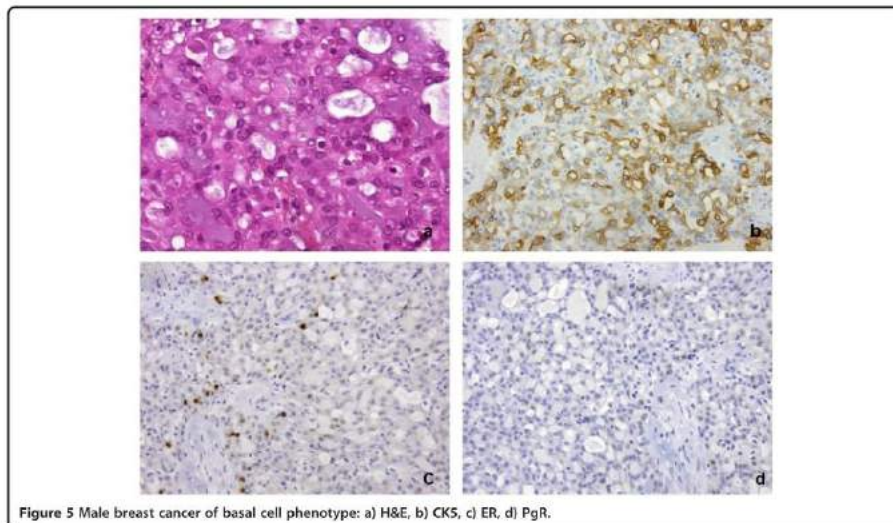
Figure 5 Male breast cancer of basal cell phenotype: a) H&E, b) CK5, c) ER, d) PgR.

Disease specific survival

The overall 5 and 10 year disease specific survival rates were 84.6% and 40.6% for all cases, 100% and 0% for *BRCA1* case, 80.6% and 42.2% for *BRCA2* cases and 86.7% and 41.2% for BRCAX cases (Figure 6). Clinicopathological variables (Figure 7) that were of prognostic significance for DSS included a primary tumour size >2.0 cm (HR:4.26 95%CI 1.63-11.11, p=0.003), age at diagnosis > 65 years (HR:4.09 95%CI 1.65 -10.12, p=0.002), lymphovascular invasion (HR:3.25 95%CI 1.21-8.74, p=0.019) and PNI (HR:2.82 95% CI 1.13-7.06, p=0.027) (Table 4). A strong adverse trend for loss or low progesterone receptor expression was also seen (HR:2.59 95%CI 0.86-7.80, p=0.091) but fell short of being statistically significance.

Comparisons of mutation carrier status, tumour grade, presence of nodal disease, involvement of surgical margins and multifocality were not prognositically significant (all p>0.05).

Second cancers

Ten patients had a second major malignancy (5/25 *BRCA2* mutation carriers, 5/31 BRCAX cases) (Table 5). No *BRCA1* patients developed a second malignancy. In eight (80%) cases, the diagnosis of the primary breast tumour was the sentinel event while in two cases (20%) another malignancy was diagnosed preceding the breast cancer. The median time to diagnosis was 3.8 years after the diagnosis of the breast cancer (range 3 years previous to 15.5 years after). The most common second malignancy was prostatic acinar adenocarinoma (50%, 5/10). Of note, one patient had an adenocarcinoma of the abdominal wall of unknown primary origin with exclusion of a breast metastasis. Mutation carrier status was not prognostic of development of a second malignancy when comparing *BRCA2* and BRCAX cohorts (Figure 8).
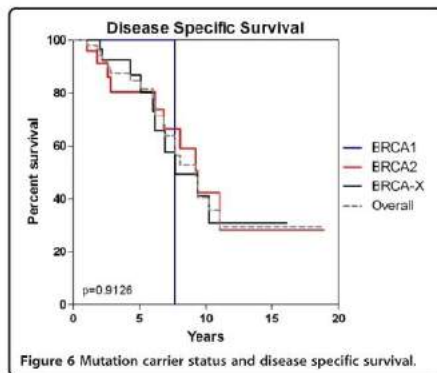
Figure 6 Mutation carrier status and disease specific survival.

Discussion

To the best of our knowledge this is the largest high-risk population based study to date describing the genotypic, conventional clinicopathological and intrinsic phenotypic characteristics of MBCs arising within breast cancer families. Previous studies have either not contained large numbers of patients with a significant family history [30,34,35,37,43,47], not commented or examined family history [6-8,28,29,36,39,40], or have contained large numbers of such cases with strong family pedigree but not described clinicopathological features [32] (Table 4). As a large proportion of MBCs are purported to arise in families with breast cancer and in particular *BRCA2* mutation carriers, further description of this cohort is of significance in understanding and characterising the disease.

The incidence of MBC in *BRCA2, BRCA1* and BRCAX males is significantly higher than the lifetime cumulative incidence of 0.1% in the general population [17,48] confirming this group as a high risk for MBC. However, the representation of carriers is different to that of familial FBC with direct comparison within the kConFab registry [41] showing an increased proportion of *BRCA2* male carriers and underrepresentation of *BRCA1* male tumours. This suggests that significant gender associated modifiers such as high estrogen levels may affect *BRCA1* penetrance over *BRCA2*. Comparing studies of sporadic MBC [6-8, 28-32,35,37-40,44], the median and mean age of onset in our patients is also younger, and this together with the observation of frequent multifocality or bilateral disease reflects the pattern of cancer often seen with underlying genetic predisposition as seen in familial FBC. A recent large population based study by Ottini *et al.* [45] containing 46 *BRCA2* mutation carriers also observed a high rate (15.2%) of contralateral breast cancer in these carriers, thus supporting this observed pattern.

Compared with other MBC groups, our study appeared to have a higher proportion of high grade tumours with only 3.3% of tumours of BRE grade I, the lowest within any MBC cohort reported to date. We also reported the highest proportion of invasive papillary carcinomas with 6.7% of cases, the next highest in the literature being 5.5% by Ottini *et al.* [45]. The histopathological tumour characteristics of our group otherwise is comparable to that seen in previous studies of sporadic MBC with the majority of cancers being invasive ductal carcinoma. This is higher than that seen in FBCs from kConFab [41]. Unlike FBC, we also observed proportionately less lobular carcinoma which is thought to reflect paucity of lobular and acinar units in males [49].

We also report a relatively higher proportion of tumours with invasive micropapillary areas particularly within *BRCA2*-associated tumours, an association not previously reported. Recent studies suggest that these

Figure 7 (See legend on next page.)

100

(See figure on previous page.)
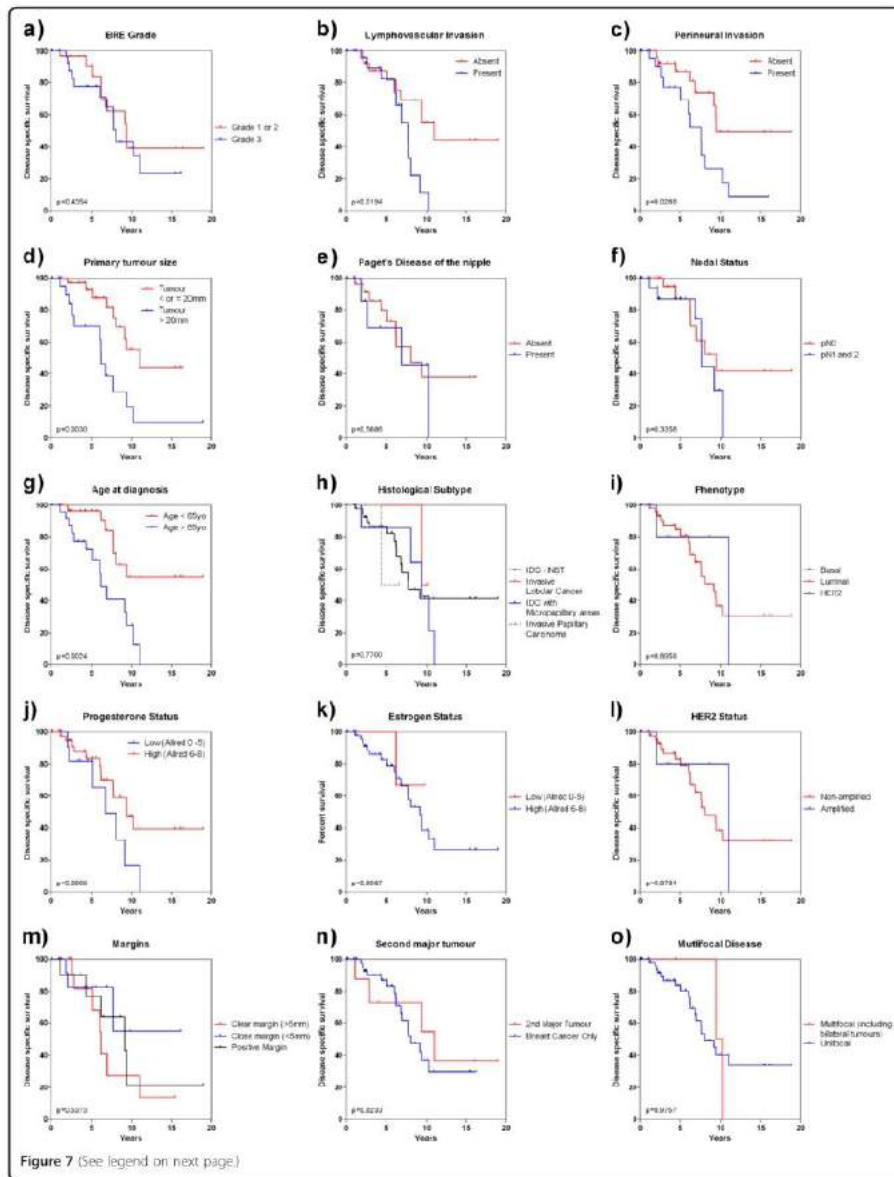**Figure 7** Clinicopathological variables and disease specific survival: (a) BRE grade, (b) lymphovascular invasion, (c) perineural invasion, (d) primary tumour size, (e) Paget's disease of the nipple, (f) nodal status, (g) age at diagnosis, (h) histological subtype, (i) Intrinsic phenotype, (j) PgR immunohistochemical expression, (k) ER immunohistochemical expression, (l) HER2 amplification, (m) involvement of margins, (n) diagnosis of second non breast primary malignancy, (o) multifocal disease.

lesions are a distinct entity with more aggressive behaviour than IDC-NST [50]. The distinct histological features of these tumours correlate with distinct molecular genetic profiles [42], however, in female cancer a correlation with *BRCA2* mutation has not been described or suggested [10]. Ottini *et al* [45], also describe a *BRCA2* MBC phenotype with a high proportion of BRE grade 3 tumours (54.8%), loss of PgR expression (67.9%) and HER2 amplification (63.2%). Similar to them, our *BRCA2* carriers contained a large proportion of BRE grade 3 but was not significantly different to the *BRCA1* and BRCAX population. The expression of ER and PgR in our familial MBCs is similar to that seen in sporadic MBC, with proportionally higher levels than seen in FBC, and absence of PgR expression did not discriminate a *BRCA2* phenotype. Subsequently, the majority of our cases were also of the luminal subtype. Reported HER2 amplification in MBC has been more variable than ER and PgR with studies demonstrating between 3.3% [40] to 28.4% [45] of cases showing HER2 amplification. While our study and Ottini are the only to date to examine the association with *BRCA* status, using routine diagnostic testing for HER2 we see lower frequency of HER2 amplification both overall (9.1%) and within our *BRCA2* carriers (8.3%) as a subgroup. Our results are consistent with most MBC studies that suggest HER2 amplification is seen half as frequently as that in FBC [41].

The few numbers of *BRCA1* MBCs in our cohort precludes extensive clinicopathological analysis, however, in contrast and unlike tumours seen in *BRCA1* female carriers [27,51], cancers of medullary/basal cell phenotype in *BRCA1* males has not been reported in the literature and was also not observed in our cohort of *BRCA1* males. The paucity of tumours of basal phenotype in our

cohort overall also reflected observations of other MBC studies.

Several prognostic markers in our study are also reported in both FBC and sporadic MBC. In our study, we confirmed many but also identified PNI as being of prognostic significance, which has not been reported previously in MBC. Its presence, being double most rates reported in FBC [52,53], may be due to frequent subareolar tumour location which is less frequently seen in women, and comparable to frequent perineural involvement seen in other epithelial tumours such as pancreatic [54] and prostatic [55] adenocarcinoma where the organs have closer proximity to nerve bundles. While mixed prognostic significance of PNI has been seen in FBC studies [53], PNI positive tumours have been shown to be more often associated with positive nodal status and hormonal positivity [53], both of which are more commonly seen in MBC in general, and in our study cohort when compared with FBC.

While our numbers are not large, a considerable proportion (16.6%) of the *BRCA2* and BRCAX patients developed a second non-breast primary malignancy. The onset or histological type of these tumours did not correlate with mutation carrier status. These findings are consistent with those previously reported in MBC cohorts where the range of second cancer incidence varies between 5.9% to 22.8% when reported [8,28,30,31,34,35]. Notably, the studies with higher rates of breast cancer families such as Ding [31] (60% either *BRCA2* pathogenic mutation carrier or strong family history of breast cancer), Liukkonen[35] (33.1% with significant familial history) and Kiluk [34] (29% with significant familial history) had 22.8%, 19% and 19.4% of their patients reporting a second primary respectively. Of the types reported, prostate cancer was the most common followed by bladder cancer, a tumour type not seen in our cohort. In recent studies we and others have demonstrated the relative risk for developing prostate cancer in male *BRCA2* mutation carriers as between 2.9 to 4.8 times the general population [56-59]. Comparing our study with the age related rate of Australian males in the 60–64 year age group, there is an increased relative risk of prostate cancer of 19.08 (p<0.0001, 95%CI 4.50-80.91) and 20.56 (p<0.0001, 95%CI 6.30-67.12) times the normal population for *BRCA2* and BRCAX male patients with breast cancer respectively. These data show that patients with MBC may be a high-risk group for developing second malignancies, even when comparing with BRCA2 carriers

Table 4 Clinicopathological variables of prognostic significance

Variable	P-value	Hazard's ratio	95% confidence interval
Lymphovascular Invasion	0.0194	3.25	1.21 - 8.74
Perineural Invasion	0.0266	2.82	1.13 - 7.06
Tumour Size > 20mm	0.0030	4.26	1.63 - 11.11
Age of Diagnosis > 65 years	0.0024	4.09	1.65 - 10.12
Low Progesterone Receptor Expression	0.0909	2.59	0.86 - 7.80

101

Table 5 Second non-breast primary malignancies

Gene	Mutation	2nd tumour	Diagnosis relative to Breast Primary (years)
BRCA2	BRCA2 2988 del C (STOP 959)	Ascending Colon - Adenocarcinoma	15.5
BRCA2	BRCA2 698_702 del AGTCA (STOP 180)	Prostate - Acinar Adenocarcinoma	0.8
BRCA2	BRCA2 8168_8169 ins C (STOP 2661)	Prostate - Acinar Adenocarcinoma	1.4
BRCA2	BRCA2 del exons 1_27	Parotid gland - Oxyphilic adenocarcinoma	6.2
BRCA2	BRCA2 IVS 7–1 G>A	Lung - squamous cell carcinoma	13.8
BRCAX		Adenocarcinoma - unknown primary	3.0
BRCAX		Lung – Carcinoma not otherwise specified.	9.4
BRCAX		Prostate - Acinar Adenocarcinoma	9.5
BRCAX		Prostate - Acinar Adenocarcinoma	3.0 years prior to breast cancer
BRCAX		Prostate - Acinar Adenocarcinoma	1.3 years prior to breast cancer

without MBC. Whether this is due to hormonal influence driving both tumour types or underlying genetic factors requires further study in a larger data set.

Conclusions

This is the largest clinicopathological study of male breast cancers arising in breast cancer families. It identifies three high-risk population groups (*BRCA1/2*, BRCAX) which may be important for screening for male breast cancer. The clinical and pathological characteristics are different to familial female breast cancer but similar to previously described male breast cancer studies which have contained but not separately analysed sporadic and familial breast cancers. Notably, our study in comparison contains proportionately more multifocal disease, a younger age of onset and a significant proportion with a second major malignancy, features often seen in tumours that arise with a genetic predisposition. *BRCA2* mutation status did not appear to correlate with a distinct clinicopathological phenotype or disease behaviour, and a strong trend was seen within *BRCA2* carrier tumours containing areas of micropapillary carcinoma possible suggesting a possible *BRCA2* male breast cancer phenotype. Further subgroup analysis, in particular of *BRCA1* tumours, was limited by the number of cases available. Further recruitment of well characterised tumours in breast cancer families, in particular a focused collection of *BRCA1* cases, is warranted to validate and characterise familial MBC further.

Additional files

Additional file 1: Table S1. Eligibility criteria for recruitment of families into kConFab.

Additional file 2: Figure S1. Distribution of ER Allred histoscores.

Additional file 3: Figure S2. Distribution of PgR Allred histoscores.

Additional file 4: Table 2. Comparison of previous MBC studies.

Abbreviations
MBC: Male Breast Cancer; FBC: Female Breast Cancer; DSS: Disease Specific Survival; ER: Estrogen Receptor; PgR: Progesterone Receptor; CK: Cytokeratin; EGFR: Epidermal Growth Factor Receptor; IDC-NST: Invasive Ductal Carcinoma of No Special Type; PNI: Perineural Invasion; BRE: Bloom Richardson Ellis.

Competing interests
The authors declare they have no competing interests.

Authors' contributions
SD – Pathology review of all cases, conception and design of study, analysis and interpretation of data, statistical analysis, manuscript preparation, NJ – Immunohistochemistry and ISH, kConFab Investigators – BRCA1/2 testing, acquisition of all data, manuscript preparation, SBF – Conception and design of study, analysis and interpretation of data, manuscript preparation. All authors read and approved the final manuscript.
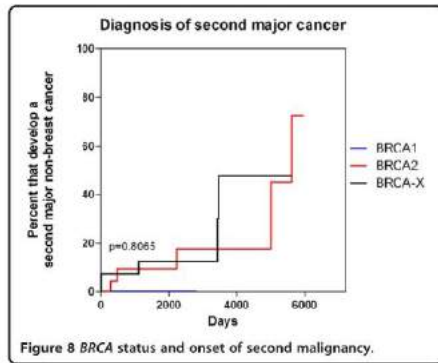
Figure 8 *BRCA* status and onset of second malignancy.

102

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103

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104

3.3.2 Additional file 1: Eligibility criteria for recruitment of families into kConFab.

<u>CATEGORY 1.</u> **Families in which no predisposition mutation has been identified.** All criteria are required:	At least one member of the family at high risk according to the National Breast Cancer Centre Category 111 guidelines (www.nbcc.org.au)
	Four or more cases of breast or ovarian cancer (on one side of the family)
	Two or more living affected with breast or ovarian cancer
<u>CATEGORY 2.</u> **Families in which a *BRCA1* or *BRCA2* mutation has been identified (pathogenic, splice site or unclassified variant).** kConFab will recruit all families in which there are at least two or more living potential female mutation carriers (affected and/or unaffected) amongst first- and second-degree relatives from the informative side of the family.	Mutation status of potential carries may be unproven, but simply predicted by Mendelian inheritance
	There does not need to be a living affected potential carrier
	One or both potential carriers can be unaffected
<u>CATEGORY 3.</u> **Families with mutations in other breast cancer predisposition genes.** A small number of pedigrees submitted by the FCC have some features of other cancer syndromes that include breast cancer and are of interest to kConFab because they carry mutations in PTEN, TP53 or ATM. Families that carry pathogenic mutations in	

these genes and have two or more living carriers, or potential carriers are eligible for enrolment into kConFab. Families must carry a mutation to be enrolled into Category 3. *If the clinical features suggest a relevant cancer syndrome (e.g. LiFraumeni Syndrome) but no mutation has been identified, the family can only be enrolled if they fulfil the Category 1 criteria.*	
CATEGORY 4. High risk breast cancer families from which tumour is available but who do not fit other kConFab criteria. Families that fit the National Breast Cancer Centre Category 111 guidelines but do not fit category 1, 2 or 3 above are of value to kConFab if a family member wishes to enroll in kConFab and consent for a portion of their tumour (breast and ovarian) to be collected from surgery and used for research.	Families are recruited through category 4 if a woman is having surgery for a <u>suspected tumour</u>. Normal tissue is collected that is prophylactically removed at the time of this surgery for a suspected tumour
	Prophylactic mastectomies are collected from women already enrolled in kConFab
	Prophylactic oophorectomies have been previously collected but no longer currently.

3.3.3 Additional file 2: Figure S1. Distribution of ER Allred histoscores.
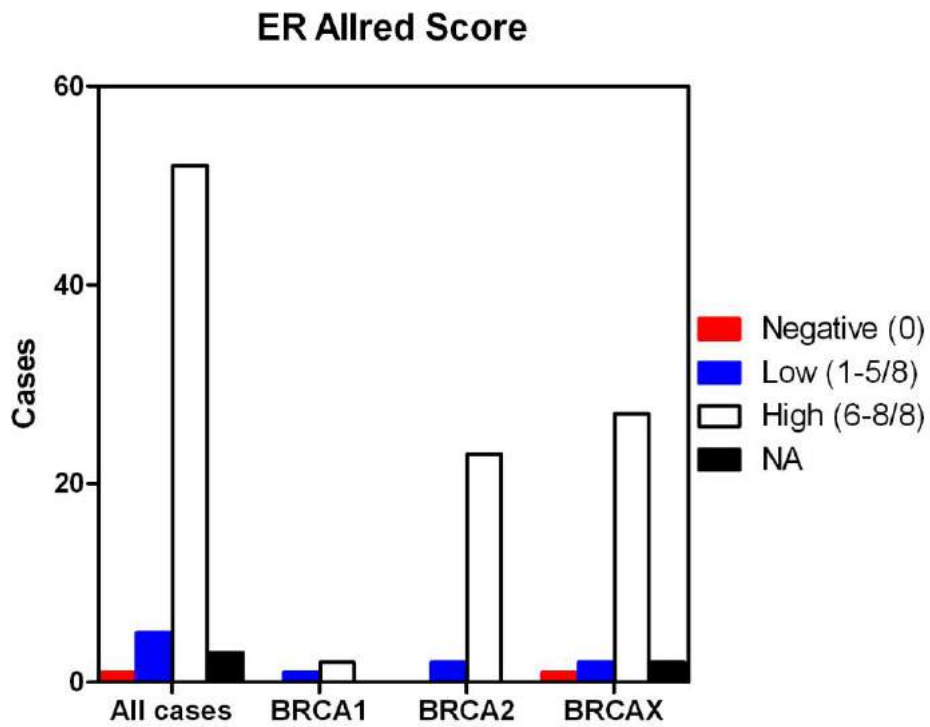
ER Allred Score

3.3.4 Additional file 3: Figure S2. Distribution of PgR Allred histoscores

PgR Allred Score
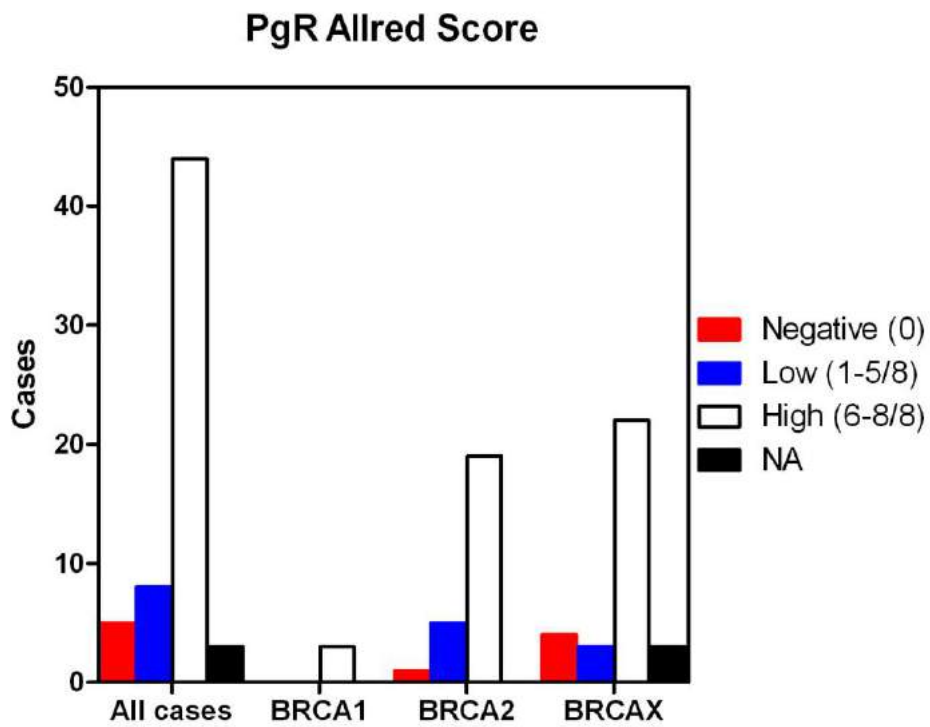
1.3.3 Additional file 4: Table 6. Comparison of previous MBC studies.

Study	Loughrey [49] - FBC	Current Study	p-value (Loughrey vs current study)	Ottini [38]	Arsalan [28]	Anderson [7]	Nahleh [37]	Foerster R [8]	Cutuli [30]	Kiluk [34]	Nilsson [6]	Evans [32]	Ding [31]	Bourhafour [29]	Shaaban [39]	Miao [36]	Johansson [43]	Zhou [40]	Liukkonen [35]	Marchal [45]	Ottini [46]
Database/Location	kCONFAB, Australia/New Zealand	kCONFAB, Australia/New Zealand		Italy	Turkey	SEER Database, USA	Veterans Affairs Central Cancer Registry, USA	Germany	France	USA	Sweden	UK	USA	Morocco	UK	Multi national**	Sweden	China	Finland	France	Italy
Patient Accrual	High-risk population	High-risk population		Population based	Population based	Population based	Veterans	Population based	Population based	Population based	Population based	High-risk population	Population based	Population based	Population based	Population based	Population based	Population based	Population based	Population based	Population based
Cases	180	60		108	118	5,494	612	108	489	62	99	64	115	127	251	2,665	56	72	58	58	382
Familial Cancers	100.0%	100.0%		25.9% (breast/orvarian)	-	-	26.5%	-	18.0%	29.0%	-	-	47.80%	-	-	-	-	-	31.4%	-	36.8%
BRCA1 mutation carriers	14.4%	5.0%	0.0001	1.9%	-	-	-	-	0.6%	-	-	6.3%	-	-	-	-	-	-	0.0%	-	1.1%
BRCA2 mutation carriers	8.3%	41.7%	0.0008	7.4%	-	-	-	-	0.4%	-	-	26.6%	39.1% (13.9% Pathogenic, 12.2% Unknown, 13.0% Neutral)	-	-	-	1.8%	-	1.7%	-	12.2%
Age																					
Median	-	62.5	-	65	60.5	-	-	67	66	68.8	-	-	68.1	-	66	-	-	61	63	-	-
Mean	-	60.0	-	63.2	-	65.8	67	-	-	-	-	62.5	-	62	-	69.6	68	-	-	-	-
Range	25-90	30-86	-	24-90	29-83	10-104	-	43-89	24-94	29-85	-	-	28-93	32-91	30-94	-	42-92	-	35-91	31-86	-
Survival	-	5yr DSS - 85%	-	-	5yr OS - 82%	-	-	5yr OS - 71.4%	5yr DSS - 89%, 5yr OS - 81%	5 yr OS - 80.0%	5 yr DSS - 74%, OS - 41%	-	-	5 yr OS - 63%	-	5 yr DSS - 72%	-	5 yr DSS - 72%	5yr - 72.4%	5yr - 75%	5yr DSS - 73.0%	-
Multifocal/Bilateral Cancer	-	6.7%	-	2.8%	-	-	0.5%	-	1.1%	1.6%	-	-	-	-	-	-	-	0	-	1.7%	4.1%
Size	-	Mean 18.3mm, Median 17mm	-	-	-	Mean - 24mm, Median 20mm	Median - 20mm	-	-	Mean - 22mm, Median 16mm	-	-	32.1% > 20mm	-	-	-	-	-	Median 18mm	-	-
Tumour Grade							6870.0%														
G1	20.6%	3.3%	0.0010	19.0%	10.7%	-	16.2%	6.5%	22.4%	16.4%	16.2%	-	26.5%	18.0%	10.7%	-	8.2%	-	-	5.2%	12.5%
G2	36.1%	51.7%	0.0473	52.6%	59.5%	-	51.8%	63.3%	50.6%	50.9%	47.1%	-	48.0%	G2+3=82.0%	54.7%	-	59.5%	-	-	48.3%	57.7%
G3	43.3%	45.0%	NS	28.4%	29.8%	-	31.9%	29.6%	20.0%	32.7%	36.8%	-	25.5%		34.7%	-	32.4%	-	-	36.2%	29.8%
Histological Subtype																					
IDC	63.9% (40-75%)*	90.0%	0.0001	78.2%	90.6%	-	74.2%	79.6%	94.5%	93.5%	-	-	79.1%	96.1%	82.9%	-	-	81.9%	94.8%	86.2%	87.0%
Papillary	- (<1-2%)*	6.7%	-	5.5%	-	-	4.0%	-	1.6%	-	-	-	-	-	4.4%	-	-	2.8%	-	-	-
Lobular	10.5% (5-15%)*	3.3%	NS	2.7%	2.5%	-	4.2%	4.6%	1.6%	-	-	-	1.8%	0.8%	0.4%	-	-	4.2%	3.4%	-	1.4%
Medullary/Atypical Medullary	12.8% (1-7%)*	0.0%	0.0016	-	-	-	0.5%	-	0	-	-	-	0.9%	-	-	-	-	2.8%	-	-	0.3%
Not specified/Others	-	-	-	13.60%	6.9%	-	-	12.0%	-	3.2%	-	-	14.6%	-	6.8%	-	-	8.4%	-	13.8%	4.0%
ER positive***	62.6%	89.7%	0.0001	87.5%	82.9%	92.40%	60.1%	65.7%	94.3%	100%	93.2%	-	92.3%	63.9%	90.1%	-	78.6%	90.9%	100%	95.2%	91.4%
PR positive***	58.2%	77.2%	0.0132	79.2%	75.8%	-	53.1%	63.9%	89.0%	-	79.7%	-	82.0%	63.9%	81.9%	-	69.6%	84.8%	79%	85.4%	83.9%
HER2 Amplification	-	9.1%	-	18.7%	23.4%	-	-	6.5%	-	-	12.7%	-	24.0%	-	-	-	3.6%	3.3% IHC 3+	10.5%	-	28.4%
Triple Negative (ER-,PR-,HER2-)	-	0%	-	-	-	5.9%	-	-	4.8%	-	-	-	-	-	1.6%	-	-	6.6%	-	-	-
Lymph node involvement	-	43.4%	-	-	-	50.0%	42%	46.5%	43.6%	52.8%	39.7%	37.3%	-	64.5%	48.7%	-	-	33.9%	47% (1.7 nodes involved on average)	-	42.0%
2nd Non-Breast Primary Tumour	-	16.6% (Prostate most common)	-	14.8% (Prostate, Bladder most common)	-	5.9%	-	19.4%	9.2%	-	-	-	19.4% (Prostate most common)	22.8% (Prostate, Bladder most common)	-	-	-	-	19%	12.1% (prostate most common)	15.1%
Other	-	15.4% Paget's Disease, 11.6% Gynaecomastia, 75.0% DCIS or intraductal component	-	1.8% Paget's Disease	-	-	-	-	41% of invasive tumours had DCIS/Intraductal component	14.3% Gynaecomastia	-	-	-	2.5% Paget's Disease, 4% Gynaecomastia	-	-	19.6% of invasive tumours had DCIS	27.4% Skin or nipple involvement	-	-	-

Chapter 4 - Evaluation in male breast cancer of common known drivers from female breast cancer.

This chapter is composed of two papers:

1) *PIK3CA* mutations are frequently observed in BRCAX but not *BRCA2* –associated male breast cancer. Siddhartha Deb, David Byrne, Nicolas Jene, kConFab Investigators, Alexander Dobrovic, Stephen B Fox. Breast Cancer Research. 2013 August 23; 15(4): R69.

2) Nuclear HIF1A expression is strongly prognostic in sporadic but not familial male breast cancer. Siddhartha Deb, Ida Johansson, David Byrne, Cecilia Nilsson, kConFab Investigators, Leonie Constable, Marie-Louise Fjällskog, Alexander Dobrovic, Ingrid Hedenfalk, Stephen B. Fox. Mod Pathol. 2014 Sep;27(9):1223-30. doi: 10.1038/modpathol.2013.231. Epub 2014 Jan 24.

Supplementary/additional figures and tables from the articles are present at the end of each published article.

4.1 Aims and rationale

A large proportion of MBC research to date has focused on cancer predisposition in MBCs. Other studies suggest that MBC and FBC may be different with distinct MBC subsets and with unique genophenotypic correlations. As the management and treatment of MBCs is largely extrapolated from practices used in FBC, and the

outcome of MBCs has remained stagnant and may be worse than FBCs, characterization and identification of significant drivers in MBCs also has important implications for the screening and treatment of MBCs.

The aim was to characterize the *PIK3CA*/mTOR pathway and hypoxia inducible factors in a series of familial and sporadic MBCs. As the RAS/RAF/MAPK pathway also intersects the *PIK3CA*/mTOR pathway at multiple points(235), MBCs were evaluated for activating *KRAS* and *BRAF* mutations.

Of the several well characterized oncogenic drivers in FBC, the most frequent gain of function mutation is seen in *PIK3CA(150)*. Subsequent activation of the mTOR pathway is seen leading to cell proliferation, angiogenesis and promotion of metastasis(236).

Clinically, *PIK3CA* mutations are more frequently seen in ER-α positive luminal A tumors, the phenotype which is most commonly seen in MBCs. It may be that *PIK3CA* mutations may also have clinical implications in *BRCA1*/2 deficient tumors, as *in vitro* studies propose activation of the *PIK3CA*/mTOR pathway may confer resistance to the PARP inhibitors commonly used to treat these tumors. Prior to this study, only one study has evaluated *PIK3CA* mutation in a series of 39 MBC with unknown *BRCA* status(140). The authors showed exclusively exon 20 mutations in their MBCs occurring at a comparable frequency to the FBC cohort from their study, which showed an even distribution of exon 9 and 20 mutations.

The aims in the study were to: 1) identify *PIK3CA*, *AKT1*, *KRAS* and *BRAF* mutations in familial male breast cancer, 2) assess the relationship between such somatic gene mutations and clinicopathological factors including *BRCA1*/2 mutation carrier status, and 3) identify and characterize the *PIK3CA*/mTOR and MAPK pathway and correlate with clinicopathological factors and survival.

It is now well established that cancer hypoxia is an important contributor to carcinogenesis and cancer phenotype. As a response to hypoxia in the tissue microenvironment, induction of hypoxia-related proteins leads to transcription of a myriad of genes involved in iron metabolism, erythropoiesis, angiogenesis, activation of the glycolytic pathway and activation of MAPK and PI3K signaling pathways(237). This leads to cell proliferation and survival but also paradoxically apoptosis in some instances. Clinically, overexpression of these proteins in cancer is associated with poor prognosis, increased treatment resistance and increased tumor associated mortality(238, 239). Of the multiple known proteins, HIF1A and CA9 expression was studied in MBCs. These proteins have been shown to be prognostic and are associated with *BRCA1* loss, basal-like breast cancer and HER amplification in FBCs(240). A single MBC study on unselected patients with unknown *BRCA* status has been performed where these proteins have been shown to have prognostic significance(176). As there is no data from separate familial and sporadic MBC cohorts, and with ongoing development of hypoxia protein based targeted therapies, the aim was to: 1) characterize HIF-1a and CAIX levels in a large cohort of sporadic and familial MBCs, 2) correlate expression with conventional clinicopathological parameters and intrinsic phenotypes, 3) investigate expression in familial breast

cancers stratified by cancer mutation status, and 4) evaluate the prognostic significance of HIF-1a and CAIX expression on disease specific survival.

4.2 Summary

At the time of the publication of these two papers, they were the largest studies examining components of the mTOR/MAPK pathway and expression of hypoxia proteins in MBCs. They are also the only articles examining these two areas in familial MBC.

The findings show that the mTOR pathway may be a significant driver in non-*BRCA2* cancers. Activating mutations of *PIK3CA* were seen in MBCs, however, there were no *AKT1*, *KRAS* or *BRAF* mutations seen. Several novel observations were made: 1) Exon 9 *PIK3CA* mutations in MBCs were seen in addition to previously demonstrated exon 20 mutations, 2) mutation profiling in BRCAX MBCs showed a frequency of *PIK3CA* mutations similar to that seen in sporadic MBC and slightly lower than in FBCs, 3) *PIK3CA* mutations may be infrequent in MBCs arising in *BRCA2* mutation carriers, 4) two rare E547K mutations were detected which has only been detected in one FBC previously(142), and 5) and a case with two concurrent exon 9 mutations was also seen, which has not been previously reported in male breast cancers. Given the high penetrance of MBCs seen in *BRCA2* mutations carriers and the emergence of a *BRCA2* phenotype, these data support the proposition that *BRCA2* MBCs may be a distinct subgroup and that *BRCA2* loss is a significant and powerful driver in MBC development. Interestingly, the study showed an upregulation of phosphorylated 4EBP1 in *BRCA2* mutation carriers compared to BRCAX patients, suggesting alternate *PIK3CA*/mTOR pathway activation may occur in *BRCA2* cases. This is

possibly due to disordered homologous recombination, which *in vitro* has been shown to activate *PIK3CA*/mTOR pathway interactions to maintain homologous recombination steady state.

The absence of *AKT1*, *BRAF* and *KRAS* mutations is in line with the relatively low frequency seen in FBC. However, it is in contrast to one other study of MBCs by Dawson *et al*. which showed a *KRAS* mutation rate of 12% in MBCs(141). This may be due to methodological reasons and improved sequencing techniques for paraffin embedded materials.

The study of hypoxia markers HIF1a and CA9 showed differences in hypoxic effect between male and female breast cancers and between familial and sporadic male breast cancers. Compared to FBC(240), the frequency of HIF1a and CA9 expression is almost half in MBCs. The association with *BRCA1* tumors in FBC is not seen in this MBC cohort, albeit with only 3 *BRCA1* MBCs examined. The association between hypoxia protein expression and tumors with basal cell phenotype seen in FBCs(240) also appears to be present in MBCs. These data suggest that hypoxia does not occur as frequently in the male breast microenvironment as it does in the female breast, and may contribute to the less frequent HER2 and basal phenotypes that are seen in MBCs (both phenotypes which are associated with hypoxic drive in FBCs), and to the lower than expected penetrance of tumors in *BRCA1* male mutations carriers (when compared to *BRCA2* mutation carriers). seen.

Expression of CA9 correlated with an older age of onset and increased tumor size while HIF1a expression was inversely correlated with luminal phenotype.

Interestingly, males with high HIF1a and/or CA9 expression in their tumors were twice more likely to develop a second malignancy. This phenomenon had not been noted in any tumor studies to date and while patients with MBCs have been shown to have relatively high rates of second malignancies, the frequency of second malignancies reported in the hypoxia marker positive tumors is almost 50% higher than the highest reported rates. Differences are also seen between sporadic and familial MBC subgroups, with more frequent expression seen in sporadic MBCs. HIF1a was also only shown to be prognostic in sporadic MBCs, having no effect in familial MBCs.

Breast Cancer
R E S E A R C H

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PIK3CA mutations are frequently observed in BRCAX but not BRCA2 -associated male breast cancer

Siddhartha Deb[1,2*], Hongdo Do[1], David Byrne[1], Nicholas Jene[1], kConFab Investigators[3], Alexander Dobrovic[1,2] and Stephen B Fox[1,2]

Abstract

Introduction: Although a substantial proportion of male breast cancers (MBCs) are hereditary, the molecular pathways that are activated are unknown. We therefore examined the frequency and clinicopathological associations of the PIK3CA/mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase (MAPK) pathways and their regulatory genes in familial MBC.

Methods: High resolution melting analysis and confirmatory sequencing was used to determine the presence of somatic mutations in PIK3CA (exon 9 and 20), AKT1 (exon 4), KRAS (exon 2) and BRAF (exon 15) genes in 57 familial MBCs. Further analysis of the PIK3CA/mTOR pathway was performed using immunohistochemistry for the pAKT1, pS6 and p4EBP1 biomarkers.

Results: PIK3CA somatic mutations were identified in 10.5% (6 of 57) of cases; there were no AKT1, KRAS or BRAF somatic mutations. PIK3CA mutations were significantly more frequent in cancers from BRCAX patients (17.2%, 5/29) than BRCA2 (0%, 0/25) carriers ($P = 0.030$). Two BRCAX patients had an E547K mutation which has only been reported in one female breast cancer previously. PIK3CA mutation was significantly correlated with positive pS6 (83.3% vs. 32.0%, $P = 0.024$) and negative p4EBP1 (100% vs. 38.0%, $P = 0.006$) expression, but not pAKT expression. Expression of nuclear p4EBP1 correlated with BRCA2 mutation carrier status (68.0% vs. 38.7%, $P = 0.035$).

Conclusions: Somatic PIK3CA mutation is present in familial male breast cancer but absent in BRCA2 carriers. The presence of two of the extremely rare E547K PIK3CA mutations in our cohort may have specific relevance in MBCs. Further study of PIK3CA in MBCs, and in particular BRCAX patients, may contribute to further establishing the relevance of specific PIK3CA mutations in MBC aetiology and in the identification of particular patient groups most likely to benefit from therapeutic targeting with the novel PIK3CA inhibitors that are currently in development.

Keywords: PIK3CA, E547K, mTOR, familial, male breast cancer, BRCA2, BRCAX

Introduction

Recent studies characterising male breast cancer (MBC) show that these rare tumours are very different to their female counterparts [1,2]. In particular, there are notable distinctions between familial female and MBC with a different pattern of penetrance and genotypic phenotypic correlation in BRCA1, BRCA2 and BRCAX subsets [1]. While it is likely that hormonal influence is a significant

contributor, as yet, the characterisation of oncogenic drivers by mutation analysis of even the most common gene mutations in MBCs has not been undertaken.

Several significant targetable oncogenes are known and relatively well described in female breast cancer (FBC). The most frequent gain of function mutations is seen in phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha 9 (PIK3CA) which forms one of the catalytic subunits of the phosphatidylinositol 3-kinase (PI3K) holoenzyme [3,4]. Mutations of the helical or kinase domain lead to activation of the p110a kinase with subsequent downstream triggering of the mammalian target of

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rapamycin (mTOR) leading to cell proliferation, angiogenesis and promotion of the metastatic process [5,6]. Additional regulators of the PIK3CA/mTOR pathway include *AKT1* and the RAS/RAF/mitogen-activated protein kinase (MAPK) pathway that intersect at multiple points [7-13].

Within FBC, the prevalence and prognostic significance of tumours with these driving mutations are unclear and may be dependent on both tumour histological type and estrogen receptor (ERα) status [14-17]. Notably, *in vitro* studies propose that activation of the PIK3CA/mTOR pathway may be important in tumours with deficient homologous recombination [18], suggesting a possible role in gaining resistance to poly ADP ribose polymerase (PARP) inhibitors in *BRCA1/2* deficient tumours. However, although there are limited data ($n = 22$), an association between *BRCA1/2* loss and activation of the PIK3CA/mTOR pathway in human tumours has not been confirmed [15].

Despite accruing data in FBC as to the significance of these oncogenes, there are few studies examining somatic mutation in sporadic MBC only [19-23], with the majority of studies focused on gene expression profiling [24-26] and germ-line mutational analysis [27-32].

Since the PIK3CA/mTOR pathway is more frequently associated with ERα positive FBC, and MBC is largely characterised by ERα positive disease, we have examined the frequency of activation of the PIK3CA/mTOR pathway and its regulators in a cohort of 57 familial MBCs. While the reported frequency of *KRAS* and *BRAF* mutations in female breast cancer is generally low (<5%) reference [33,34], a single sporadic MBC study showing a markedly high percentage of *KRAS* mutations (12%) also encouraged investigation of the mitogen-activated protein kinase (MAPK) pathway, which also interacts with the PIK3CA/mTOR pathway. Our aims were to; (1) identify *PIK3CA, AKT1, KRAS* and *BRAF* mutations in familial male breast cancer, (2) assess the relationship between such somatic gene mutations and clinicopathological factors, including *BRCA1/2* mutation carrier status, and (3) identify and characterise the PIK3CA/mTOR and MAPK pathway and correlate with any clinicopathological factors and survival.

Materials and methods
Patient samples

Only primary breast cancers were examined in this study. Cases were obtained from the kConFab repository [35]. Prerequisites for cases to be included into kConFab are a strong family history of breast and ovarian cancer (Breast and Ovarian Analysis of Disease Incidence and Carrier Estimation Algorithm (BOADICEA) scores [36] generated from family pedigree and stratified by *BRCA1/2* mutation carrier status included as Additional file 1: Supplementary figure 1) with criteria for admission to the kConFab study

as outlined previously [37]. Cases were from Australia and New Zealand and diagnosed between 1980 and 2009.

The flow of patients through the study according to the REMARK criteria [38] is listed in Additional file 2: Supplementary table 1. Of the 118 cases within the kConFab registry, 58 cases were excluded due to unavailability of tissue. Of the 60 cases where tissue was available, 2 cases had insufficient tumour tissue for DNA extraction or for a core to be taken for assembly of a tissue microarray (TMA) and a further single case had an extremely low DNA yield and insufficient material for tissue microarray. Fifty seven cases had sufficient material at an appropriate DNA concentration for somatic mutation testing and one case did not have adequate tissue for TMA construction with all tissue committed to DNA extraction. Clinical parameters, including disease specific mortality were obtained from referring clinical centres, kConFab questionnaires and state death registries. Information on pedigree, mutational status and testing were available from the kConFab central registry. Histological classification was based on criteria set by the World Health Organization 2012 [39] and all slides and pathological records from all cases were reviewed for tumour size, tumour grade, lymphovascular and perineural invasion. Immunohistochemistry for ERα, progesterone receptor (PgR), basal markers (cytokeratin (CK) 5, epidermal growth factor receptor (EGFR)) and HER2 silver *in situ* hybridisation (SISH) had been performed as previously reported [1]. Using stratification of intrinsic phenotypes based on Nielsen *et al.* [40], tumours were placed into luminal (ERα positive, HER2 negative, CK5 and/or EGFR negative or positive), basal (HER2 and ERα negative; CK5 and/or EGFR positive), HER2 (HER2 positive, ERα, CK5 and EGFR negative or positive) and null/negative (HER2, ERα, CK5 and EGFR negative) phenotypes. This work was carried out with approval from the Peter MacCallum Cancer Centre Ethics Committee (Project No: 11/61). The approval included waiver of patient consent.

Germline *BRCA1/2* testing

Mutation testing for *BRCA1* and *BRCA2* mutations was performed as reported previously [1]. Testing of index cases in kConFab families was carried out by denaturing high performance liquid chromatography or multiplex ligation-dependent probe amplification. Once the family mutation had been identified, all pathogenic (including splice site) variants of *BRCA1* and *BRCA2* were genotyped by kConFab in all available family members' DNA.

High-Resolution Melting (HRM) assay

Genomic DNA was extracted from formalin-fixed, paraffin embedded (FFPE) samples. A 3 µM haematoxylin and eosin (H&E) stained slide was cut from FFPE blocks and stained to identify tumour enriched areas. From the

116

relevant area on the FFPE block, a 2 mm punch biopsy core was taken. The cores were then dewaxed and hydrated through gradient alcohol. Genomic DNA was then extracted using the DNeasy Tissue kit (Qiagen, Valencia, CA, USA)) following proteinase K digestion at 56°C for three days.

The PIK3CA, AKT1, BRAF and KRAS primer sequences are shown in Additional file 3: Supplementary table 2. PIK3CA exon 9 and 20 primers produced amplicons with 104 base pairs (bp) and 102 bp, respectively. AKT1 exon 4, BRAF exon 15 and KRAS exon 4 primers produced 78 bp, 144 bp and 92 bp amplicons, respectively. PCR for HRM analysis was performed in 0.1 ml tubes on a Rotor-Gene Q (Qiagen) utilising the fluorescent DNA intercalating dye, SYTO 9 (Invitrogen, Carlsbad, CA, USA). A 20 μL final reaction volume contained 1 × PCR buffer, 0.5 to 2.0 mM MgCl2, 200 to 400 nM of forward and reverse primer, 200 μM of dNTPs, 5 μM of SYTO 9, 0.5 U of HotStarTaq polymerase (Qiagen), 5 ng of genomic DNA, Uracil-DNA glycosylase (UDG) (0.5 units/reaction), UDG buffer (New England BioLabs, Ipswich, MA, USA) and PCR grade water. The cycling and melting conditions are shown in Additional file 3: Supplementary table 2. All reactions had initial UDG treatment for FFPE artefacts at 37°C for 30 minutes [41], followed by an incubation step at 95°C for 15 minutes, denaturation step at 95°C, annealing steps at the temperatures listed in Additional file 3: Supplementary table 2, and an elongation step at 72°C. A single cycle of 97°C for one minute preceded a melt phase run between temperatures listed in Additional file 3: Supplementary table 2 and rising 0.2°C per step. Samples were run in duplicate. HRM analysis was performed on the Rotor-Gene Q Software (v1.7) (Qiagen, Valencia, CA, USA).

DNA sequencing

All samples with either or both duplicates showing abnormal melt were sequenced for detection of mutations. PIK3CA exon 9 and 20 HRM products were amplified using M13 tagged primers (Additional file 3: Supplementary table 2) initially and then M13 primers for a second step for PIK3CA exon 9 (amplicon 185 bp) and a single step PCR reaction for PIK3CA exon 20 (amplicon 149 bp) using primers listed in Additional file 3: Supplementary table 2. The composition of a total reaction mixture of 20 μL contained; 1 × PCR buffer, 2.5 mM MgCl2, 400 nM of each primer, 200 μM of dNTPs, 0.5 U of HotStarTaq polymerase (Qiagen), 5 ng of HRM DNA products and PCR grade water. The PCR conditions were as follows: an initial incubation at 95°C for 1 minute, followed by 35 cycles of 95°C for 10 seconds, 55°C for 10 seconds and 72°C for 4 minutes. The sequencing reaction was then performed using the Big Dye

Terminator v3.1 chemistry according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA) using 6 μL of the PCR products that were purified with 2 μL of ExoSapIT (GE Healthcare, Little Chalfont, Buckinghamshire, UK). After ethanol precipitation, the sequencing products were run on a 3700 Genetic Analyser (Applied Biosystems). The sequencing data were then analysed using Sequencher 4.6 (Gene Codes Corporation, Ann Arbor, MI, USA). Each mutation was confirmed by sequencing a second independent PCR reaction. The work flow is outlined in Figure 1.

Immunohistochemistry

Tumour-tissue microarrays (1-mm cores), with a two-fold redundancy, were prepared from archival FFPE tissue blocks. TMA sections were cut from each block at 4 μm thick intervals, dewaxed, placed through graded alcohol and then into water.

For phosphorylated 4EBP1 (p4EBP1) and phosphorylated S6 (pS6), antigen retrieval was performed using high pH antigen retrieval buffer (DAKO, Glostrup, Denmark) in pressure cooker for three minutes at 125°C. For phosphorylated AKT1 (pAKT), antigen retrieval was performed with CC1 high pH retrieval solution (Roche, Basel, Switzerland) at 100°C for 36 minutes. Staining for p4EBP1 (dilution 1:400, clone 2855, Cell Signalling Technology, Danvers, MA, USA) and pS6 (dilution 1:200, clone 2211, Cell Signalling Technology) was performed using a monoclonal and polyclonal rabbit antibodies respectively. Antigen-antibody complex was detected using the Envision FLEX system (EnVision FLEX/HRP and EnVision FLEX DAB + Chromogen, DAKO). Staining for pAKT1 (dilution 1:1,000, clone LP18, Novocastra, Newcastle upon Tyne, UK) was performed using a monoclonal mouse antibody with secondary detection using Ventana Ultraview detection reagents (Roche). Slides were then counterstained with haematoxylin, dehydrated, cleared and mounted for assessment. Phosphorylated 4EBP1 expression was assessed for both cytoplasmic and nuclear expression, nuclear expression for pAKT1 and cytoplasmic expression for pS6 (Figure 2a). A histoscore was generated by multiplying staining intensity (0, no staining; 1, weak; 2, moderate; 3, strong) by the percentage of positive tumour cells (0, 0; 1, < or = to 25%; 2, >25% to 50%; 3, >50% to 75%, 4, >75%). The histoscores ranged between 0 and 12. For subsequent analysis, histoscores were categorised into either absent (histoscore = 0) or present (1 to 12) or low (0 to 3) and high (4 to 12) to differentiate from baseline staining of adjacent normal breast epithelium.

A PIK3CA mutation phenotype was defined by either a tumour harbouring a somatic PIK3CA activating mutation or showing an absence of p4EBP1 expression

117

Figure 1 Work flow of sample analysis. Initial High-Resolution Melting (HRM) analysis was used to screen mutation by abnormal melt. Sanger sequencing was then performed to detect specific mutations.

and moderate to strong pS6 expression (histoscore 4-12/12) on immunohistochemistry.

Statistical analysis

Comparison of groups was made using Mann-Whitney U for non-parametric continuous distributions and chi-square test for threshold data. Kaplan-Meier survival curves were plotted using breast cancer related death as the endpoint and compared using a log rank test. Analysis was performed with GraphPad Prism 5 software (GraphPad Prism version 5.04 for Windows, GraphPad Software, La Jolla, CA, USA). A two-tailed P-value test was used in all analyses and a P-value of less than 0.05 was considered statistically significant.

Results

PIK3CA is commonly mutated in familial male breast cancer

Seven *PIK3CA* mutations were identified and confirmed in six samples (Table 1). Four activating mutations were

Figure 2 PIK3CA/mTOR pathway (# 4EBP1 is inactivated by phosphorylation) a). Immunohistochemistry was performed for phosphorylated AKT, pS6 and p4EBP1. **b)** Outcome of immunohistochemical staining integrating *PIK3CA* mutation status. Numbers indicate the amount of cases showing positive immunohistochemical staining. Mutations are signified by (n*), with n = number of cases. mTOR, mammalian target of rapamycin.

118

Table 1 Somatic PIK3CA mutations in familial male breast cancer

Nucleotide change	Amino acid change	BRCA status
c.1624G>A	p.E542K	BRCAX
c.1639G>A	p.E547K	BRCAX
c.1624G>A, c.1639G>A	p.E542K, p.E547K	BRCAX
c.3140A>G	p.H1047R	BRCAX
c.3140A>G	p.H1047R	BRCAX
c.3140A>G	p.H1047R	BRCA1 del exons 21_24

Table 2 Correlation of PIK3CA mutation status with clinicopathological parameters

	PIK3CA Mutation ($n = 6$)	PIK3CA Wild type ($n = 51$)	P-value
Age - mean (years)	62.2	63.0	0.899
Overall DSS	33.3%	33.3%	1.000
Carrier mutation status			
BRCA1	1	2	0.288
BRCA2	0	25	**0.030**
BRCAX	5	24	0.194
Primary tumour size (mm)	18.8	19.1	0.948
Histological type			
Invasive carcinoma - no special type (IC - NST)	6	37	0.319
IC-NST with micropapillary areas	0	9	0.575
Invasive Papillary Carcinoma	0	3	1.000
Invasive Lobular Carcinoma	0	2	1.000
Tumour grade			
1	0	3	1.000
2	3	26	1.000
3	3	22	1.000
Lymphovascular invasion	33.3%	39.2%	1.000
Perineural invasion	50.0%	41.2%	0.689
Intrinsic subtype			
Luminal	6	45	1.000
HER2	0	5	1.000
Basal	0	1	1.000
Null	0	0	1.000

P-values < 0.05 in bold text.

identified in exon 9, with two cases of E547K mutation and one sample demonstrated concurrent E542K and E547K mutations in exon 9. Three further mutations were identified in exon 20, all of which were H1047R mutations. Screening of *AKT1*, *BRAF* and *KRAS* showed no evidence of somatic mutations.

PIK3CA mutation is uncommonly seen in BRCA2 mutation carriers

One tumour arising in a *BRCA1* carrier had an exon 20 *PIK3CA* mutation, five *PIK3CA* mutations occurred in BRCAX males whereas no *PIK3CA* mutation were identified in tumours from *BRCA2* mutation carriers. There was a significant positive association between *PIK3CA* mutation incidence and BRCACX (17.2%) compared with *BRCA2* (0%) associated tumours ($P = 0.030$). There was otherwise no correlation between the presence of somatic *PIK3CA* mutation and age of diagnosis, primary tumour size, tumour histological subtype, tumour grade, intrinsic phenotype, lymphovascular or perineural invasion ($P > 0.05$) (Table 2). The presence of *PIK3CA* mutation was not associated with a significant difference in Disease Specific Survival (DSS) (Figure 3).

Co expression and clinicopathological correlation of p4EBP1, pS6, pAKT biomarkers

Cytoplasmic expression of p4EBP1 was present (histoscore 1 to 12) in 55.4% (31/56) of cases, nuclear p4EBP1 expression (histoscore 1 to 12) in 51.8% (29/56) of cases and either nuclear or cytoplasmic expression in 58.9% (33/56) of cases. High expression of both pS6 and pAKT1 (histoscore 4 to 12) was seen in 37.5% (21/56) of cases each. A pattern of co-expression of any of the markers was not seen ($P > 0.05$) (Figure 2b). Clinicopathological correlation showed that nuclear expression of p4EBP1 correlated with BRCA2 carrier status (17/25 (68.0%) $P = 0.035$) and inversely with BRCAX cases (11/30 (36.7%) $P = 0.0184$). There was no correlation between DSS and expression of any markers (Additional file 4: Supplementary figure 2).

PIK3CA mutation phenotype

All tumours with *PIK3CA* mutation showed differences in some downstream pathway members. Expression of
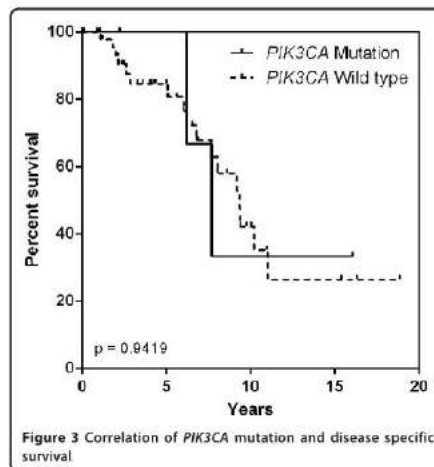

Figure 3 Correlation of PIK3CA mutation and disease specific survival

p4EBP1, pS6 and pAKT was observed in 0/6 (0%), 5/6 (83.5%) and 2/6 (33.3%) of cases respectively (Figure 2b). There was significant absence of p4EBP1 nuclear ($P = 0.009$) or cytoplasmic ($P = 0.006$) staining and up-regulation of pS6 ($P = 0.024$) in tumours with PI3KCA somatic mutations when compared with PIK3CA wild type (Table 3).

Discussion

This study is the first to characterise biomarkers and mutations in the PIK3CA/mTOR pathway in familial male breast cancer noting several novel observations. We identified a PIK3CA mutation rate of 10.5% in familial MBCs but an absence of common activating mutations of AKT1, KRAS and BRAF. While limited by moderate numbers in our study, the absence of KRAS mutation contrasts with the only other study performed in sporadic MBCs by Dawson et al. who reported an overall incidence of 12% [20]. Methodological reasons may be underlying these difference but in our experience, HRM is a highly sensitive and robust technique [42,43]. The absence of BRAF mutation is also somewhat expected and is supported by the stronger association between basal cell breast cancer lines and BRAF mutation [44] (since the majority of MBCs are of a luminal subtype). While a true frequency of these mutations requires further testing in a much larger cohort, these data suggest frequency is unlikely to be high and should parallel the range (0.7 to 5%) that is observed in female breast cancer.

The mutation rate of PIK3CA in this series is lower than the reported 17.9% (7/39) in the only other study performed, although this was in a population-based cohort of MBCs patients [19]. It is also less frequent than that reported in FBC (16.3% [19] to 40.0% [3]) (Table 4), which supports the notion that male breast cancer is biologically different from female breast cancer and that therapies that rely on the experience of the female disease are likely to be suboptimal. Furthermore, evidence from our data demonstrating that differences in this PIK3CA/mTOR pathway is dependent on the germline genotypes of male breast cancer, shows the basis of male breast cancer in BRCA2 mutation carriers is very different to that of BRCAX giving further credence to personalising breast cancer treatment whether

Table 3 Correlation of p4EBP1, pS6 and pAKT immunohistochemistry with BRCA status and clinicopathological factors

| | 4EBP1 | | | | | | pS6 | | | pAKT | | |
| | Cytoplasmic | | | Nuclear | | | Cytoplasmic | | | Nuclear | | |
	Present	Absent	P-value	Present	Absent	P-value	High	Low	P-value	High	Low	P-value
PIK3CA Mutation	0	6		0	6		5	1		2	4	
PIK3CA Wild-Type	31	19	0.006	29	21	0.009	16	34	0.024	19	31	1.000
Carrier mutation status												
BRCA1	1	2	0.581	1	2	0.605	1	2	1.000	0	3	0.284
BRCA2	15	10	0.596	17	8	0.035	9	16	0.582	10	15	0.786
BRCAX	15	13	0.456	11	19	0.018	11	17	1.000	11	17	1.000
Age - mean (years)	60.3	64.5	0.240	59.1	65.3	0.078	63.4	61.4	0.503	62.0	62.2	0.940
Primary tumour size (mm)	20.3	17.2	0.169	19.5	18.3	0.589	17.21	20.03	0.312	20.05	18.25	0.437
Histological type												
Invasive carcinoma - no special type (IC - NST)	21	19	0.563	22	19	0.766	13	28	0.212	15	26	1.000
IC-NST with micropapillary areas	6	3	0.716	3	6	0.288	6	3	0.066	3	6	1.000
Invasive Papillary Carcinoma	2	2	1.000	3	1	0.612	2	2	0.626	2	2	0.626
Invasive Lobular Carcinoma	2	1	1.000	1	1	1.000	0	2	0.523	1	1	1.000
Tumour Grade												
1	2	1	1.000	2	1	1.000	2	1	0.549	1	2	1.000
2	16	13	1.000	16	13	0.789	9	20	0.409	11	18	1.000
3	13	11	1.000	11	13	0.590	10	14	0.591	9	15	1.000
LVI	40.7%	36.0%	0.781	28.0%	48.2%	0.163	31.8%	43.3%	0.565	33.3%	41.9%	0.780
PNI	51.7%	45.8%	0.575	40.7%	57.6%	0.436	36.4%	58.1%	0.166	33.3%	59.0%	0.093
Intrinsic subtype												
Luminal	26	24	0.210	24	26	0.195	17	33	0.183	20	30	0.393
HER2	4	1	0.367	4	1	0.353	3	2	0.352	1	4	0.640
Basal	1	0	1.000	1	0	1.000	1	0	0.375	0	1	1.000
Null	0	0	1.000	0	0	1.000	0	0	1.000	0	0	1.000

Table 4 Comparison of *PIK3CA* mutation studies in male and female breast cancer

	Male Breast Cancer		Female Breast Cancer			
	Current Study	Benvenuti S et al. [20]	Benvenuti S et al. [20]	Buttitta F et al. [14]	Campbell IG et al. [3]	Saal H et al. [16]
Study population	High risk - familial	Population based	Population based	Population based	Population based	Population based
Frequency	6/57 (10.5%)	7/39 (17.9%)	14/86 (16.3%)	46/180 (25.6%)	28/70 (40.0%)	77/292 (26.4%)
Mutation Locus	3 exon 9, 3 exon 20	7 exon 20	6 exon 9, 8 exon 20	23 exon 9, 23 exon 20	15 exon 9, 9 exon 20, 3 exon 7, 1 exon 6	31 exon 9, 49 exon 20, 7 exon 7, 7 others
Clinicopathological association	Inverse correlation with *BRCA2* mutation carrier status	No clinicopathological association	No clinicopathological association	Mutation seen more frequently in lobular carcinoma (46%, $P < 0.001$). Exon 9 more frequently seen in lobular carcinoma (30% of cases, $P < 0.001$).	No clinicopathological association	Association with ER positivity ($P = 0.0001$), PgR Positivity (0.0063) and lymph node positivity ($P = 0.0375$).

P-values < 0.05 in bold text

male or female using individual patient and tumour characteristics. Thus, as the incidence of *PIK3CA* mutations in tumours from in *BRCA2* carriers is likely to be negligible, these patients are unlikely to derive benefits from the PIK3CA inhibitors that are now entering clinical trials for female breast cancer [19].

The distribution of mutations of *PIK3CA* in male breast cancer reported by Benvenuti *et al.* (Table 4) showed exclusively exon 20 mutations in MBC, supporting the suggestion that the frequency of exon 9 and 20 mutations may be gender and tissue specific. We, however, noted an equal distribution of exon 9 and 20 mutations, which is more reflective of the distribution seen by others in FBC [3,14]. Furthermore, the E547K mutation noted in two of our BRCAX patients has only once previously been reported in a single female breast cancer suggestive of a unique hot spot preferentially within male cancers. This mutation was detected and confirmed using HRM and Sanger sequencing in duplicate for each case using methodologies optimised for FFPE material. We have extensive experience with this methodology and feel it to be well suited and robust for formalin fixed paraffin embedded material. While we also acknowledge the occurrence of artifactual changes, the E547K mutation has not been detected in over 300 FFPE tumour samples we have screened to date (unpublished data) and thus, we feel that this mutation may be particular to a subset of MBC. The E547K mutation itself is found in the highly conserved helical domain of PIK3CA and possibly confers increased catalytic activity. The mutation is not unique to breast cancer, and has also been reported previously in one colorectal adenocarcinoma [45] and in seven neuroendocrine tumours of the lung [46] lending support for a true pathogenic mutation. Targeted sequencing of further MBC, and in particular non-BRCA2 tumours, may help determine a

more accurate incidence and potential relevance of this uncommon mutation. We also observed a case with two concurrent exon 9 mutations, which has not been previously reported in MBC. While there is some suggestion of a more aggressive phenotype or of tumour heterogeneity in cases with dual *PIK3CA* mutations [16,47,48], the clinical significance of this is also unclear due to the infrequency of this observation.

Recent data show that *BRCA2* appears to be a significant driver in MBC, with a considerably higher penetrance within male *BRCA2* carriers compared with males in BRCAX families and *BRCA1* male mutation carriers [1]. It is also noteworthy that *BRCA2* somatic mutations have also been reported in 21.8% of sporadic MBCs [22]. Furthermore, unlike in FBC, studies by Ottini *et al.* [49] and ourselves [1] intimate a distinct *BRCA2* phenotype in MBCs, which more commonly contain areas of micropapillary histology, are of a higher grade, are PgR negative and are HER2 amplified. The genomic findings of this study emphasize that *BRCA2* tumours may be a distinct subgroup in familial MBC and as such *BRCA2* mutation may be a significant driver in MBC. Further support for a strong inherent *BRCA2* associated drive independent of gender and estrogenic influence in male breast cancer is the association of *PIK3CA* mutation and ERα positive female breast cancer [14-17], a phenotype which is common to *BRCA2* associated male tumours (92%) [1], but without the associated rate of *PIK3CA* mutation. These data suggest that gender and hormonal dimorphism may not be so significant in *BRCA2* carriers and that *BRCA2* male breast cancers align with the non-*PIK3CA* mutated ERα positive group of female breast cancer.

PIK3CA oncogenic drive, however, may be more important in non-*BRCA2* MBCs where estrogenic influences may be more prominent. While our previous studies have shown that ERα and PgR positive tumours

121

were seen at a similar frequency across all *BRCA1*, *BRCA2* and BRCAX cohorts and more commonly than in FBC [1], based on this genotypic analysis, the mechanism and effect of *PIK3CA* mutation is likely to be different between the subgroups. Overall, given the association between ERα positive tumours and increased *PIK3CA* mutation frequency in FBC, one would assume an increased rate of *PIK3CA* mutation in MBCs. This is not seen and may suggest alternate receptor and PIK3CA/mTOR interaction in male breast cancer or a dose-based relationship differentiated by male cancers with low estrogen at one end of the spectrum and higher levels of estrogen in females at the opposite end. While studies have extensively examined the correlation between hormone receptor status and incidence of *PIK3CA* mutation, as yet there are very limited data on the effect of circulating oestradiol on *PIK3CA* mutation rate with some suggestion that PIK3CA/mTOR activation may contribute to tamoxifen-resistance. Further evidence of estrogen influence is also provided by Benvenuti *et al.* who observed a gender bias for *PIK3CA* mutations in colorectal cancer with a higher incidence of mutations in women (23%) compared with men (9%) [19] (Table 4), which reflect the findings of our study. Further study correlating serum oestradiol, testosterone levels and *PIK3CA* mutation frequency in MBCs are required to further elaborate on a possible association.

Recent *in vitro* studies showing increased sensitization of cancers with defects in DNA homologous recombination (as seen in *BRCA1/2* deficient cancers), to PARP inhibition by targeting of PIK3CA [18,50] suggest that PIK3CA/mTOR pathway interactions result in homologous recombination steady state. Support for the model is not yet seen *in vivo* with only one study to date to have examined a correlation between *BRCA* mutation carriers' status and *PIK3CA* mutation incidence in FBC. Limited by numbers, Michelucci *et al.* describe two mutations (one codon 9 and one codon 20) in 12 *BRCA2* mutation carriers and no mutations in 10 *BRCA1* mutation carriers [15]. The clinical value of this dual targeting is unknown in *BRCA1/2* FBCs and whether it is male or female, this study is also the first to describe a *PIK3CA* somatic mutation in a *BRCA1* mutation carrier. The low numbers of MBCs in *BRCA1* mutation carriers in our study reflects the paucity of these tumours in this particular cohort, and in *BRCA1* carriers in general [51-54]. What is apparent is that *BRCA1*-associated tumours in males appear to be more similar to the tumours seen in post-menopausal female *BRCA1* carriers, with an absence of tumours arising in young patients and an absence of an association with basal cell phenotype. Notwithstanding, carrying a *BRCA1* mutation does appears to be a risk factor for MBC with a higher incidence than that of the general population but at much lower penetrance than seen in female *BRCA1* carriers and it is still unclear as to the role *BRCA1* plays in MBC. While the findings in this study are novel, true incidence and relevance of *PIK3CA* mutations in this cohort require further investigation of larger numbers of *BRCA1* patients, if these can be acquired for study.

The alignment of *PIK3CA* mutation with elevated pS6 expression and absent p4EBP1 expression is different to the expected model. Theoretically, *PIK3CA* mutational activation of the pathway should only lead to an elevated pS6, as is seen, but not an elevated p4EBP1 (the phosphorylated form being inactive) and pAKT, which is not observed. This is in part likely to be due to the complexity of the PIK3CA/mTOR pathway. Indeed, a correlation between *PIK3CA* mutation in luminal A FBC (the phenotype most similar to MBC) and combined up-regulation of pAKT, p4EBP1 and pS6 is not seen [55]. The association seen in the series between *PIK3CA* mutation and elevated pS6 ($P = 0.024$) may suggest partial activation of the PIK3CA/mTOR pathway in MBC and reflect the variability of pS6 and p4EBP1 and pAKT levels seen *in vitro* with dose dependent inhibition of mTORC1 [56], or interactions of mTORC2, other pathways and feedback loops.

Nevertheless, we observed up-regulation of p4EBP1 in *BRCA2* mutation carriers (68.0%) more frequently than in BRCAX carriers (36.7%), an association not reported in FBC, giving further evidence to the difference in male and female breast cancers. It may be that an alternate mechanism of PIK3CA/mTOR pathway activation may be present in *BRCA2* cases linked to disordered homologous recombination, as mentioned previously, through p4EBP1 and eIF4e.

Conclusion

The results of this study indicate that somatic *PIK3CA* mutation are a frequent alteration in familial MBC of BRCAX families, the incidence and type of which is comparable to that seen in sporadic male and slightly lower than FBCs. Conversely, the absence of *PIK3CA* mutation in *BRCA2* associated MBCs suggests that alternate oncogenic drivers minimally contribute to tumour drive in this group, thus supporting distinct male breast cancer types. The study has also revealed differences of MBC to FBC and between sporadic and familial MBC which are of importance in optimising treatment strategies and underlying relevance of the PIK3CA/mTOR pathway in tumour biology. Indeed, the therapeutic implications of these findings support the delineation of significant molecular pathways, such as PIK3CA/mTOR and MAPK cascades for subsequent targeted therapies within specific populations.

122

Additional material

Additional file 1: Supplementary figure 1: BOADICEA scores for patients included in study. Probability (Prob) score (0 to 1) is generated for *BRCA1* and *BRCA2* mutations for each case, stratified by known BRCA status.

Additional file 2: Supplementary table 1. REMARK criteria leading to cases recruitment.

Additional file 3: Supplementary table 2. HRM and Sequence specific *PIK3CA, AKT1, KRAS* and *BRAF* primers.

Additional file 4: Supplementary figure 2 Disease specific survival stratified by 1a) nuclear p4EBP1 expression, 1b) cytoplasmic p4EBP1 expression, 1c) pS6 expression and 1d) pAKT expression.

Abbreviations

BOADICEA: Breast and Ovarian Analysis of Disease Incidence and Carrier Estimation Algorithm; Bp: base pairs; CK: cytokeratin; DSS: disease specific survival; EGFR: epidermal growth factor receptor; ERα: estrogen receptor alpha; FBC: female breast cancer; FFPE: formalin fixed paraffin embedded; H&E: haematoxylin and eosin; HRM: high resolution melt; IC-NST: invasive carcinoma of no special type; MAPK: mitogen activated pathway kinase; MBC: male breast cancer; mTOR: mammalian target of rapamycin; mTORC: mammalian target of rapamycin complex; pAKT: phosphorylated AKT; PARP: poly ADP ribose polymerase; PI3K: phosphatidylinositol 3-kinase; PIK3CA: phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha; PgR: progesterone receptor; pS6: phosphorylated S6; p4EBP1: phosphorylated 4EBP1; SISH: silver in *situ* hybridization; TMA: tissue microarray; UDG: uracil DNA glycosylase.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AD performed the manuscript review, contributed to study concept and design and the HRM assay design. DB performed p4EBP1 and pS6 immunohistochemistry. HD developed HRM assays, and assisted in performing and interpreting Sanger sequencing. The kConFab Investigators performed germ-line *BRCA1/2* testing on all patients, and acquired clinical data. NJ performed pAKT immunohistochemistry. SBF prepared the manuscript and contributed to study concept and design. SD performed HRM and Sanger Sequencing of samples, interpretation of pAKT, p4EBP1 and pS6 immunohistochemistry, statistical analysis and manuscript preparation. All authors read and approved the final manuscript.

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125

4.3.2 Additional file 1: Supplementary figure 1: BOADICEA scores for patients included in study. Probability (Prob) score (0 to 1) is generated for *BRCA1* and *BRCA2* mutations for each case, stratified by known BRCA status.

BOADICEA score stratified by BRCA status

4.3.3 Additional file 2: Supplementary table 1. REMARK criteria leading to cases recruitment.

	BRCA1	*BRCA2*	BRCAX	Total
1. Males present in kConFab registry	5	35	78	118
2. Patients with tissue available	3	25	32	60
3. Tumour tissue available for mutational analysis	3	25	29	57
4. Tumour tissue present on microarray	3	25	28	56

4.3.4 Additional file 3: Supplementary table 2. HRM and Sequence specific *PIK3CA, AKT1, KRAS* and *BRAF* primers.

HRM Primers	Primer (Sequence 5' to 3')	Cycles	Annealing temperature	Melt
AKT1 - exon 4	*AKT1* - HRM - exon 4 – Forward: CGAGGGTCTGACGGGTAGAGTG *AKT1* - HRM - exon 4 – Reverse: GGCCGCCAGGTCTTGATGT	55	55°C	70-95°C
BRAF - exon 15	*BRAF* - HRM - exon 15 – Forward: CAGGAAACAGCTATGACCCATGAAGACCTCACAGTAAAAATAGGT *BRAF* - HRM - exon 15 – Reverse: TGTAAAACGACGGCCAGTCATCCACAAAATGGATCCAGACAAC	60	55°C	72-95°C
KRAS exon 2	*KRAS* - HRM - exon 2 – Forward: TTATAAGGCCTGCTGAAAATGACTGAA *KRAS* - HRM - exon 2 – Reverse: TGAATTAGCTGTATCGTCAAGGCACT	55	68°C	70-90°C
PIK3CA - exon 9	*PIK3CA* - HRM - exon 9 – Forward: AAAGAACAGCTCAAAGCAATTTCTACAC *PIK3CA* - HRM - exon 9 – Reverse: TGCTGTTTAATTGTGTGGAAGATCC	60	55°C	70-90°C
PIK3CA – exon 20	*PIK3CA* - HRM - exon 20 – Forward: TGAGCAAGAGGCTTTGGAGTATTTC *PIK3CA* - HRM - exon 20 – Reverse: TGCTGTTTAATTGTGTGGAAGATCC	55	55°C	70-85°C
Sequencing	**Primer (Sequence 5' to 3')**			

Primers

PIK3CA – exon 9	*PIK3CA* - exon 9 – Forward: TGTAAAACGACGGCCAGTCAGAGTAACAGACTAGCTAGAGACAATG *PIK3CA* - exon 9 – Reverse: CAGGAAACAGCTATGACCAATCTCCATTTTAGCACTTACCTGTGAC
PIK3CA – exon 20	*PIK3CA* - exon 20 – Forward: TCGACAGCATGCCAATCTCTTC *PIK3CA* - exon 20 – Reverse: TGCTGTTTAATTGTGTGGAAGATCC
M13 Primers	M13 Forward: TGTAAAACGACGGCCAGT M13 Reverse: TGTAAAACGACGGCCAGT

4.3.5 Additional file 4: Supplementary figure 2. Disease specific survival stratified by 1a) nuclear p4EBP1 expression, 1b) cytoplasmic p4EBP1 expression, 1c) pS6 expression and 1d) pAKT expression.
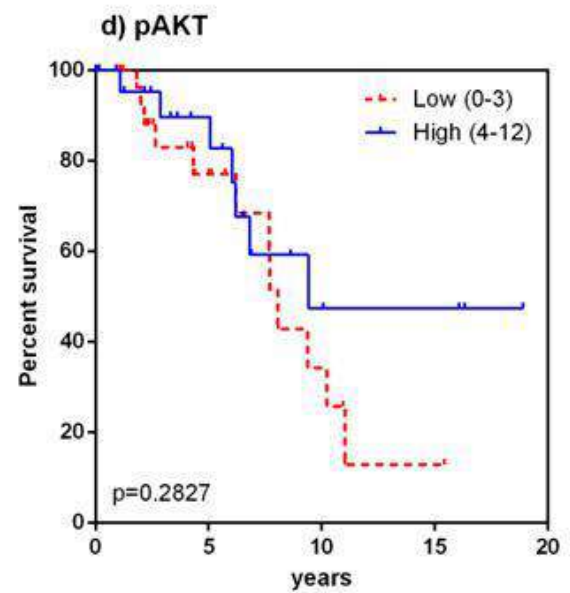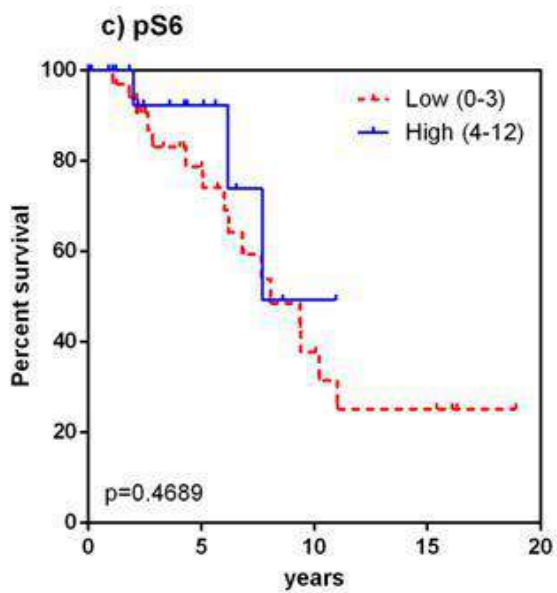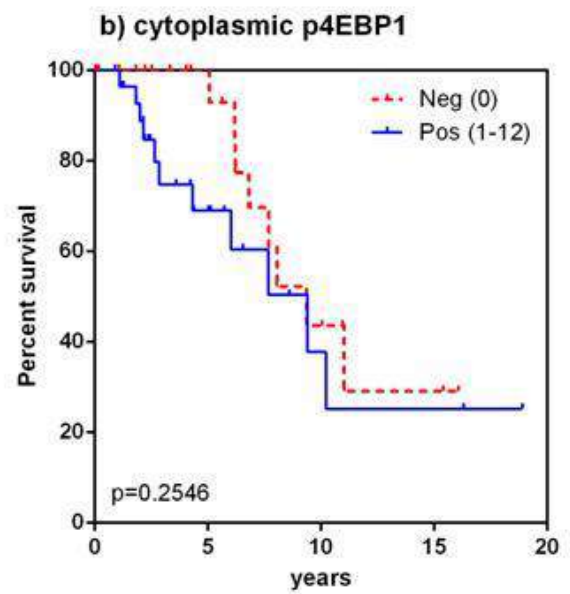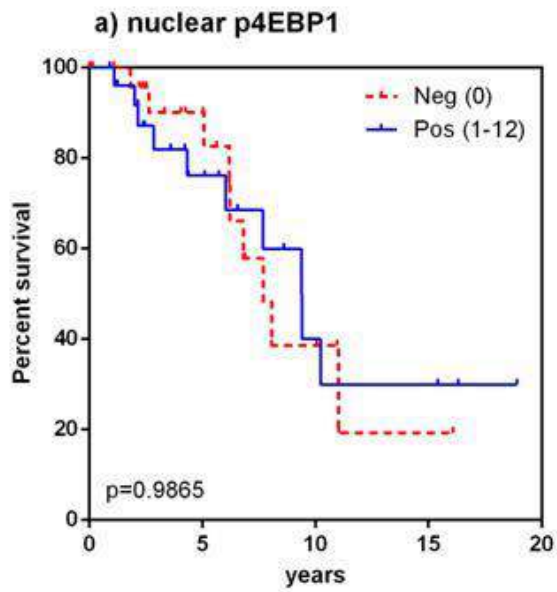
Nuclear HIF1A expression is strongly prognostic in sporadic but not familial male breast cancer

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Male breast cancer is poorly understood with a large proportion arising in the familial context particularly with the BRCA2 germline mutation. As phenotypic and genotypic differences between sporadic and familial male breast cancers have been noted, we investigated the importance of a hypoxic drive in these cancers as this pathway has been shown to be of importance in familial female breast cancer. Expression of two major hypoxia-induced proteins, the hypoxia-inducible factor-1α (HIF1A) and the carbonic anhydrase IX (CA9), examined within a large cohort including 61 familial (3 BRCA1, 28 BRCA2, 30 BRCAX) and 225 sporadic male breast cancers showed that 31% of all male breast cancers expressed either HIF1A (25%) and/or CA9 (8%) in the combined cohort. Expression of HIF1A correlated with an increased incidence of a second-major malignancy ($P = 0.04$), histological tumor type ($P = 0.005$) and basal phenotype ($P = 0.02$). Expression of CA9 correlated with age ($P = 0.004$) in sporadic cases and an increased tumor size ($P = 0.003$). Expression of HIF1A was prognostic for disease-specific survival in sporadic male breast cancers (HR: 3.8, 95% CI: 1.5–9.8, $P = 0.006$) but not within familial male breast cancer, whereas CA9 was only prognostic in familial male breast cancers (HR: 358.0, 95% CI: 9.3–13781.7, $P = 0.002$) and not in sporadic male breast cancer. This study found that hypoxic drive is less prevalent in male breast cancer compared with female breast cancer, possibly due to a different breast microenvironment. The prognostic impact of HIF1A is greatest in sporadic male breast cancers with an alternate dominant mechanism for the oncogenic drivers suggested in high risk familial male breast cancers.

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Male breast cancer is an uncommon and relatively uncharacterized disease entity. There is gathering evidence showing genetic and phenotypic differences and subsets distinct from female breast cancer.[1,2] Although male carriers of germline mutations in BRCA1 and BRCA2 and males from BRCAX families form a substantial proportion of male breast cancers,

they are at a higher risk of developing breast cancer when compared with the general population,[1] factors affecting tumorigenesis and phenotype are still relatively unclear but may be through distinct neoplastic pathways as is suggested by an emerging distinct BRCA2 phenotype of male breast cancer, characterized by a higher mitotic rate, higher grade, HER2 amplification, and an absence of PI3K pathway activation.[1,3,4]

In cancer, hypoxia is an important contributor to carcinogenesis[5] and cancer phenotype.[6,7] Factors contributing to an imbalance of oxygen demand and supply lead to adaptive cellular mechanisms being initiated and involving vast transcriptional and

Hypoxia in male breast cancer
S Deb et al
1224

post-transcriptional changes in gene expression, of which the hypoxia-inducible factor 1 (HIF1) is a master regulator.[8] HIF1 is composed of two subunits; the HIF1α (HIF1A) and the aryl hydrocarbon receptor nuclear translator (ARNT), with HIF1A induced and continuously degraded via an association with the von Hippel–Lindau protein ubiquitin E3 ligase complex leading to degradation via the ubiquitin–proteosome pathway in normoxia.[9] Under hypoxic conditions, however, HIF1A is translocated to the nucleus where it heterodimerizes with ARNT,[10–12] which is constitutively expressed and independent of oxygen levels. The subsequent HIF1 complex regulates transcription of more than 100 target genes, and may account for >2% of all human genes either directly or indirectly.[13] The result is an increase in erythropoietin, iron metabolism, angiogenesis, activation of the glycolytic pathway, activation of MAPK and PI3K signaling pathways promoting cell proliferation and survival and paradoxically apoptosis in some circumstances. Clinically, the HIF1A overexpression consequently correlates with poor prognosis, increased treatment resistance, and tumor associated mortality.[14,15]

In female breast cancer, we have previously demonstrated that increased frequency of HIF1A expression is seen in BRCA1 type, basal-like cancers, and HER2 amplified female breast cancers,[16] subsets that are infrequently seen in male breast cancer. Similarly, expression of the carbonic anhydrase IX (CA9) in female breast cancer has also been correlated with higher grade and poorer survival and is also overexpressed in triple negative (estrogen receptor negative, progesterone receptor negative, HER2 negative) tumors and associated with somatic loss of the BRCA1 protein.[17] There is only one study of hypoxic markers in male breast cancer,[18] comprising of 134 cases, which showed that 27% (34/125) and 7% (9/132) of cases demonstrated HIF1A and CA9 expression with HIF1A also associated with prognosis.[18] The study was not stratified into sporadic and familial cancers, and currently there are no data in familial male breast cancer, or whether similar changes of the hypoxic pathway are present in particular intrinsic phenotypes as shown in female breast cancer.[6] As both sporadic and familial male breast cancer patients also appear to have an increased predisposition to develop second malignancies, the effect or intrinsic response to hypoxia in these individuals may also be significant and has not been described in any study to date. Given the paucity of data in sporadic male breast cancer and absence of reports in the familial context, our aims were to (1) characterize HIF1A and CA9 levels in a large cohort of sporadic and familial male breast cancers, (2) correlate expression with conventional clinicopathological parameters and intrinsic phenotypes, (3) investigate expression in familial breast cancer stratified by carrier mutation status and (4) evaluate the prognostic significance of HIF1A and CA9 expression on disease-specific survival.

Materials and methods

Patient Accrual

An Australian based cohort of cases included familial male cases obtained from the KConFab resource (http://www.kconfab.org: criteria for admission to the kConFab study has been previously published)[1] and sporadic male breast cancers obtained from the Peter MacCallum Cancer Centre and Melbourne Pathology. These cases were ascertained following a search of the relevant kConFab registry and pathology databases, and were diagnosed between 1980 and 2009 in Australia or New Zealand. Patients forming the Swedish cohort were identified through the Swedish National Cancer Registry.[19] Males diagnosed between 1990 and 2007 within the Lund and Uppsala-Orebro regions that had available formalin-fixed paraffin-embedded tumor blocks, clinicopathological data and outcome data were included in the study. This work was carried out with approval from the Peter MacCallum Cancer Center Ethics Committee (Project No: 11/61) and the local ethics committee in Uppsala, Sweden (i2007/254), and the Lund University (2012/89).

Clinical parameters, including the American Joint Committee on Cancer 7th ed TNM staging, tumor recurrence, occurrence of non-breast primary tumors, and death were obtained from referring clinical centers, kConFab questionnaires and state death registries when available. Information on pedigree, mutational status and testing were available from the kConFab central registry. All available slides from cases were reviewed by a pathologist for relevant histopathological parameters. Histological classification was based on criteria set by the World Health Organization (2012).[20]

Germline BRCA1/2 Testing

Any Australian and New Zealand cases of male breast cancer with a strong family pedigree were referred to kConFab preceding this study. Mutation testing for BRCA1 and BRCA2 mutations was performed as reported previously[1] on kConFab referred cases. Once the family mutation had been identified, all pathogenic (including splice site) variants of BRCA1 and BRCA2 were genotyped by kConFab in all available family member's DNA. BRCAX cases were defined by cases with a strong family history meeting kConFab eligibility criteria (http://www.kconfab.org/Collection/Eligibility.shtml), but with absent BRCA1 or BRCA2 mutations within family members. In the Swedish cohort, only patients with a strong family history of breast and ovarian cancer had germline BRCA1/2 testing.

Tissue-Microarray Construction and Immunohistochemistry

Tumor tissue microarrays (1-mm cores), with a twofold redundancy, were prepared from archival

132

formalin-fixed paraffin-embedded tissue blocks. Patient flow/use was as per the REMARK criterion[21] is listed in Table 1. All 104 cases that were excluded were due to; blocks not being available, an absence of clinical and pathological information, or an absence of adequate material (ie, core biopsy diagnosis of breast cancer) for tissue-microarray construction.

Tissue-microarray sections were cut from each block at 4 µm thick intervals, dewaxed, placed through graded alcohol, and then into water. Antigen retrieval was performed using high pH EnVision FLEX Target Retrieval Solution (Dako) for 4 min at 124 °C for HIF1A and CA9. Staining for HIF1A (1:50 overnight incubation at 4 °C, Novus Biologicals) and CA9 (1:4000, 30 min at room temperature, Novus Biologicals) was performed using rabbit polyclonal antibodies. Antigen–antibody complex was detected using the Envision FLEX system (EnVision FLEX/HRP and EnVision FLEX DAB + Chromogen, DAKO) (Figure 1).

Scoring Criteria and Cut-offs

Scoring was performed according to a previously used semi-quantitative system.[6,16,22–24] Briefly, HIF1A was scored only according to the presence (1+) or absence (0) of nuclear expression. Only

tumors showing a strong membranous staining in ≥10% cells were considered positive for CA9.[6]

Statistical Analysis

Comparison of groups was made with using Mann–Whitney U for non-parametric continuous distributions and χ^2- test for threshold data. Kaplan–Meier survival curves were plotted using breast cancer related death as the endpoint and compared using a log rank test. Analysis was performed with Graph-Pad Prism 5 software (GraphPad Prism version 5.04 for Windows, GraphPad Software, La Jolla CA, USA). A two-tailed P-value test was used in all analyses and a P-value of less than 0.05 was considered statistically significant.

Results

Expression of HIF1A and CA9 in Male Breast Cancer

In the overall combined sporadic and familial cohort, 25% (68/271) of cases were positive for HIF1A (Table 2). There was no statistically significant difference in HIF1A positivity between sporadic male breast cancers (28%, 59/213) compared with familial male breast cancers (16%, 9/58, $P=0.06$) with similar frequencies within the familial with

Table 1 Study summary according to REMARK criteria

	Sweden	MP	PMCC	kConFab	Total
1. Males present in clinical registry	249	14	7	118	388
2. Cases with material available for use in tissue microarray	205	14	7	58	284
3. Cases present after TMA drop out- HIF1A	198	13	6	54	271
4. Cases present after TMA drop out –CA9	201	14	6	54	276

Abbreviations: MP, Melbourne Pathology; PMCC, Peter MacCallum Cancer Centre.
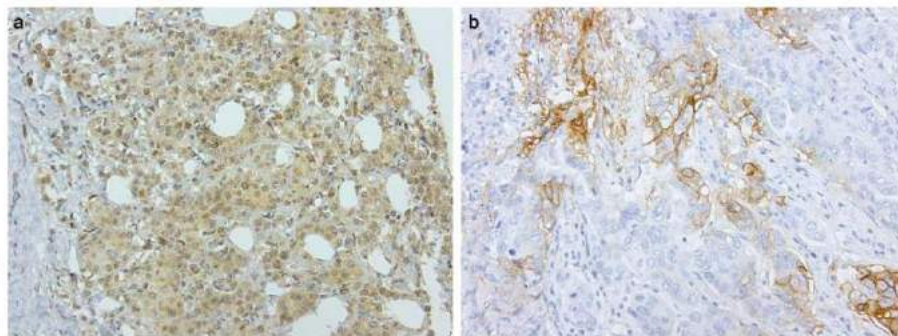
Figure 1 Immunohistochemistry: (a) HIF1A immunohistochemistry showed both activated nuclear and basal cytoplasmic staining. (b) CA9 immunohistochemistry showed heterogeneous membrane staining.

133

BRCAX (14%, 4/28) and *BRCA2* (15%, 4/27) males, and one of three (33%) *BRCA1* male cancers. CA9 was less frequently observed in the overall combined cohort (8%, 22/276) (Table 2), with a higher proportion of positive cases in sporadic male breast cancers (9%, 19/218) when compared with familial male breast cancers (5%, 3/58, *P* = 0.59); although numbers for *BRCA1* are small, there was no statistically significant difference between *BRCA1* (0%, 0/3), *BRCA2* (7%, 2/28), and BRCAX (4%, 1/27) males.

In total, 31% (83/266) of all cases had expression of either marker with a higher frequency seen in sporadic male breast cancers (33%, 71/213) compared with familial male breast cancers (23%, 12/53, *P* = 0.14) (Table 2), and the lowest frequency seen in BRCAX patients (19%, 5/26) (compared with *BRCA1* (33%, 1/3) and *BRCA2* (23%, 6/26) cohorts). Only seven cases had both CA9 and HIF1A positive expression, of which all were sporadic male breast cancers (7/213, 3% *vs* 0/53, 0% *P* = 0.35).

Clinicopathological Correlation and Disease-Specific Survival.

HIF1A. Overall in the combined sporadic and familial cohorts, HIF1A expression correlated with specific histological subtype (invasive carcinoma of no special type)(100 *vs* 90%, *P* = 0.005) and basal cell intrinsic phenotype (100% 4/4, *P* = 0.02) (Table 1a). Expression of HIF1A also correlated with

a history of second-major malignancy (35 *vs* 22%, *P* = 0.04) (Supplementary Table 2) but was not specific to a particular type of cancer. There was no correlation between HIF1A expression and patient age, tumor size, grade, stage or presence of lymph node, and distant metastasis (all *P* > 0.05). There was no association between HIF1A expression and disease-specific survival (Figure 2a).

The association between HIF1A expression with invasive carcinoma of no special type (100 *vs* 92%, *P* = 0.03) and basal cell intrinsic phenotype (100 *vs* 0%, *P* = 0.02) was maintained within the sporadic male breast cancer cohort, as was the correlation with a history of second-major malignancy (37 *vs* 22%, *P* = 0.04). In this group, HIF1A expression was associated with statistically significantly shorter disease-specific survival (HR: 3.8, 95% CI: 1.5–9.8, *P* = 0.006) (Figure 3a).

Within familial male breast cancers, expression of HIF1A inversely correlated with a luminal intrinsic phenotype (67 *vs* 96%, *P* = 0.02). Unlike in sporadic male breast cancer, a direct correlation between HIF1A nuclear expression and basal phenotype (1/9, 11% *vs* 0/49, 0%, *P* = 0.17) was not seen, as only one case of basal phenotype was present within this cohort. There was no association between HIF1A expression and disease-specific survival (Figure 3a).

CA9. Overall, CA9 expression showed a correlation with older age (median 75.5 years *vs* 69.2 years,

Table 2 Expression of HIF1A and CA9 stratified by *BRCA* status

	HIF1A positive cases (%)	P-value	CA9 positive cases (%)	P-value	HIF1A and/or CA9 positive cases (%)	P-value
Overall	68 (25.1%)		22 (8.0%)		83 (31.0%)	
Sporadic	59 (27.7%)	0.06	19 (8.7%)	0.59	71 (33.3%)	0.14
Familial	9 (15.5%)	0.06	3 (5.2%)	0.59	12 (21.8%)	0.14
BRCA1	1 (33.3%)	1.00	0 (0%)	1.00	1 (33.3%)	1.00
BRCA2	4 (14.3%)	0.25	2 (7.1%)	1.00	6 (23.1%)	0.50
BRCAX	4 (14.8%)	0.25	1 (3.7%)	0.71	5 (19.2%)	0.19
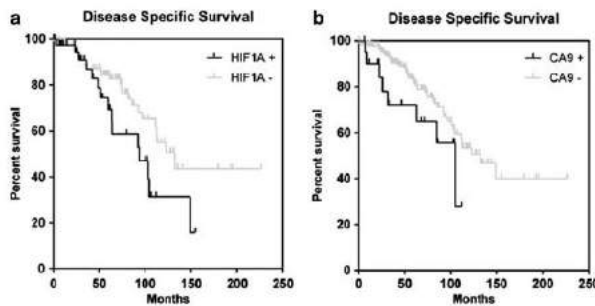
Figure 2 Kaplan–Meier curve for disease-specific survival of: (a) HIF1A and (b) CA9 in the overall cohort.

134
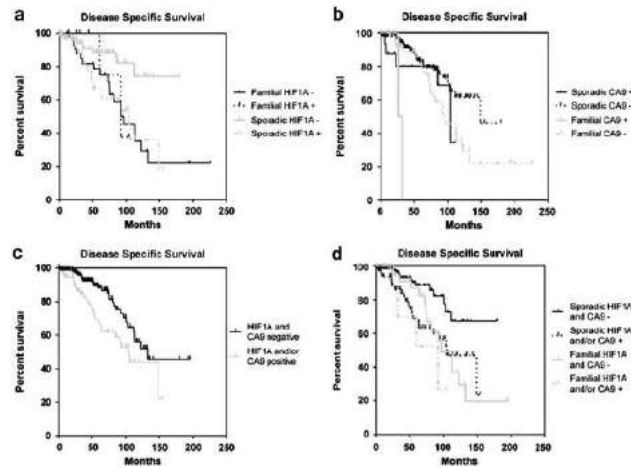
Figure 3 Kaplan–Meier curves for disease-specific survival: (a) HIF1A expression in sporadic and familial male breast cancer, (b) CA9 expression in sporadic and familial male breast cancer, (c) HIF1A and/or CA9 expression—overall cohort, (d) HIF1A and/or CA9 expression in sporadic and familial male breast cancer.

$P = 0.004$) and an increased tumor size demonstrated by association with an advanced T-stage (stage 2–4) (81 vs 46%, $P = 0.003$) (Supplementary Table 1b). Expression of CA9 was not prognostic for disease-specific survival (Figure 2b).

Within sporadic male breast cancers, expression of CA9 maintained a correlation with later age of onset (median 72.2 years vs 70.9 years, $P = 0.02$) and an advanced T-stage (stage 2–4) (78 vs 48%, $P = 0.02$). There were no other clinicopathological associations and no association with disease-specific survival (Figure 3b).

In familial male breast cancers, expression of CA9 correlated with a larger tumor size (median 26.7 mm (CA9 positive) vs 17.3 mm (CA9 negative), $P = 0.046$) but no other clinicopathological factors. CA9 expression correlated with worse disease-specific survival (HR: 358.0, 95% CI: 9.3–13781.7, $P = 0.002$) (Table 3, Figure 3b).

HIF1A and/or CA9. Overall, expression of any hypoxic marker (HIF1A and/or CA9) correlated with specific histological subtype (IC-NST)(99 vs 90%, $P = 0.010$) and a basal cell intrinsic phenotype (100% 4/4, $P = 0.03$) (Supplementary Table 1c). Expression of HIF1A also correlated with a history of a second-major malignancy (34 vs 21%, $P = 0.001$). Prognostically, expression of any hypoxic marker was associated with a statistically significantly shorter disease-specific survival (HR: 2.2, 95% CI: 1.2–3.8, $P = 0.008$) (Figure 3c, Table 3).

In sporadic male breast cancer, expression of any hypoxic marker correlated with a basal cell phenotype (100 vs 0%, $P = 0.04$) and a history of second malignancy (37 vs 20%, $P = 0.01$). Expression was also prognostically significant (Figure 2d) and associated with worse disease-specific survival (HR: 2.9, 95% CI: 1.5–5.8, $P = 0.002$) (Figure 3d). In familial male breast cancer, there was no correlation with clinicopathological factors (Supplementary Table 1c) or disease-specific survival (Table 3, Figure 3d).

Discussion

This study is the first to examine the role of HIF1A and CA9 in a large cohort of sporadic and familial male breast cancers, including a small number of highly infrequent *BRCA1* male breast cancers. Previous studies in female breast cancer show activation of the hypoxic pathway occurs early in tumorigenesis,[25] promoting adaptive mechanism and conferring enhanced survival, invasive and metastatic capabilities. A strong geno-phenotypic association between pathway activation and *BRCA1* associated tumors with a basal cell phenotype is seen in familial female breast cancers,[16] and has yet to be comprehensively evaluated in male breast cancer. This study is the first to evaluate the effect of hypoxia is a cohort of male breast cancer segregated into familial and sporadic subgroups. All cases with a strong family history suggestive of an inherited

135

Table 3 Median disease-specific survival stratified by hypoxia marker and *BRCA* status

Group	Median Survival (months)		Hazards Ratio	95% CI of ratio	P-value
	Positive	Negative			
HIF1A					
All patients	93.4	132.4	2.0	0.99–4.2	0.053
Sporadic	**103.5**	Undefined	**3.8**	**1.5–9.8**	**0.006**
All Familial	63.9	92.4	0.7	0.3–8.4	0.65
BRCA1	Undefined	92.0	NS		
BRCA2	63.9	112.9	2.5	0.3–19.1	0.37
BRCAX	Undefined	92.4	0.3	0.007–12.5	NS
CA9					
All patients	104.6	132.4	2.2	0.8–5.7	0.11
Sporadic	104.6	148.9	1.6	0.5–4.7	0.42
All Familial	**28.8**	**96.7**	**358.0**	**9.3–13781.7**	**0.002**
BRCA1	Undefined	92.0	NS		
BRCA2	31.7	96.7	13.1	0.3–557.4	0.18
BRCAX	**25.9**	**92.4**	**20999.6**	**31.5–13986014.0**	**0.003**
CA9 and/or HIF1A					
All patients	**103.5**	**132.4**	**2.2**	**1.2–3.8**	**0.008**
Sporadic	**104.6**	Undefined	**2.9**	**1.5–5.8**	**0.002**
All Familial	92.0	96.7	2.7	0.6–11.5	0.18
BRCA1	92.0	Undefined	NS		
BRCA2	96.7	127.9	1.6	0.3–10.1	0.60
BRCAX	110.8	112.5	32.2	0.5–2169.5	0.11

Statistically significant *P*-values <0.05 are in bold.

predisposition were tested for *BRCA1/2* germline mutation, the remainder of cases were categorized into 'sporadic male breast cancers'. Although we cannot be entirely confident of the true sporadic nature, we believe the unparalleled large number of cases we present in the study, as the sporadic cohort should overcome the potential effect of any unknown germline mutation carriers, and thus contamination of the sporadic cases with hereditary cases should be minimal.

When compared with female breast cancer, the frequency of HIF1A expression is lower in all of our male breast cancer cohorts, being most marked is seen in familial male breast cancer, compared with familial female breast cancer[16] (72, 38, and 41% of *BRCA1*, *BRCA2*, and BRCAX cases for female breast cancer compared with 33%, 14, and 15% in the respective male breast cancer cohorts). Similarly, sporadic female breast cancers[6] showed a higher frequency of HIF1A positive tumors (50%) compared with sporadic male breast cancers in our study (28%), and comparable to the study by Kornegoor *et al*[18] (27%). A similar but less extreme difference is seen when comparing CA9 expression in male breast cancer and female breast cancer,[6,26] again in keeping with the findings of Kornegoor *et al*.[18] We also only found seven cases (3%) that expressed both HIF1A and CA9. Although this is below the range seen in female breast cancer (11–23%),[6,27] it is not unexpected given the decreased staining of either marker when compared with female breast cancer and the known

variability in HIF1A and CA9 half-lives. HIF1A is rapidly degraded within minutes of oxygenation,[28] whereas CA9 has a half-life of up to 38 h.[29,30]

Importantly, using comparable staining and immunohistochemical scoring, our methodology has been closely addressed to minimize any potential differences to these comparable female breast cancer studies. Most of the staining observed was either 'diffuse' or 'scattered' for both markers, with no cases of perinecrotic staining seen mainly due to targeted sampling of non-necrotic areas for tissue-microarray assembly. Although the differentiation of staining patterns (perinecrotic vs diffuse) by Vleugel *et al* has been shown to be prognostically relevant and thought to correspond with greater activation of the hypoxic pathway, these parameters are not as consistently significant as the dichotomisation into expressing and non-expressing groups that we have used. Notably, although we also used a cut-off of ≥10% positive cells to define tumor CA9 positivity in an attempt to compare with female breast cancer studies, we found that all of our cases either showed completely absent staining or staining above this 10% threshold. We have also critically compared the use of tissue microarrays for the analysis of hypoxia markers in female breast cancer and found equivalent frequencies of expression with other studies that had used whole sections.[31,32] We feel this is a robust and well established methodology, and as our staining frequencies in male breast cancer are similar to Kornegoor *et al*,[18] our detection rates were not substantially compromised.

136

Although there are low numbers of *BRCA1* cases, reflecting the general paucity of these tumors within male breast cancer registries, our findings show that a decreased expression of hypoxia markers is characteristic of male breast cancers. This is in contrast to familial female breast cancer, which has an enhanced hypoxic drive.[6] This may be due to a different breast microenvironment in males and may also be of critical importance in protecting male *BRCA1* carriers from the development of breast cancers generally,[6,16,25] and partly account for the low frequency of basal type in male breast cancer, even in *BRCA1* associated tumors.[1] Thus male breast cancer *BRCA1* tumors that arise are likely to be through alternate mechanisms. The corollary of this is the observation that male breast cancers have a high frequency of hormone receptor expression. Thus, in female breast cancer, activation of the hypoxic pathway is associated with degradation of hormone receptor expression,[33] but the low frequency of HIF1A in male breast cancers, enable retention of estrogen receptor and progesterone receptor.[1]

Nevertheless, overall male breast cancers that express HIF1A demonstrate a shorter disease-specific survival, as with female breast cancer, with a less clear association with the clinicopathological factors noted in female breast cancer. Interestingly in our study, when a male breast cancer had a strong identifiable driver, such as hypoxia in sporadic tumors or a predisposing *BRCA2* mutation, there was a clear association with worse disease-specific survival. This perhaps suggests that high and low risk groups may exist in male breast cancers with prognostic relevance.

An interesting observation was the association between HIF1A expression and the occurrence of a second malignancy in both familial and sporadic male breast cancer. This is also novel and not previously described in female breast cancer. We and others have previously shown a high rate of second malignancies in patients with male breast cancer[11,34,35] but the rates of second malignancies is significantly higher in HIF1A positive tumors (35%), prominent particularly in the prostate (Supplementary Table 2) and not dependent on *BRCA2* status, and is higher than the range of reported rates of second malignancies (12–23%) in large male breast cancer series. It is unclear as to whether in these males, the effect of hypoxia is more significant due to inheritable modifiers or acquired lifestyle factors that may predispose to the onset of cancers.

Conclusion

Our findings suggest that oncogenic mechanisms resulting in overexpression of hypoxic markers are different between female and male breast cancer, and furthermore between sporadic and familial male breast cancer. Overall activation of the pathway is less frequently observed than in female breast

cancer, and the association with *BRCA1*, basal-type tumors and HER2 amplification seen in female breast cancers is not clearly seen in male breast cancers within this study. The study also alludes to the prognostic importance of oncogenic drivers on disease-specific survival. As targeted therapies directed against HIF1A and downstream targets emerge,[36–38] these observation may also have important therapeutic implications for identifying specific populations for screening and explaining treatment efficacy in familial male breast cancers.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/modpathol)

4.4.2 Additional file 1 - Supplementary Table 1a: HIF1A expression and clinicopathological correlation (statistically significant p-values <0.05 are in bold).

	ALL CASES			SPORADIC CASES			ALL FAMILIAL			BRCA2			BRCAX			BRCA1		
	HIF1A+ (n=60)	HIF1A- (n=203)	p-value	HIF1A+ (n=59)	HIF1A- (n=154)	p-value	HIF1A+ (n=9)	HIF1A- (n=49)	p-value	HIF1A+ (n=4)	HIF1A- (n=24)	p-value	HIF1A+ (n=4)	HIF1A- (n=23)	p-value	HIF1A+ (n=1)	HIF1A- (n=2)	p-value
Median Age	68.9	71.1	0.11	70.2	73.1	0.79	54.3	55.4	8.04	54.0	65.0	0.26	55.6	54.1	0.19	49.5	72.8	NA
History of 2nd major malignancy (including breast cancer)	34 / 56.3%	44 / 21.7%	0.03	22 / 37.3%	34 / 22.1%	0.04	2 / 22.2%	10 / 20.4%	1.00	1 / 25.0%	5 / 20.8%	1.00	1 / 25.0%	5 / 21.7%	1.00	0 / 0.0%	0 / 0.0%	1.00
Median tumour size (mm)	17.9	18.7	0.78	17.8	17.0	0.91	18.3	19.5	0.01	16.0	20.8	0.41	18.4	18.6	0.96	25.0	12.0	NA
Tumour stage																		
1	33 / 49.3%	105 / 54.1%		27 / 46.0%	75 / 51.7%		6 / 66.7%	29 / 59.2%		2 / 50.0%	11 / 45.8%		3 / 75.0%	16 / 69.6%		1 / 100.0%	2 / 100.0%	
2-4	34 / 50.7%	89 / 45.9%	0.46	31 / 53.4%	69 / 47.0%	0.51	3 / 33.3%	20 / 40.8%	0.29	2 / 50.0%	13 / 54.2%	1.00	1 / 25.0%	7 / 30.4%	1.00	0 / 0.0%	0 / 0.0%	1.00
Unknown	1	9		1	9		0	0		0	0		0	0		0	0	
Nodal stage																		
0	25 / 45.5%	92 / 55.1%		20 / 43.5%	74 / 56.1%		5 / 55.6%	18 / 51.4%		3 / 50.0%	9 / 52.9%		2 / 50.0%	8 / 50.0%		1 / 100.0%	1 / 50.0%	
1-8	30 / 54.5%	75 / 44.9%	0.28	26 / 56.5%	58 / 43.9%	0.17	4 / 44.4%	17 / 48.6%	1.00	2 / 50.0%	8 / 47.1%	1.00	2 / 50.0%	8 / 50.0%	1.00	0 / 0.0%	1 / 50.0%	1.00
Unknown	13	36		13	22		0	14		0	7		0	7		0	0	
M stage																		
0	63 / 96.9%	188 / 96.9%		54 / 96.4%	136 / 95.9%		9 / 100.0%	49 / 100.0%		4 / 100.0%	24 / 100.0%		4 / 100.0%	23 / 100.0%		1 / 100.0%	2 / 100.0%	
1	2 / 3.1%	6 / 3.1%	1.000	2 / 3.6%	6 / 4.1%	1.00	0 / 0.0%	0 / 0.0%	1.00	0 / 0.0%	0 / 0.0%	1.00	0 / 0.0%	0 / 0.0%	1.00	0 / 0.0%	0 / 0.0%	1.00
Unknown	3	9		3	9		0	0		0	0		0	0		0	0	
Histological type																		
IDC	62 / 100.0%	193 / 96.1%		59 / 100.0%	141 / 91.6%		9 / 100.0%	42 / 85.7%		4 / 100.0%	21 / 87.5%		4 / 100.0%	19 / 82.6%		1 / 100.0%	2 / 100.0%	
Lobular	0 / 0.0%	9 / 4.4%		0 / 0.0%	6 / 3.9%		0 / 0.0%	3 / 6.1%		0 / 0.0%	1 / 4.2%		0 / 0.0%	2 / 8.7%		0 / 0.0%	0 / 0.0%	
Papillary	0 / 0.0%	3 / 1.5%		0 / 0.0%	0 / 0.0%		0 / 0.0%	3 / 6.1%		0 / 0.0%	1 / 4.2%		0 / 0.0%	2 / 8.7%		0 / 0.0%	0 / 0.0%	
Other	0 / 0.0%	8 / 3.9%	0.006	0 / 0.0%	7 / 4.5%	<0.001	0 / 0.0%	1 / 2.0%	0.58	0 / 0.0%	1 / 4.2%	1.00	0 / 0.0%	0 / 0.0%	1.00	0 / 0.0%	0 / 0.0%	1.00
Grade																		
1	0 / 0.0%	14 / 8.9%		0 / 10.2%	14 / 9.1%		0 / 0.0%	2 / 4.1%		0 / 0.0%	1 / 4.2%		0 / 0.0%	1 / 4.3%		0 / 0.0%	0 / 0.0%	
2	29 / 43.6%	100 / 49.5%		25 / 42.4%	77 / 50.0%		4 / 44.4%	23 / 46.9%		1 / 25.0%	9 / 37.5%		3 / 75.0%	14 / 60.9%		0 / 0.0%	0 / 0.0%	
3	35 / 48.5%	89 / 43.0%	0.57	29 / 47.5%	65 / 42.2%	0.54	5 / 55.6%	24 / 49.0%	1.00	3 / 75.0%	14 / 58.3%	1.00	1 / 25.0%	8 / 34.0%	1.00	1 / 100.0%	2 / 100.0%	1.00
Intrinsic Subtype																		
Luminal	56 / 85.3%	184 / 90.0%		52 / 88.1%	137 / 89.0%		6 / 66.7%	47 / 95.9%		3 / 75.0%	23 / 95.8%		2 / 50.0%	22 / 95.7%		1 / 100.0%	2 / 100.0%	
HER2	6 / 8.8%	19 / 9.4%		4 / 6.8%	17 / 11.0%		2 / 22.2%	2 / 4.1%		1 / 25.0%	1 / 4.2%		1 / 25.0%	1 / 4.3%		0 / 0.0%	0 / 0.0%	
Basal	4 / 6.9%	0 / 0.0%		3 / 5.1%	0 / 0.0%		1 / 11.1%	0 / 0.0%		0 / 0.0%	0 / 0.0%		1 / 25.0%	0 / 0.0%		0 / 0.0%	0 / 0.0%	
Null	0 / 0.0%	0 / 0.0%	0.02	0 / 0.0%	0 / 0.0%	0.02	0 / 0.0%	0 / 0.0%	0.02	0 / 0.0%	0 / 0.0%	0.27	0 / 0.0%	0 / 0.0%	0.049	0 / 0.0%	0 / 0.0%	1.00

4.4.3 Additional file 2 - Supplementary Table 1b - CA9 expression and clinicopathological correlation (statistically significant p-values <0.05 are in bold).

	ALL CASES					SPORADIC CASES					ALL FAMILIAL					BRCA2					BRCAX					BRCA1				
	CAIX+ (n=22)		CAIX- (n=254)		p-value	CAIX+ (n=19)		CAIX- (n=199)		p-value	CAIX+ (n=3)		CAIX- (n=55)		p-value	CAIX+ (n=2)		CAIX- (n=26)		p-value	CAIX+ (n=1)		CAIX- (n=26)		p-value	CAIX+ (n=0)		CAIX- (n=3)		p-value
Median Age	75.5		69.2		**0.004**	72.2		70.9		**0.02**	74.7		61.7		0.09	78.9		61.0		0.08	78.9		62.6		NA	NA		65.4		NA
History of 2nd major malignancy (including breast cancer)	4	18.2%	65	25.6%	0.51	4	21.1%	53	26.6%	0.79	0	0.0%	12	21.8%	1.00	0	0.0%	6	23.1%	1.00	0	0.0%	6	23.1%	1.00	0	0.0%	0	0.0%	1.00
Median tumour size (mm)	24.0		18.2		0.14	20.0		17.2		0.87	26.7		17.3		**0.046**	25.0		16.8		0.13	30.0		18.1		NA	NA		16.3		NA
Tumour stage																														
1	4	19.0%	137	54.5%		4	22.2%	100	52.4%		0	0.0%	37	67.3%		0	0.0%	16	61.5%		0	0.0%	18	69.2%		0	0.0%	3	100.0%	
2-4	17	81.0%	109	43.5%	**0.003**	14	77.8%	91	47.6%	**0.02**	3	100.0%	18	32.7%	1.00	2	100.0%	10	38.5%	0.17	1	100.0%	8	30.8%	0.33	0	0.0%	0	0.0%	1.00
Unknown	1		8			1		8			0		0			0		0			0		0			0		0		
Nodal stage																														
0	9	45.0%	108	52.7%		8	44.4%	87	53.4%		1	50.0%	21	50.0%		0	0.0%	11	52.4%		1	100.0%	8	44.4%		0	0.0%	2	66.7%	
1-4	11	55.0%	97	47.3%	0.84	10	55.6%	76	46.6%	0.82	1	50.0%	21	50.0%	1.00	1	100.0%	10	47.6%	1.00	0	0.0%	10	55.6%	0.47	0	0.0%	1	33.3%	1.00
Unknown	2		49			1		36			1		13			1		5			0		8			0		0		
M stage																														
0	19	90.5%	237	97.5%		16	88.9%	182	96.3%		3	100.0%	55	100.0%		2	100.0%	26	100.0%		1	100.0%	26	100.0%		0	0.0%	3	100.0%	
1	2	9.5%	6	2.5%	0.13	2	11.1%	6	3.2%	0.15	0	0.0%	0	0.0%	1.00	0	0.0%	0	0.0%	1.00	0	0.0%	0	0.0%	1.00	0	0.0%	0	0.0%	1.00
Unknown	1		11			1		11			0		0			0		0			0		0			0		0		
Histological type																														
IDC	21	95.5%	236	92.9%		18	94.7%	188	94.5%		3	100.0%	48	87.3%		2	100.0%	22	84.6%		1	100.0%	23	88.5%		0	0.0%	3	100.0%	
Lobular	0	0.0%	8	3.1%		0	0.0%	5	2.5%		0	0.0%	3	5.5%		0	0.0%	1	3.8%		0	0.0%	2	7.7%		0	0.0%	0	0.0%	
Papillary	0	0.0%	3	1.2%		0	0.0%	0	0.0%		0	0.0%	3	5.5%		0	0.0%	2	7.7%		0	0.0%	1	3.8%		0	0.0%	0	0.0%	
Other	1	4.5%	7	2.8%	1.00	1	5.3%	6	3.0%	1.00	0	0.0%	1	1.8%	1.00	0	0.0%	1	3.8%	1.00	0	0.0%	0	0.0%	1.00	0	0.0%	0	0.0%	1.00
Grade																														
1	1	4.5%	20	7.9%		1	5.3%	18	9.0%		0	0.0%	2	3.6%		0	0.0%	1	3.8%		0	0.0%	1	3.8%		0	0.0%	0	0.0%	
2	9	40.9%	122	48.0%		9	47.4%	95	47.7%		0	0.0%	27	49.1%		0	0.0%	12	46.2%		0	0.0%	15	57.7%		0	0.0%	0	0.0%	
3	12	54.5%	112	44.1%	0.51	9	47.4%	86	43.2%	1.00	3	100.0%	26	47.3%	0.24	2	100.0%	13	50.0%	0.48	1	100.0%	10	38.5%	0.41	0	0.0%	3	100.0%	1.00
Intrinsic Subtype																														
Luminal	22	100.0%	225	88.6%		19	100.0%	173	86.9%		3	100.0%	52	94.5%		2	100.0%	24	92.3%		1	100.0%	25	96.2%		0	0.0%	3	100.0%	
HER2	0	0.0%	25	9.8%		0	0.0%	22	11.1%		0	0.0%	3	5.5%		0	0.0%	2	7.7%		0	0.0%	1	3.8%		0	0.0%	0	0.0%	
Basal	0	0.0%	4	1.6%		0	0.0%	4	2.0%		0	0.0%	0	0.0%		0	0.0%	0	0.0%		0	0.0%	0	0.0%		0	0.0%	0	0.0%	
Null	0	0.0%	0	0.0%	0.14	0	0.0%	0	0.0%	0.14	0	0.0%	0	0.0%	1.00	0	0.0%	0	0.0%	1.00	0	0.0%	0	0.0%	1.00	0	0.0%	0	0.0%	1.00

4.4.4 Additional file 3- Supplementary Table 1c: HIF1A and/or CA9 expression and clinicopathological correlation (statistically significant p-value <0.05 are in bold).

	ALL CASES HIF1a and/or CA IX +	(n=83)	ALL CASES HIF1a and CA IX -	(n=185)	p-value	SPORADIC HIF1a and/or CA IX +	(n=71)	SPORADIC HIF1a and CA IX -	(n=142)	p-value	FAMILIAL HIF1a and/or CA IX +	(n=12)	FAMILIAL HIF1a and CA IX -	(n=43)	p-value	BRCA2 HIF1a and/or CA IX +	(n=6)	BRCA2 HIF1a and CA IX -	(n=20)	p-value	BRCAX HIF1a and/or CA IX +	(n=5)	BRCAX HIF1a and CA IX -	(n=21)	p-value	BRCA1 HIF1a and/or CA IX +	(n=1)	BRCA1 HIF1a and CA IX -	(n=2)	p-value
Mean Age	70.1		69.8		0.25	71.9		71.8		0.22	56.2		63.9		0.45	62.3		61.1		0.71	59.6		62.7		0.32	49.5		72.8		NA
History of 2nd major malignancy (including breast cancer)	28	33.7%	38	20.5%	**0.001**	26	36.6%	28	19.7%	**0.01**	2	16.7%	10	23.3%	1.00	1	16.7%	5	25.0%	1.00	1	20.0%	5	23.8%	1.00	0	0.0%	0	0.0%	1.00
Median tumour size (mm)	19.7		18.4		0.55	18.0		19.0		0.81	20.6		17.8		0.27	19.6		17.4		0.56	21.0		18.2		0.57	25.0		12.0		NA
Stage at diagnosis																														
1	36	44.4%	101	56.4%		30	43.5%	73	53.7%		6	50.0%	28	65.1%		2	33.3%	11	55.0%		3	60.0%	15	71.4%		1	100.0%	2	100.0%	
2 - 4	45	55.6%	78	43.6%	0.06	39	56.5%	63	46.3%	0.19	6	50.0%	15	34.9%	0.50	4	66.7%	9	45.0%	0.64	2	40.0%	6	28.6%	0.63	0	0.0%	0	0.0%	
Unknown	2		6			2		6			0		0			0		0			0		0			0				
Nodal stage																														
0	32	47.1%	85	55.9%		26	45.6%	69	57.5%		6	54.5%	16	50.0%		2	40.0%	7	46.7%		3	60.0%	8	53.3%		1	100.0%	1	50.0%	
1 - 3	36	52.9%	67	44.1%	0.24	31	54.4%	51	42.5%	0.15	5	45.5%	16	50.0%	1.00	3	60.0%	8	53.3%	1.00	2	40.0%	7	46.7%	1.00	0	0.0%	1	50.0%	
Unknown	15		33			14		22			1		11			1		5			0		6			0	0.0%	0	0.0%	1.00
M stage																														
0	76	96.2%	172	97.2%		64	95.5%	129	96.3%		12	100.0%	43	100.0%		6	100.0%	20	100.0%		5	100.0%	21	100.0%		1	100.0%	2	100.0%	
1	3	3.8%	5	2.8%	1.00	3	4.5%	5	3.7%	1.00	0	0.0%	0	0.0%	1.00	0	0.0%	0	0.0%	1.00	0	0.0%	0	0.0%	1.00	0	0.0%	0	0.0%	1.00
Unknown	4		8			4		8			0		0			0		0			0		0			0				
Histological type																														
IDC	82*	98.8%	167*	90.3%		70	98.6%	131	92.3%		12	100.0%	36	83.7%		6	100.0%	17	85.0%		5	100.0%	17	81.0%		1	100.0%	2	100.0%	
Lobular	0	0.0%	8	4.3%		0	0.0%	5	3.5%		0	0.0%	3	7.0%		0	0.0%	1	5.0%		0	0.0%	2	9.5%		0	0.0%	0	0.0%	
Papillary	0	0.0%	3	1.6%		0	0.0%	0	0.0%		0	0.0%	3	7.0%		0	0.0%	1	5.0%		0	0.0%	2	9.5%		0	0.0%	0	0.0%	
Other	1	1.2%	7	3.8%	**0.01**	1	1.4%	6	4.2%	0.07	0	0.0%	1	2.3%	0.32	0	0.0%	1	5.0%	1.00	0	0.0%	0	0.0%	0.56	0	0.0%	0	0.0%	1.00
Grade																														
1	6	7.2%	13	7.0%		6	8.5%	11	7.7%		0	0.0%	2	4.7%		0	0.0%	1	5.0%		0	0.0%	1	4.8%		0	0.0%	0	0.0%	
2	36	43.4%	91	49.2%		32	45.1%	71	50.0%		4	33.3%	20	46.5%		1	16.7%	8	40.0%		3	60.0%	12	57.1%		0	0.0%	0	0.0%	
3	41	49.4%	81	43.8%	0.43	33	46.5%	60	42.3%	1.00	8	66.7%	21	48.8%	0.34	5	83.3%	11	55.0%	0.35	2	40.0%	8	38.1%	1.00	1	100.0%	2	100.0%	1.00
Intrinsic Subtype																														
Luminal	73	88.0%	166	89.7%		64	90.1%	125	88.0%		9	75.0%	41	95.3%		5	83.3%	19	95.0%		3	60.0%	20	95.2%		1	100.0%	2	100.0%	
HER2	6	7.2%	19	10.3%		4	5.6%	17	12.0%		2	16.7%	2	4.7%		1	16.7%	1	5.0%		1	20.0%	1	4.8%		0	0.0%	0	0.0%	
Basal	4*	4.8%	0*	0.0%		3*	4.2%	8*	0.0%		1	8.3%	0	0.0%		0	0.0%	0	0.0%		1	20.0%	0	0.0%		0	0.0%	0	0.0%	
Null	0	0.0%	0	0.0%	**0.03**	0	0.0%	0	0.0%	**0.04**	0	0.0%	0	0.0%	0.06	0	0.0%	0	0.0%	0.42	0	0.0%	0	0.0%	0.08	0	0.0%	0	0.0%	1.00

4.4.5 Additional file 4 - Supplementary Table 2: Incidence of 2nd tumors in patients, stratified by expression of HIF1A and CA9. All p-values for statistical association were >0.05, the incidence of the cancer within the subgroup is expressed as a percentage.

	HIF-1a positive	HIF-1a negative	CAIX positive	CAIX negative	HIF1a and/or CAIX positive	HIF-1a and CAIX negative
Prostate	10 (14.7%)	17 (8.4%)	1 (4.6%)	28 (11.0%)	11 (13.3%)	15 (8.1%)
Non-melanoma skin	2 (2.9%)	4 (2.0%)	1 (4.6%)	5 (2.0%)	3 (3.6%)	2 (1.1%)
Colorectal	2 (2.9%)	4 (2.0%)	1 (4.6%)	5 (2.0%)	3 (3.6%)	3 (1.6%)
Breast	1 (1.5%)	4 (2.0%)	1 (4.6%)	4 (1.6%)	2 (2.4%)	3 (1.6%)
Melanoma	2 (2.9%)	2 (1.0%)	0	4 (1.6%)	2 (2.4%)	2 (1.1%)
Bladder	1 (1.5%)	3 (1.5%)	0	4 (1.6%)	1 (1.2%)	3 (1.6%)
Lung	2 (2.9%)	1 (0.5%)	0	3 (1.2%)	2 (2.4%)	1 (0.5%)
Lymphoma	0	2 (1.0%)	0	1 (0.4%)	0	2 (1.1%)
Head/Neck	0	1 (0.5%)	0	1 (0.4%)	0	1 (0.5%)
Gastric	0	1 (0.5%)	0	1 (0.4%)	0	1 (0.5%)
Sarcoma	1 (1.5%)	0	0	1 (0.4%)	1 (1.2%)	0
Pancreatic	0	1 (0.5%)	0	1 (0.4%)	0	1 (0.5%)
Other	3 (4.4%)	4 (2.0%)	0	7 (2.8%)	3 (3.6%)	4 (2.2%)

Chapter 5 – Profiling male breast cancer.

This chapter is composed of two papers:

1) Mutational profiling of familial male breast cancers reveals similarities with luminal A female breast cancer with rare TP53 mutations. Siddhartha Deb, Stephen Q Wong, Jason Li, Hongdo Do, Jonathan Weiss, David Byrne, Anannya Chakrabarti, Trent Bosma, kConFab Investigators, Andrew Fellowes, Alexander Dobrovic, Stephen B Fox. Br J Cancer. 2014 Dec 9;111(12):2351-60. doi: 10.1038/bjc.2014.511

2) *BRCA2* carriers with male breast cancer show elevated tumour methylation. Siddhartha Deb, Kylie L Gorringe, Jai-Min B Pang, David J Byrne, Elena A Takano, kConFab Investigators, Alexander Dobrovic. Stephen B Fox. BMC Cancer. 2017 Sep 11;17(1):641. doi: 10.1186/s12885-017-3632-7.

Supplementary/additional figures and tables from the articles are present at the end of each published article.

5.1 Aims and rationale.

The genomic study of male breast cancer has largely neglected sporadic changes contributing to breast cancer development, rather focusing on cancer predisposition. The studies that have been performed(140-150) show differences to female breast cancer and the presence of unique molecular subsets. Very few cases of familial male breast cancer are either included or reported in these analyses.

The aim is to interrogate a series of familial MBCs for somatic mutations and copy number alterations in common cancer genes, and to examine methylation of well characterized female breast cancer-associated candidate tumor suppressor genes.

Using the Illunina TruSeq Amplicon Cancer Panel, presence of somatic mutations in 48 common cancer genes, including 15 of the 20 most commonly mutated genes in female breast cancer, was investigated. Copy number analysis of these genes was also performed. Among female breast cancers, there is correlation between the intrinsic phenotypes of breast cancer and the spectrum of genes mutated(150). Up until recently, male breast cancer has been thought of as a relatively homogenous entity, composed primarily of ER/PgR positive, luminal type A cancers most similar to post-menopausal female breast cancer. Phenotypic studies and gene expression profiling of male breast cancer confirm the reduced frequency of HER2 and basal phenotypes but reveal two stable male breast cancer subtypes (luminal M1 and luminal M2) different to known characterized female breast cancer subtypes(157, 159). Correlation with chromosomal complexity and clinicopathological factors such as HER2 expression has been seen(42, 178), however, association with somatic mutations, BRCA mutation carrier status or specific phenotypic markers has not been established. Clinical implications for management are also not known. Conversely, , little is known about the mutational landscape of male breast cancer with only seven studies to date examining sporadic mutation, almost all of which have looked at small numbers of targeted candidate genes, and only one study(142) correlating with *BRCA* mutation carrier status.

Tumor suppressor gene promoter methylation is now a well-recognized early and common event in tumorigenesis(241). Aberrant methylation of CpG sites within promoter regions results in gene silencing and loss of gene function akin to inactivating mutations. The clinical implications are multiple, with increasing utility of methylation assays for tumor screening, analyzing tumor progression and monitoring of relapse as methylation of many of these genes is not seen in normal tissues. Furthermore, therapies targeting gene methylation are also in development and may become novel therapies of the future(242).

At the time of publication only 3 MBC studies(168-170), composed of a total of 182 male breast cancers, had evaluated methylation in MBCs, and had shown some difference to female breast cancer. The largest study by Johansen *et al*(170) examined methylation of 47 MBCs and 188 FBCs using a 450k Infinium array. Two distinct MBC epitypes were described with the ME1 epitype correlating with a more proliferative tumor, with a tendency for inferior survival in comparison to the ME2 group. When compared to FBCs, the ME1 cluster most closely correlated to luminal B tumors, while ME2 tumors showed a stronger correlation with luminal A FBCs. There was no significant clustering of MBCs with HER2 and Basal FBC subgroups. Differences between MBCs and FBC were alluded to with the MBCs grouping together within clusters rather than being interspersed among the FBC tumours. MBCs were not segregated into BRCA or familial and sporadic subtypes.

The study by Koornegoor *et al*.(168) examined candidate methylation of 25 genes in 108 MBCs by MS-MLPA. This study does not segregate MBC into sporadic and familial groups, which have been shown to contain distinct geno-phenotypic

characteristics. The study by Pinto *et al*.(169) evaluated *RASSF1A* and *RARB* in 27 familial MBCs, 29 familial FBCs and 16 sporadic FBCs using QMS-PCR. At best this was a comparative study between some of the study cohorts as it introduced bias by defining hypermethylation as greater or equal to the median value and is relatively underpowered in subgroups such as *BRCA2* MBCs.

5.2 Summary

At the time of the publication of these two papers, these were the largest studies examining methylation and somatic mutations in familial MBC.

Sequencing of 48 familial male breast cancers, including 28 MBCs arising in males from BRCAX families, 17 MBCs in *BRCA2* mutation carriers and 3 MBCs in *BRCA1* mutation carriers showed 12 missense mutations present in three genes: *PIK3CA* (9 mutations, incidence 18.8%), *TP53* (2 mutations, incidence 4.2%) and *PTEN* (1 mutation, incidence 2.1%). This profile is similar to that seen in luminal A female breast cancers(150), where *PIK3CA* abnormalities are the most common mutations seen, with rarer TP53 mutations. Interestingly, most *PIK3CA* mutations occurred in BRCAX MBCs, and all the TP53 and PTEN mutations occurred in *BRCA2* MBCs, suggesting alternate drivers between these subsets. The E547K mutation, rarely demonstrated in FBC, was observed in two cases of male breast cancer suggesting a possible gender bias. This study, Piscuglio *et al*.(146) and a recent study by Rizzolo *et al*.(243) showed several cases of dual *PIK3CA* mutations occurring in MBC cases, perhaps more frequently than observed in FBCs. An absence of somatic CDH1 mutations was seen corresponding with less frequent lobular MBCs.

Copy number analysis showed some frequent alterations also seen in FBC as well as differences between BRCA subsets. The only common gain seen in >30% of cases was for *GNAS* (34.1%, chromosome position 20q13.3). Losses were seen in *GNAQ* (36.4%, 9q21), *ABL1* (47.7%, 9q34.1, *ATM* (34.1%, 11q22-q23) and the *C11orf65* reporter (38.6%, 11q22.3). Analysis stratified by *BRCA* status showed differences between groups with an association of *BRCA2* mutation carrier status with gains of *HRAS* (37.5% vs 3%, p=0.006), *STK11* (25.0% vs 0%, p=0.01) and *SMARCB1* (18.8% vs 0%, p=0.04), and the loss of *RB1* (43.8% vs 13%, p=0.03). Rank comparison between copy number changes also showed difference in patterns of genes that were co-amplified between *BRCA2* (supplementary table 5) and BRCAX MBCs.

Examination of allelic variants showed one hundred and twelve allelic variants identified comprising of 17 SNPs present in 11 genes. Of these the most common were the homozygous P72R (rs1042522) *TP53* variant (37.5% frequency), heterozygous T1493T (rs41115) *APC* variant (43.8%), homozygous T1493T (rs41115) *APC* variant (43.8%) and the heterozygous V824V (rs2228230) *PDGFRA* variant (31.3%). Variant frequencies were similar to those reported in the general population, thus suggesting no modifier risk to MBC predisposition in familial MBCs.

Methylation of 10 genes commonly observed to be methylated in female breast cancers (*RASSF1A*, *TWIST1*, *APC*, *WIF1*, *MAL*, *RARβ*, *CDH1*, *RUNX3*, *FOXC1* and *GSTP1*)(244-246) was semi quantitated in 60 tumors from 3 *BRCA1* and 25 *BRCA2* male mutation carriers plus 32 males from BRCAX families using methylation-

sensitive high resolution melting. A cumulative methylation index (CMI) was calculated for each case as the average of the 10 genes as an indicator of overall methylation within the tumor.

Methylation analysis also demonstrated differences between MBC and FBC and between MBC subgroups. Overall, methylation was more frequent in *BRCA2* MBCs when compared to the other subgroups. Within these cancers, two distinct patterns were observed, one characterized by predominantly *GSTP1* methylation with an earlier age of diagnosis, and a second cluster of cancers with more predominant *RASSF1A* methylation. Methylation patterns in BRCAX cancers were more heterogenous, and in particular, cluster analysis of all MBCs showed a distinct pattern characterized by *RASSF1A, WIF1A, RARB* and *GSTP1* methylation more particular to *BRCA2* tumors. Similar to FBC, methylation was less frequently observed in *BRCA1* MBCs(244-246).

Similar to observations in FBC(247), clinicopathological correlation showed *TWIST1* methylation (HR:3.7, 95%CI:2.0-12.0, p=0.001) and high overall methylation (CMI) (HR:3.3, 95%CI: 1.3-7.0, p=0.01) were prognostic for disease specific survival. *RARB* methylation and CMI high status were significantly associated with tumor size (p=0.01 and p=0.02 respectively), *RUNX3* methylation correlated with invasive carcinomas of no special type (94% vs 69%, p=0.048), while *RASSF1A* methylation correlated with coexistence of high grade DCIS (33% vs 6%, p=0.04).

This cohort showed higher frequency of *GSTP1* methylation than seen in MBC by Koornegoor *et al*.(168) or seen in other FBC studies(247). These assays have been

used in other female DCIS and cancer studies and show similar methylation levels to other MBC studies, suggesting the results may be true and not greatly affected by assay bias. Interestingly, the other cancer within which a high frequency of *GSTP1* methylation is seen is prostate adenocarcinoma, a cancer frequently seen in MBC patients as a second malignancy, often at higher rates than the general population.

Mutational and methylation analysis show that familial MBCs may be a unique cohort among which differences exist between *BRCA2* and BRCAX cancers. Furthermore, from a future treatment perspective, the findings suggest that different pathways may be screened and targeted depending on the BRCA status of MBC patients. There are also potential clinical applications in screening for circulating methylated genes in high risk populations, such as the *GSTP1* gene in *BRCA2* mutation carriers.

Mutational profiling of familial male breast cancers reveals similarities with luminal A female breast cancer with rare *TP53* mutations

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Background: Male breast cancer (MBC) is still poorly understood with a large proportion arising in families with a history of breast cancer. Genomic studies have focused on germline determinants of MBC risk, with minimal knowledge of somatic changes in these cancers.

Methods: Using a TruSeq amplicon cancer panel, this study evaluated 48 familial MBCs (3 *BRCA1* germline mutant, 17 *BRCA2* germline mutant and 28 BRCAX) for hotspot somatic mutations and copy number changes in 48 common cancer genes.

Results: Twelve missense mutations included nine *PIK3CA* mutations (seven in BRCAX patients), two *TP53* mutations (both in *BRCA2* patients) and one *PTEN* mutation. Common gains were seen in *GNAS* (34.1%) and losses were seen in *GNAQ* (36.4%), *ABL1* (47.7%) and *ATM* (34.1%). Gains of *HRAS* (37.5% vs 3%, P = 0.006), *STK11* (25.0% vs 0%, P = 0.01) and *SMARCB1* (18.8% vs 0%, P = 0.04) and the loss of *RB1* (43.8% vs 13%, P = 0.03) were specific to *BRCA2* tumours.

Conclusions: This study is the first to perform high-throughput somatic sequencing on familial MBCs. Overall, *PIK3CA* mutations are most commonly seen, with fewer *TP53* and *PTEN* mutations, similar to the profile seen in luminal A female breast cancers. Differences in mutation profiles and patterns of gene gains/losses are seen between *BRCA2* (associated with *TP53/PTEN* mutations, loss of *RB1* and gain of *HRAS*, *STK11* and *SMARCB1*) and BRCAX (associated with *PIK3CA* mutations) tumours, suggesting that *BRCA2* and BRCAX MBCs may be distinct and arise from different tumour pathways. This has implications on potential therapies, depending on the *BRCA* status of MBC patients.

Recent advances in next-generation sequencing technologies have made it possible to interrogate the molecular characteristics of individual cancers. Within breast cancer research, perhaps, the best contemporary example is the recent analysis of 466 breast cancers by The Cancer Genome Atlas Network that integrated analysis from various molecular platforms to produce a comprehensive

portrait of genetic and epigenetic alterations (Cancer Genome Atlas Network, 2012). Analysis revealed convergent changes leading to common gene circuits that correlated with luminal, HER2 and basal phenotypes as defined by mRNA profiling. Furthermore and importantly, from a therapeutic standpoint, a greater knowledge of genomic and potentially targetable drivers was ascertained.

While the TCGA study contained eight male breast cancers (MBCs), the analysis neither segregated nor commented on specific alterations in males. This is reflective of much of MBC research where the study and treatment of these rarer tumours have been extrapolated from findings concluded from female breast cancer (FBC) studies. Overall, MBC cancers comprise <1% of all breast cancers but account for greater mortality (Weiss et al, 2005; Korde et al, 2010). Traditionally, these tumours are thought to be most similar to peri/postmenopausal FBC with a mean/median age at diagnosis 5–10 years later than FBC with a high proportion of invasive ductal carcinomas of no special type and high frequency of oestrogen/progesterone receptor (ER/PgR) positivity (Giordano et al, 2002; Deb et al, 2012a). However, unlike FBC, there is a lower proportion of tumours of basal and possibly HER2 phenotypes (Bloom et al, 2001; Muir et al, 2003) and an absence of early onset cancers (<40 years of age) (Deb et al, 2012a). Although a significant proportion of MBCs arise in a setting of familial breast and ovarian cancer, the effect of being a BRCA mutation carrier is different from female gene carriers with a relative high penetrance seen in BRCA2 male carriers (10.3%) but very low penetrance in BRCA1 male carriers (1.2%) (Deb et al, 2012a).

Little is known about the risk factors and biology for MBC, and to date most molecular studies have examined the germline for specific predisposing genes. There are few somatic studies that have interrogated chromosomal changes largely through array-based CGH, with some reported differences compared with FBC (Tirkkonen et al, 1999; Rudlowski et al, 2006; Johansson et al, 2011; Tommasi et al, 2011). Only seven studies have specifically investigated MBCs comprising a total of 208 males (Anelli et al, 1995; Dawson et al, 1996; Hiort et al, 1996; Kwiatkowska et al, 2002; Benvenuti et al, 2008; Cancer Genome Atlas Network, 2012; Deb et al, 2013), with all but one study not reporting on BRCA status or family history of the patients. Furthermore, only a limited panel of genes have been examined, including PIK3CA, TP53, KRAS, BRAF, androgen receptor (AR) and BRCA2 mutations with some differences again noted from FBC. While older studies suggest a similar frequency of TP53 mutations (25–41% range in MBCs) (Anelli et al, 1995; Dawson et al, 1996) and BRCA2 alterations (21%) (Kwiatkowska et al, 2002) between MBC and FBCs, other MBCs studies have been inconsistent with regard to the frequency of KRAS mutations (0% vs 12%) (Dawson et al, 1996; Deb et al, 2013). Furthermore, some mutations, such as the PIK3CA E547K mutation, appear to be overrepresented and potentially specific to MBCs (Deb et al, 2013). As an extension from our previous study, we have therefore taken advantage of new technologies that are able to parallel sequence formalin-fixed, paraffin-embedded tissue and have profiled 48 familial MBCs (28 BRCAX, 17 BRCA2 and 3 BRCA1) using a 48 gene panel that includes hotspot regions of 15 of the 20 most commonly mutated genes in FBC, including those above in addition to AKT1, ALK1, APC, ATM, CDH1, CTNBB1, NOTCH1, PTEN, RB1 and SMAD4. Although the somatic mutation landscape of MBC is relatively unknown, the panel also includes genes commonly mutated in other cancers to test against. This is the most comprehensive mutational analysis performed on familial MBC to date and aims to: (1) report the type and frequency of these mutations in MBC, (2) identify the number of driver mutations in MBC and compare these with FBC, (3) identify potential mutations specific to MBC, (4) examine copy number variation (CNV) of these gene and (5) determine the genomic relationship with MBC phenotype and

assess whether there are any clinicopathologic correlates. The aim of this study is to improve our understanding of the genomic landscape and architecture of MBC and to identify potential novel targets for therapy specific to this tumour type and assess whether similar targets is present in a subset of FBC. We also aim to further define familial MBC genomically, compare familial MBC with sporadic MBC and identify potential MBC subsets.

MATERIALS AND METHODS

Patients. Males with breast cancers were obtained from the kConFab repository (http://www.kconfab.org) and included cases from Australia and New Zealand diagnosed between 1980 and 2009. The criteria for admission to the kConFab study has been published previously (Loughrey et al, 2008). The flow of patients through the study, according to the REMARK criteria, is listed in Supplementary Table 1. Of the 118 cases within the kConFab registry, 58 cases were excluded because of the unavailability of tissue. Of the 60 cases where tissue was available, 12 cases had poor quality DNA or insufficient tumour tissue for DNA extraction. Clinical parameters, including disease-specific mortality, were obtained from referring clinical centres, kConFab questionnaires and state death registries. Information on pedigree, mutational status and testing were available from the kConFab central registry. Histologic classification was based on the criteria set by the World Health Organisation 2012 (Cleton-Jansen et al, 1995), and all slides and pathologic records from all cases were reviewed centrally within a single institute for tumour size, tumour grade, lymphovascular and perineural invasion. Immunohistochemistry was performed centrally for ERα, PgR, basal markers (cytokeratin (CK) 5, EGFR) and HER2 silver in situ hybridisation (SISH) and scored as per scoring systems described by Harvey et al (1999) and Wolff et al (2007) as reported previously (Deb et al, 2012b) and also listed in Supplementary Table 2. While a consensus on positive CK5 and EGFR scoring is not presently defined, all tumours that were positive showed strong staining in >10% of tumour cells. Using stratification of intrinsic phenotypes based on Nielsen et al (2004), tumours were placed into luminal (ERα positive, HER2 negative, CK5 and/or EGFR negative or positive), basal (ERα and ERα negative; CK5 and/or EGFR positive), HER2 (HER2 positive, ERα, CK5 and EGFR negative or positive) and null/negative (HER2, ERα, CK5 and EGFR negative) phenotypes. This work was carried out with approval from the Peter MacCallum Cancer Centre Ethics Committee (Project No: 11/61).

Germline BRCA1/2 testing. Mutation testing for BRCA1 and BRCA2 mutations was performed as reported previously (Loughrey et al, 2008). Once the family mutation had been identified, all pathogenic (including splice site) variants of BRCA1 and BRCA2 were genotyped by kConFab in all available family members' DNA.

DNA extraction. Genomic DNA was extracted from formalin-fixed, paraffin embedded (FFPE) samples. A 3 μm haematoxylin-and eosin-stained slide was cut from FFPE blocks and stained to identify for tumour-enriched areas (>80% tumour content). From the relevant area on the FFPE block, at least one 2 mm punch biopsy core was taken with 85% of samples having two cores extracted. The cores were then dewaxed and hydrated through gradient alcohol. Genomic DNA was then extracted using the DNeasy Tissue Kit (Qiagen, Hilden, Germany) following proteinase K digestion at 56 °C for 3 days.

UDG treatment. The treatment of FFPE DNA with uracil-DNA glycosylase (New England Biolabs, Ipswich, MA, USA) was performed on the MyCycler instrument (Bio-Rad, Hercules, CA, USA). This has been demonstrated to significantly reduce sequence artefact induced by formalin fixation (Do et al, 2013). One unit of

151

UDG was added for each 20 ng of FFPE DNA with $0.5 \times$ of UDG buffer. The treatment conditions had two incubation steps: an initial activation at 37 °C for 2 h and an inactivation of UDG enzyme at 97 °C for 10 min.

Illumina TruSeq amplicon cancer panel. The TruSeq Amplicon Cancer Panel comprises a total of 212 amplicons from 48 genes (Supplementary Table 3) and 6 amplicons from reporter sequences (*RP5-1091E12.1*, *RP11-286H14.8*, *RP11-530I17.1*, *RP11-350N15.4*, *CTC-554D6.1*, *C11orf65*) that are simultaneously amplified in a highly multiplexed and single-tube reaction. Five microlitres at a concentration of $25\,\mathrm{ng}\,\mu\mathrm{l}^{-1}$ of each DNA sample was used for the experiment according to the manufacturer's instructions. The MiSeq system was used for paired end sequencing using a v1 150 bp kit (Illumina, San Diego, CA, USA). Forty-eight cases were able to examine gene mutation completely and 44 cases were able to assessCNV.

Sequencing validation. Within all samples, hot spots on *TP53* (exons 5–7) *PIK3CA* (exons 9 and 20), *AKT1* (exon 1), *BRAF* (exon 15) and *KRAS* (exon 2) genes were analysed for mutation by high-resolution melting and Sanger sequencing. The *PIK3CA*, *AKT1*, *BRAF* and *KRAS* data using Sanger sequencing for these exons in these patients has been published previously (Deb *et al*, 2013) (Supplementary Table 4 and Supplementary Figure 1). Mutations of other cancer samples on the same runs were also validated by Sequenom MassARRAY platform (San Diego, CA, USA) (Supplementary Table 4 and Supplementary Figure 1). Three MBC samples were also run at least two times across multiple sequence runs to examine for run-specific variation.

Bioinformatics. Primer sequences prefixing the short reads were used to assign each read to an amplicon. Global alignment was then performed between the reads and the amplicon reference sequences to identify sequence variations. Positive variants (in the original biologic sample) were identified using VarScan2 (http://varscan.sourceforge.net). DNA CNV was estimated by comparing sequence read depth between the breast cancer samples and a pseudocontrol. The control was created by averaging the normalised read depth from 20 random human samples that were derived from the same protocols and location as the cancer samples. The averaging and normalisation of the control group was performed using the baseline creation workflow in CONTRA (Li *et al*, 2012). Log ratios between a cancer sample and the control were then computed in 50 bp windows using CONTRA. Using >600 in-house samples, we estimated the null distribution of log ratios for each gene and each exon separately, and thereby making significant calls on genes/exons that lie at the extremes of the distributions (using a P-value cutoff of 0.05; Benjamini–Hochberg adjusted). Gains and losses were defined by a two-fold increase or decrease in reads, whereas amplification was determined by a four-fold increase. Deletions were not examined separate to losses.

Comparison of groups was made using Mann–Whitney U-test for nonparametric continuous distributions and χ^2 test for threshold data. Kaplan–Meier survival curves were plotted using breast cancer-related death as the end point and compared using a log-rank test. A two-tailed P-value test was used in all analyses and a P-value or <0.05 was considered statistically significant.

Hierarchical clustering. Unsupervised hierarchical cluster analysis of log 2 ratios of copy numbers for each gene was used to detect possible unique signatures. Analysis was performed using Cluster and Tree View software written by Michael Eisen (Stanford University, Stanford, CA, USA) as published previously (Eisen *et al*, 1998; Makretsov *et al*, 2004; van de Rijn and Gilks, 2004) and Elucidean metric distance was used.

Table 1. Clinicopathologic features of included male breast cancers		
Age at diagnosis		
Median (range) (years)	60.6 (30.1–85.7)	
Disease-specific mortality	18	38%
Multifocal	1	2%
Bilateral	1	2%
Tumour size		
Median (range) (mm)	17 (2–50)	
Histologic subtype		
Invasive carcinoma – no special type	35	73%
Invasive carcinoma with micropapillary component	7	15%
Invasive papillary carcinoma	5	10%
Invasive lobular carcinoma	1	2%
Grade		
1	2	4%
2	25	52%
3	21	44%
ER expression (Allred 0–8)		
0	1	2%
1–5	4	8%
6–8	43	90%
PgR expression (Allred 0–8)		
0	3	6%
1–5	6	13%
6–8	39	81%
HER2		
Amplified	3	6%
Non-amplified	45	94%
Phenotype		
Basal	1	2%
Luminal	44	92%
HER2	3	6%
Tumour stage		
T1a	1	2%
T1b	3	6%
T1c	24	50%
T2	19	40%
T3	1	2%
Lymphovascular invasion		
Absent	23	48%
Present	23	48%
NA	2	4%
Perineural invasion		
Absent	25	52%
Present	20	42%
NA	3	6%
Paget's disease of nipple		
Absent	37	77%
Present	7	15%
NA	4	8%
Nodal status		
N0	20	42%
N1	16	33%
N2	2	4%
NX	10	21%
Margins		
Clear	42	88%
Involved	6	13%
DCIS		
Absent	15	31%
Present	33	69%
DCIS – nuclear grade		
Low	2	6%
Intermediate	20	61%
High	11	33%

Abbreviations: DCIS = ductal *in situ* carcinoma; ER = oestrogen receptor; PgR = progesterone receptor; NA = not applicable.

152

RESULTS

Mutated genes in MBC. Overall, 48 tumours were sequenced with clinicopathologic variables as outlined in Table 1. A total of 11 373 mutations were identified and of these 479 were tested by an orthogonal method (Supplementary Table 4). There was a high artefactual/false-positive rate when total mutation reads were below one hundred counts or <5% of total reads for prospective germline and <125 mutation reads (and <150 read total coverage) for somatic mutations. Using this cutoff, 11 234 mutations were excluded with high sensitivity (98%) and specificity (99%) for mutation detection (Supplementary Figure 1). Overall, 98% of our amplicons had coverage of >150 reads. Subsequently, 112 variants, 15 nonsense mutations and 12 missense somatic mutations were identified.

No case had more than one somatic mutation present. The 12 mutations (Table 2 and Figure 1) in 48 cases (25%) were only present in three genes: PIK3CA (9 mutations, incidence 18.8%), TP53 (2 mutations, incidence 4.2%) and PTEN (1 mutation, incidence 2.1%). Of the nine PIK3CA mutations, seven were present in BRCAX patients (7 mutation, incidence 28–25%), with one each in BRCA1 (1 mutation, incidence 3–33%) and BRCA2 (1 mutation, incidence 17–5.9%) patients. Four PIK3CA mutations were present in exon 9 (E542K, E547K), three in exon 20 (H1047R) and two in exon 5 (N345K). The two TP53 mutations were found in exon 5 (A138P) and exon 8 (R306Q). The single PTEN mutation (E314*) was a truncating mutation in exon 8. All TP53 (2 mutation, incidence 17–11.8%) and PTEN (1 mutation, incidence 17–5.9%) mutations were found in BRCA2 cases.

All cases with mutations occurred in invasive carcinomas of no special type (IC-NST), with one PIK3CA mutation (N345K) and the single PTEN mutation occurring in IC-NSTs with a component of invasive micropapillary carcinoma (Table 2). No associations were observed between germline mutation groups and clinicopathologic and prognostic factors, including age at diagnosis,

Table 2. Clinicopathologic summary of somatic mutations identified with associated clinical and pathologic features

Somatic mutation	BRCA mutation status	Age (years)	Primary tumour size (mm)	Histologic type	BRE grade	LVSI	Perineural invasion	pN stage	DCIS – nuclear grade	Background breast tissue	ER (Allred score)	PR (Allred score)	HRE2 CISH	CK5	Intrinsic phenotype
PIK3CA (N345K)		30.1	15	IDC	3	N	N	x	High	Absent	8	0	9.6	Neg	HER2
PIK3CA (N345K)	BRCA2 5950_5951 del CT (STOP 1909)	43.1	17	IDC with micropapillary component	2	N	Y	N1	Low	Absent	5	5	2.0	Neg	Luminal
PIK3CA (E547K)		47.1	14	IDC	3	N	Y	x	Intermediate	Gynaecomasia	8	8	3.2	Neg	Luminal
PIK3CA (E542K, E547K)		50.3	16	IDC	3	Y	Y	N2	Intermediate	Absent	8	8	2.0	Neg	Luminal
PIK3CA (H1047R)		59.8	24	IDC	2	Y	Y	N1	High	Normal breast tissue	7	6	2.6	Neg	Luminal
PIK3CA (H1047R)		62.7	20	IDC	2	N	N	N1	Intermediate	Normal breast Tissue	8	8	2.3	Neg	Luminal
PIK3CA (E542K)		65.3	30	IDC	3	N	N	N1	Absent	Absent	8	4	2.3	Neg	Luminal
PIK3CA (E542K)		73.8	25	IDC	2	N	N	N0	High	Normal breast tissue	0	7	2.1	Neg	Luminal
PIK3CA (H1047R)	BRCA1 del exons 21_24	80.1	15	IDC	3	N	N	N1	High	Normal breast tissue	5	8	1.7	Neg	Luminal
PTEN (E314*)	BRCA2 9161 C>A (S2978X)	58.7	22* (contralateral carcinoma later)	IDC with micropapillary component	2	N	N	N0	Absent	Absent	7	7	3.2	Neg	Luminal
TP53 (A318P)	BRCA2 del exons 1_2	60.2	28	IDC	3	N	N	N1	Absent	Absent	7	7	2.2	Neg	Luminal
TP53 (R306Q)	BRCA2 983 986 del ACAG (STOP 275)	61.1	25	IDC	2	Y	Y	N0	Intermediate	Normal breast tissue	8	8	2.2	Neg	Luminal

Abbreviations: CISH = carcinoma in situ hybridisation; DCIS = ductal in situ carcinoma; ER = oestrogen receptor; IDC = invasive ductal carcinoma; MBC = male breast cancer; NA = not applicable; Neg = negative; PgR = progesterone receptor.
*Multifocal MBCs with contralateral carcinoma diagnosed subsequently.
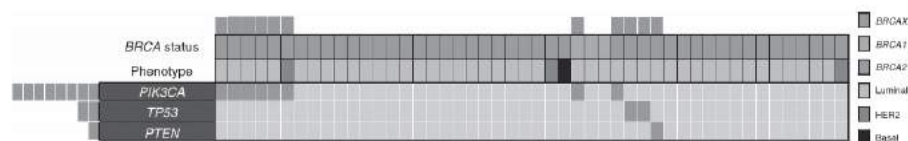

Figure 1. Mutations (red squares) present in MBC. Tumours are classified by BRCA status (orange = BRCAX; yellow = BRCA1; green = BRCA2) and phenotype (light grey = luminal; dark grey = HER2; black = basal). A full color version of this figure is available at British Journal of Cancer journal online.

153

tumour size, grade, histologic subtype, hormone and HER2 receptor status, TNM stage, phenotype or disease outcome.

Allelic variants. One hundred and twelve allelic variants were identified (Table 3). There were 17 single-nucleotide polymorphisms (SNPs) present in 11 genes. Of these, the most common were the homozygous P72R (rs1042522) *TP53* variant (37.5% frequency), heterozygous T1493T (rs41115) *APC* variant (43.8%), homozygous T1493T (rs41115) *APC* variant (43.8%) and the heterozygous V824V (rs2228230) *PDGFRA* variant (31.3%). There were no variants overrepresented in any particular *BRCA* subgroups and the frequency was within that reported in the general population. There was no association between variants and the previously mentioned clinico-pathologic factors or cancer phenotype.

Copy number analysis. Satisfactory data were retrieved from 44 cases (3 *BRCA1*, 16 *BRCA2* and 25 BRCAX) for copy number analysis (Figure 2A–C). Overall, out of 54 regions (48 genes and 6 reporters), the median number of genes showing copy number changes (adjusted for multiple testing) seen per sample was 9.5 (range 0–48). This did not significantly vary between *BRCA1* (median 2, range 1–10, $P = 0.23$), *BRCA2* (median 10.5, range 2–36, $P = 0.88$) and BRCAX (median 13, range 0–48, $P = 0.31$) cases (Figure 3A). Dividing the overall cohort into three groups of low (0–4), intermediate (>4–16) and high (>16) numbers of copy number changes showed no differences

in associated clinicopathologic features or disease-specific survival (Figure 3B).

Across the MBC cohort (Table 4), the only gain seen in >30% of cases was for *GNAS* (34.1%, chromosome position 20q13.3). Losses were seen in *GNAQ* (36.4%, 9q21), *ABL1* (47.7%, 9q34.1) and *ATM* (34.1%, 11q22–q23), as well as the *C11orf65* reporter (38.6%, 11q22.3). Analysis stratified by *BRCA* status (Table 4) showed differences between groups. Only three cases of *BRCA1* MBCs were present with the most common changes noted being losses of *ABL* (67%), *NOTCH1* (67%, 9q34), *ATM* (67%) and *C11orf65* (67%). In *BRCA2* cases, aside from also harbouring the common gains and losses across all MBCs, there were also gains in *CTNNB1* (31.3%, 3p21), *FGFR3* (31.3%, 4p16.3) and *HRAS* (37.5%, 11p15.5), and losses in *NRAS* (31.3%, 1p13.2), *FBXW7* (31.3%, 4q31.3), *APC* (37.5%, 5q21–q22), *CTC-554D6.1* reporter (37.5%, 5q22.2), *RP11-286H14.8* reporter (31.3%, 7q32), *PTEN* (31.3%, 10q23.2), *KRAS* (31.3%, 12p12.1) and *RB1* (43.8%, 13q14.2). In BRCAX cases, no areas of gain were seen but losses were seen in *NRAS* (32.0%, 1p13.2), *KIT* (36.0%, 4q11–q12), *FIP1L1* (36.0%, 4q12), *PDGFRA* (36.0%, 4q12) and *MET* (32.0%, 7q31) on top of also the common losses seen in all MBCs. An association of *BRCA2* mutation carrier status was seen with gains of *HRAS* (37.5% *vs* 3%, $P = 0.006$), *STK11* (25.0% *vs* 0%, $P = 0.01$) and *SMARCB1* (18.8% *vs* 0%, $P = 0.04$), and the loss of *RB1* (43.8% *vs* 13%, $P = 0.03$). No other changes were seen specific for a *BRCA* subgroup.

Table 3. Allelic variant frequency stratified by *BRCA* status

Gene	SNP	Amino acid	Change	Codon	Allele	Total cases	%	BRCA1	%	BRCA2	%	BRCAX	%	General population
APC	Rs143638171	1129	L/S	tTg/tCg	CT	1	2.1	0	0.0	1	5.9	0	0.0	0.5–1.4
					CC	0	0.0	0	0.0	0	0.0	0	0.0	<0.1
	Rs137854579	1307	I/K	aTa/aAa	TA	1	2.1	0	0.0	0	0.0	1	3.6	0.1–5.0
					AA	0	0.0	0	0.0	0	0.0	0	0.0	<0.1
	Rs1801166	1317	E/Q	Gaa/Caa	GC	3	6.3	0	0.0	1	5.9	2	7.1	1.7–2.3
					CC	0	0.0	0	0.0	0	0.0	0	0.0	<0.1
	Rs41115	1493	T	acG/acA	GA	21	43.8	1	33.3	9	52.9	11	39.3	48.60
					AA	23	47.9	1	33.3	6	35.3	16	57.1	34.50
ATM	Rs1800056	858	F/L	Ttt/Ctt	CT	3	6.3	0	0.0	2	11.8	1	3.6	1.9–3.5
					CC	0	0.0	0	0.0	0	0.0	0	0.0	<0.1
EGFR	Rs142455912	1697	T/A	Acc/Gcc	Ag	1	2.1	0	0.0	0	0.0	1	3.6	0.20
					GG	0	0.0	0	0.0	0	0.0	0	0.0	<0.1
	Rs121913427	746	E/Q	Gaa/Caa	GC	1	2.1	0	0.0	0	0.0	1	3.6	NA
					CC	0	0.0	0	0.0	0	0.0	0	0.0	NA
ERBB4	Rs149498255	611	D/N	Gat/Aat	GA	1	2.1	0	0.0	1	5.9	0	0.0	NA
					AA	0	0.0	0	0.0	0	0.0	0	0.0	NA
KDR	Rs1870377	472	Q/H	caA/caT	AT	8	16.7	0	0.0	3	17.6	5	17.9	1.7–45
					TT	4	8.3	0	0.0	2	11.8	2	7.1	50–78.3
KIT	Rs147943899	74	T/M	aCg/aTg	CT	1	2.1	0	0.0	0	0.0	1	3.6	<0.1
					TT	0	0.0	0	0.0	0	0.0	0	0.0	<0.1
	Rs3822214	541	M/L	Atg/Ctg	AC	3	6.3	0	0.0	1	5.9	2	7.1	7.1–23.3
					CC	1	2.1	0	0.0	1	5.9	0	0.0	2.0–3.4
MET	Rs33917957	375	N/S	aAc/aGc	AG	2	4.2	0	0.0	0	0.0	2	7.1	4–4.2
					GG	0	0.0	0	0.0	0	0.0	0	0.0	2.10
	Rs56391007	1010	T/I	aCt/aTt	CT	1	2.1	0	0.0	0	0.0	1	3.6	2.40
					TT	0	0.0	0	0.0	0	0.0	0	0.0	<0.1
PDGFRA	Rs2228230	824	V	atC/gtT	CT	15	31.3	0	0.0	7	41.2	8	28.6	15.3–50
					TT	0	0.0	0	0.0	0	0.0	0	0.0	2.3–15.9
RET	Rs77711105	648	V/I	Gtc/Atc	GA	1	2.1	0	0.0	1	5.9	0	0.0	0.20
					AA	0	0.0	0	0.0	0	0.0	0	0.0	<0.1
STK11	Rs59912467	354	F/L	ttC/ttG	CG	1	2.1	0	0.0	0	0.0	1	3.6	NA
					GG	0	0.0	0	0.0	0	0.0	0	0.0	NA
TP53	Rs1042522	72	P/R	cCc/cGc	CG	2	4.2	1	33.3	0	0.0	1	3.6	30–43.6
					GG	18	37.5	0	0.0	7	41.2	11	39.3	11.9–61.7

Abbreviations: NA = not applicable; SNP = single-nucleotide polymorphism.
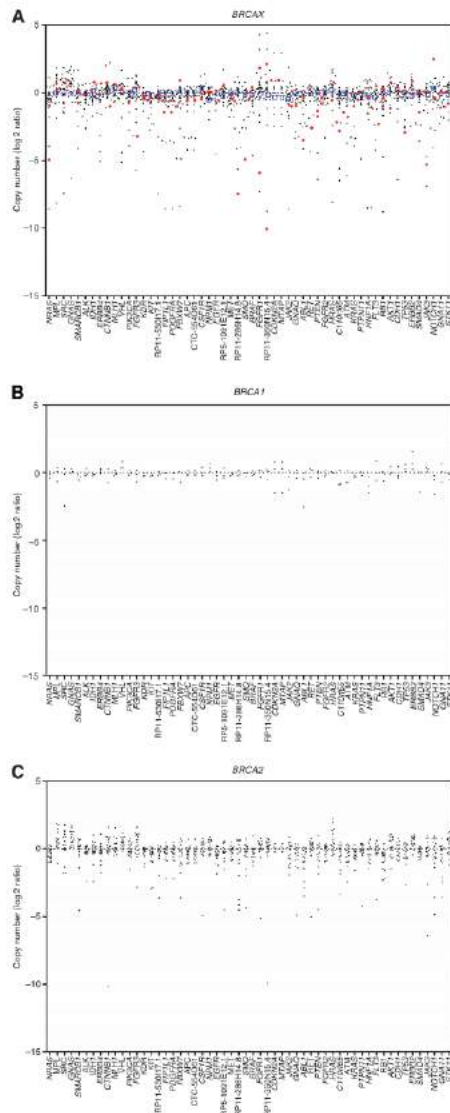
154

Figure 2. (A–C) Copy number changes in BRCAX, BRCA1 and two cohorts (black circle – luminal phenotype; blue square – HER2 phenotype; red circle – basal phenotype).

Unsupervised clustering showed two large groups (Figure 3C). One group (group A, correlation coefficient 0.359) was defined by a predominance of gene loss, whereas the second group (group B, correlation coefficient 0.360) was defined by gene gain. The CNV differences seen in group A when compared with group B included loss of *ABL1, AKT1, FGFR3, SMO, RET, FGFR1, KDR, JAK3, NOTCH1, KIT, EGFR, SMARCB1, PDGFRA, FIP1L1, SRC, FBXW7, CSF1R, STK11, FLT3, MPL, GNAQ* and *ALK* with gain of *NPM1*. Comparison of the two groups showed no association with *BRCA* status or clinicopathologic factors including disease-specific survival (Figure 3D).

Rank comparison between copy number changes was performed within the *BRCA2* (Supplementary Table 5) and BRCAX cohorts (Supplementary Table 6). Owing to the low numbers of *BRCA1* cases, this group of patients was excluded. Within *BRCA2* cases, the strongest correlation ($r > 0.9$, $P < 0.0001$) seen was between *SMO* (7q32.1) and *SMARCB1* (22q11.23), *PTPN11* (12q24.1) and *CTNNB1* (3p21), *CSF1R* (5q32) and *RET* (10q11.2) and between *RET* and *CTNNB1*. In the BRCAX cohort, a correlation was seen between *KDR* (4q11–q12) and *EGFR* (7p12), *ERBB4* (2q33.3–q34) and *FBXW7* (4q31.3), *PDGFRA/FIP1L1* (4q12) and *PTEN* (10q23.2) and between *RB1* (13q14.2) and *SMAD4* (18q21.1).

Comparison of ERBB2/HER2 SISH and copy numbers generated by MiSeq showed significant correlation ($r = 0.46$, $P < 0.01$) (Supplementary Figure 2). Only two instances of amplification (FGFR1 and FGFR3) were seen and not subanalysed further.

DISCUSSION

Our data showed somatic mutations in familial MBC occur at a lower overall frequency compared with FBC (Cancer Genome Atlas Network, 2012), which is in agreement with the limited data from TCGA. However, the profile of mutations observed in this familial MBC cohort is similar to that seen in luminal/ER-positive FBCs with which they share common phenotypic features (Cancer Genome Atlas Network, 2012; Deb *et al*, 2012a). The most common mutations identified in MBCs (where possible to compare) are similar, albeit at lower frequencies (*PIK3CA* mutations (19% *vs* 45%) followed by *TP53* (4% *vs* 12%) and then *PTEN* (2% *vs* 3%). Indeed, the similarities with FBC extend to the types and positions of mutations in MBC in *PIK3CA* with the frequencies of exon 20 mutations > exon 9 mutations > exon 5 mutations (aside from our previously reported E547K *PIK3CA* mutation (Deb *et al*, 2012a), which is rarely seen in FBC), and interestingly, the only gene mutation noted in more than one TCGA MBC sample was *PIK3CA* (two H1047R, one E545K). The overall rarity of *TP53* mutations in our MBCs and in our analysis of the TCGA data set contrast with the historic studies by Anelli *et al* (1995) and Dawson *et al* (1996) who observed 25% (5 out of 20)–41% (12 out of 29) of MBCs harbouring *TP53* mutations. Considering that these mutations are enriched in the basal and HER2 subsets of FBCs, our results are somewhat expected given that these phenotypes are more than half as frequently seen in MBCs (2% and 9%, respectively) (Deb *et al*, 2012a) when compared with FBC. Nevertheless, a notable difference is the absence of *CDH1* mutations that are frequently reported in luminal A FBC (7%) (Corso *et al*, 2012). This is likely to be because of the lower incidence of lobular carcinoma in MBCs (3%) when compared with FBC (∼10%) (Cleton-Jansen *et al*, 1995; Deb *et al*, 2012a).

To date, several studies have performed array CGH analysis of MBCs or analysed oncogene amplification by multiplex ligation-dependent probe amplification. While the array CGH studies show MBCs to contain more gains than seen in FBC and more gains than losses overall (Johansson *et al*, 2011), our MBC cohort shows relatively equal numbers of gene gains and losses. There is some overlap in the regions noted previously, with gains at the *GNAS* locus (20q13.3) and losses at the *ATM* locus (11q22–23) also seen in MBC and FBC (Rudlowski *et al*, 2006; Johansson *et al*, 2011).
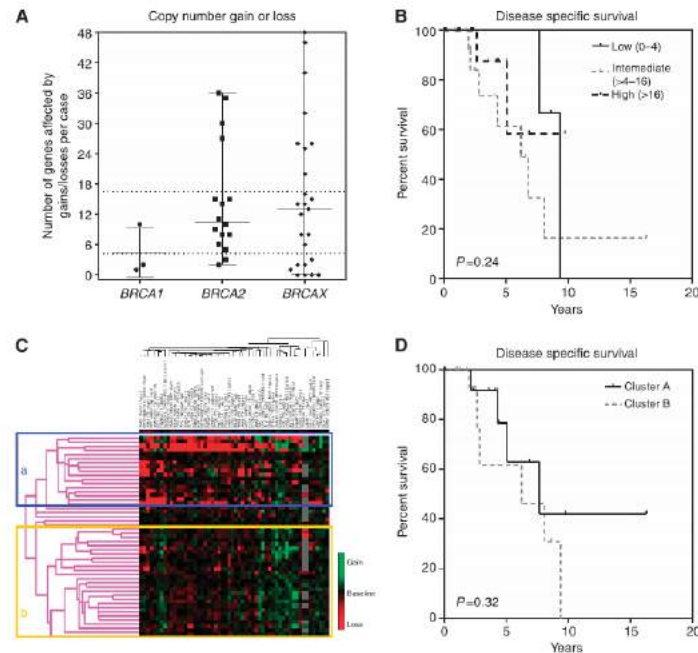
155

Figure 3. (A) Number of genes gained or lost per case, stratified by *BRCA* status, (B) disease-specific survival stratified by volume of copy number changes per case (0–4 = low, > 4–16 = intermediate, > 16 = high), (C) unsupervised cluster analysis of MBCs showing a loss predominant cluster (cluster A) and gain predominant cluster (cluster B) and (D) disease-specific survival of cluster A *vs* B.

Losses however of *GNAQ* (9q21) and *ABL1* (9q34.1) have not been reported in MBC, with 9q34 loss only noted previously in ER-negative IC-NST (Loo *et al*, 2004), a subset rarely found in MBC.

Although an association was present between loss of *ABL1* (9q34.1) and positive nodal disease (65% *vs* 25%, *P* = 0.04) and between loss of *GNAS* and the presence of invasive papillary or micropapillary carcinoma (35% *vs* 6%, *P* = 0.05), no other clinical or phenotypic association was seen either with mutations or gene CNVs. This is likely to be due to the relative homogeneity of MBCs, which are largely IC-NSTs with a luminal phenotype. Comparison with the analysis by Kornegoor *et al* (2012) of 110 MBCs for copy number changes in 21 genes showed some overlap with *AKURA* (on the same locus as *GNAS*) and *CDH1*. Unlike their study, we saw no association between *FGFR1* and a younger age of onset or between *ERBB2* and higher grade and mitotic count. This may be because of the cohort examined, as ours is exclusively familial MBCs that present earlier, or contain a large proportion of *BRCA2* that are associated with higher mitosis and grade, whereas Kornegoor *et al* (2012) did not segregate cases into familial and sporadic cases or comment on the patient's *BRCA* status.

Johansson *et al* (2011) have previously noted two subsets of MBC based on the frequency of chromosomal changes. Most MBCs fell into an MBC complex group characterised by high numbers of changes with frequent whole chromosomal arm gains/ losses. These cancers grouped well only luminal complex FBCs. Although it is difficult to compare high-resolution aCGH results with copy number changes in 54 gene loci, we noted three distinct groups of cases with low (0–4), intermediate (> 4–16) and high

(> 16) numbers of copy number gain or losses. No clinical differences were seen between these groups with relatively similar spread between *BRCA2* and BRCAX cohorts. Prognostically, a very weak trend was seen showing better outcome in the low cohort compared with the intermediate and high cohorts (*P* = 0.24). Interestingly, we also noted that genes in close chromosomal proximity shared significantly similar changes between cases consistent with the frequent whole chromosomal arm changes as seen in MBCs by Johansson *et al* (2011) and supporting the validity of our findings. Notably, samples that were run more than once clustered tightly, further indicating the analytical validity of the test (Supplementary Figure 3).

Several differences were observed between the *BRCA* subtypes. *TP53* mutations, while infrequent, were restricted to tumours arising in *BRCA2* carriers (11% *vs* 0%) with a profile more similar to luminal B cancers (Cancer Genome Atlas Network, 2012). Notably, within MBCs, *BRCA2* cancers have been associated with higher grade and increased mitotic counts (Ottini *et al*, 2003), typical of luminal B tumours and thus may represent a novel subtype in MBCs. In contrast, BRCAX tumours had a much higher incidence of activating *PIK3CA* mutations (25% *vs* 10%), suggesting that activation of the mTOR/PIK3CA pathway may be relevant in these tumours. As most of these cancers are also ER positive and of a luminal phenotype, these features are more similar genophenotypically to the luminal A FBCs. Interestingly, in contrast to familial FBCs (Greenblatt *et al*, 2001), of the three *BRCA1* MBCs, no *TP53* mutation was seen. Whiles these numbers are low, the low penetrance of MBCs in male *BRCA1* mutation

156

Table 4. Copy number variations stratified by *BRCA* status

Gene	Chromosome position	Gains				Losses			
		All cases (%)	BRCA1 (%)	BRCA2 (%)	BRCAX (%)	All cases (%)	BRCA1 (%)	BRCA2 (%)	BRCAX (%)
NRAS	1p13.2	2.3	0.0	0.0	4.0	29.5	0.0	31.3	32.0
MPL	1p34	20.5	33.3	25.0	16.0	4.5	0.0	0.0	8.0
ALK	2p23	2.3	0.0	0.0	4.0	20.5	0.0	18.8	24.0
IDH1	2q33.3	15.9	33.3	12.5	16.0	9.1	0.0	18.8	4.0
ERBB4	2q33.3–q34	6.8	0.0	6.3	8.0	20.5	33.3	12.5	24.0
CTNNB1	3p21	25.0	0.0	31.3	24.0	9.1	33.3	6.3	8.0
MLH1	3p21.3	18.2	33.3	18.8	16.0	0.0	0.0	0.0	0.0
VHL	3p25.3	6.8	0.0	12.5	4.0	6.8	0.0	0.0	12.0
PIK3CA	3q26.3	6.8	0.0	12.5	4.0	15.9	0.0	12.5	20.0
FGFR3	4p16.3	18.2	0.0	31.3	12.0	15.9	0.0	12.5	20.0
RP11-530I17.1	4q11–q12	0.0	0.0	0.0	0.0	11.4	0.0	12.5	12.0
KDR	4q11–q12	0.0	0.0	0.0	0.0	13.6	0.0	18.8	12.0
KIT	4q11–q12	0.0	0.0	0.0	0.0	29.5	0.0	25.0	36.0
FIP1L1	4q12	2.3	0.0	6.3	0.0	29.5	0.0	25.0	36.0
PDGFRA	4q12	2.3	0.0	6.3	0.0	29.5	0.0	25.0	36.0
FBXW7	4q31.3	6.8	0.0	12.5	4.0	27.3	0.0	31.3	28.0
APC	5q21–q22	2.3	0.0%	6.3	0.0	29.5	0.0	37.5	28.0
CTC-554D6.1	5q22.2	2.3	0.0	6.3	0.0	29.5	0.0	37.5	28.0
CSF1R	5q32	13.6	0.0	18.8	12.0	6.8	0.0	6.3	8.0
NPM1	5q35.1	0.0	0.0	0.0	0.0	6.8	0.0	0.0	12.0
EGFR	7p12	0.0	0.0	0.0	0.0	22.7	0.0	18.8	28.0
RP5-1091E12.1	7p12	6.8	0.0	6.3	8.0	13.6	0.0	18.8	12.0
MET	7q31	2.3	0.0	6.3	0.0	27.3	0.0	25.0	32.0
RP11-286H14.8	7q32	4.5	0.0	6.3	4.0	25.0	0.0	31.3	24.0
SMO	7q32.1	2.3	0.0	6.3	0.0	18.2	0.0	18.8	20.0
BRAF	7q34	9.1	33.3	6.3	8.0	20.5	0.0	18.8	24.0
FGFR1	8p12	18.2	0.0	12.5	24.0	11.4	0.0	6.3	16.0
RP11-350N15.4	8p12	13.6	0.0	6.3	20.0	11.4	0.0	6.3	16.0
CDKN2A	9p21	4.5	0.0	0.0	7.7	4.5	0.0	20.0	0.0
GNAQ	9q21	2.3	0.0	0.0	4.0	36.4	0.0	37.5	40.0
MTAP	9p21	2.3	0.0	0.0	4.0	2.3	0.0	20.0	0.0
JAK2	9p24	4.5	0.0	6.3	4.0	25.0	33.3	25.0	24.0
ABL1	9q34.1	0.0	0.0	0.0	0.0	47.7	66.7	43.8	48.0
RET	10q11.2	4.5	0.0	12.5	0.0	15.9	0.0	6.3	24.0
PTEN	10q23.2	2.3	0.0	6.3	0.0	27.3	0.0	31.3	28.0
FGFR2	10q26	6.8	0.0	6.3	8.0	13.6	0.0	12.5	16.0
HRAS	11p15.5	15.9	0.0	37.5^	4.0	4.5	0.0	6.3	4.0
ATM	11q22–q23	2.3	0.0	6.3	0.0	34.1	66.7	31.3	32.0
C11orf65	11q22.3	2.3	33.3	0.0	0.0	38.6	66.7	37.5	36.0
KRAS	12p12.1	0.0	0.0	0.0	0.0	27.3	0.0	31.3	28.0
PTPN11	12q24.1	2.3	0.0	0.0	4.0	11.4	0.0	6.3	16.0
HNF1A	12q24.2	11.4	0.0	6.3	16.0	25.0	0.0	25.0	28.0
FLT3	13q12	6.8	33.3	6.3	4.0	15.9	0.0	6.3	24.0
RB1	13q12	0.0	0.0	0.0	0.0	25.0	0.0	43.8**	16.0
AKT1	14q32.32	9.1	0.0	12.5	8.0	6.8	0.0	6.3	8.0
CDH1	16q22.1	6.8	0.0	6.3	8.0	13.6	0.0	18.8	12.0
ERBB2	17q12	9.1	33.3	6.3	8.0	4.5	0.0	0.0	8.0
TP53	17q13.1	0.0	0.0	0.0	0.0	15.9	0.0	12.5	20.0
SMAD4	18q21.1	0.0	0.0	0.0	0.0	22.7	33.3	25.0	20.0

157

Table 4. (Continued)

Gene	Chromosome position	Gains				Losses			
		All cases (%)	BRCA1 (%)	BRCA2 (%)	BRCAX (%)	All cases (%)	BRCA1 (%)	BRCA2 (%)	BRCAX (%)
JAK3	19p13.1	2.3	0.0	6.3	0.0	18.2	0.0	12.5	24.0
NOTCH1	19p13.2–p13.1	9.1	0.0	12.5	8.0	22.7	66.7	18.8	20.0
STK11	19p13.3	9.1	0.0	25.0***	0.0	9.1	0.0	12.5	8.0
GNA11	19p13.3	2.3	0.0	0.0	4.0	20.5	0.0	25.0	20.0
SRC	20q12–q13	11.4	0.0	12.5	12.0	13.6	33.3	12.5	12.0
GNAS	20q13.3	34.1	0.0	50.0	28.0	4.5	0.0	0.0	8.0
SMARCB1	22q11.23	6.8	0.0	18.8***	0.0	20.5	0.0	12.5	28.0

*P = 0.0061; **P = 0.0270; ***P = 0.0134; ****P = 0.0423. Light grey = 30–40% frequency; medium grey = 40–50% frequency; dark grey = > 50% frequency.

carriers and a lack of tumours with basal cell phenotype suggest that the germline mutation may not be acting as a tumour driver and emphasises difference of the *BRCA1* effect in MBCs compared with FBCs. Compared with familial FBCs stratified by subtypes, there was some similarities with luminal A cancers with frequent loss of 11q23 and 9q34.3. No overlap with other intrinsic subtypes was seen.

Two studies by Johansson *et al* (2011) and Tirkkonen *et al* (1999) have examined gene copy numbers in very small numbers of familial MBCs, reporting on copy number changes in three and five *BRCA2* MBCs, respectively. Our *BRCA2* MBCs, in comparison with other MBCs, showed novel *HRAS*, *STK11* and *SMARCB1* amplification and *RB1* loss. The loss of *RB1* may be because of its chromosomal proximity to the *BRCA2* gene, which is supported by sporadic FBC studies showing frequent contiguous loss of *RB1* and *BRCA2* on the chromosome 13q12–q14 band (Cleton-Jansen *et al*, 1995). While accurate somatic loss of heterozygosity analysis of *BRCA2* in our cases is largely restricted by availability of germline DNA, wild-type allelic loss would not be unexpected as previous studies have shown that somatic *BRCA2* mutations occur frequently (21%) in sporadic MBCs (Kwiatkowska *et al*, 2002), and thus suggesting that *BRCA2* loss is a significant driver in MBC. Interestingly, Johansson *et al* (2011) also noted gain of the 19p13 locus housing *STK11/LKB1* in their *BRCA2* MBC. As this area contains several tumour suppressor genes, it may suggest particular selection in *BRCA2*-deficient MBCs. Alternately, as the tumour suppressor *STK11/LKB1* may also enhance ERα response, it may be that *STK11/LKB1* may be oncogenic in some breast cancer subsets that may also include MBCs. Notably, in our cohort we see just as many losses of *STK11/LKB1* as FBC and imply a dual function for the protein. The amplification of the *HRAS* or the *SMARCB1* loci in *BRCA2* males has not been reported in previous MBC studies. The relevance of this finding is uncertain, but as *SMARCB1* is a tumour suppressor gene, it may be a bystander effect and may again reiterate the strength of *BRCA2* drive in MBCs. This is also supported, perhaps, by the strong correlation seen between copy number changes in tumour suppressor genes *PTEN*, *ATM*, *RB1*, *SMAD4* and *STK11* (r > 0.64), but less so with *TP53* (only with *PTEN*, *ATM* and *RB1*) within the BRCAX cohort but not within *BRCA2*, suggesting alternate drivers between these groups.

A genome-wide association study of the germline of 823 MBC patients identified 17 SNPs mapping to six independent genomic regions that were associated with predisposition to MBC (Orr *et al*, 2012). However, none of these are present on our panel. A substantial proportion of our cases were included in the above study and our findings are in keeping with previous findings in that no candidate variants within our gene panel are suggestive of MBC predisposition or are of substantial clinical relevance. More so, variant frequency appears similar across all *BRCA* subgroups, suggesting the absence of at least a strong modifier of *BRCA* affect.

CONCLUSION

This the first study to perform high-throughput somatic sequencing on familial MBC. It shows differences between *BRCA2* and BRCAX tumours, with the former harbouring *TP53* mutations and the latter containing frequent *PIK3CA* mutations similar to luminal A FBCs. Overall, mutation frequency was lower than that seen in FBC.

Analysis of gene copy number analysis also showed differences between *BRCA2* and BRCAX cohorts. While some gains and losses were similar to that reported previously in both MBC and FBC, we have identified specific gains that are particular to *BRCA2* tumours. Comparison of coexpressed genes also demonstrated differences between *BRCA2* and BRCAX cases with a distinct concordance of tumour suppressor genes with BRCAX patients and more heterogeneity in *BRCA2* cases. We also noted more gene losses than other previous MBC studies, suggesting that familial MBCs may be a unique cohort among which difference exist between *BRCA2* and BRCAX cancers. Furthermore, from a future treatment perspective, the findings suggest that different pathways may be screened and targeted depending on the *BRCA* status of MBC patients.

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158

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Supplementary Information accompanies this paper on British Journal of Cancer website (http://www.nature.com/bjc)

159

5.3.2 Additional File - Supplementary Figure 1 - Total and mutations TruSeq reads

of validated somatic mutations and variants. 1a) Test specificity, 1b) test sensitivity

and 1c) box-plot of total reads (Whiskers 5th-95th percentile, Box 25th – 7^{5th} percentile,
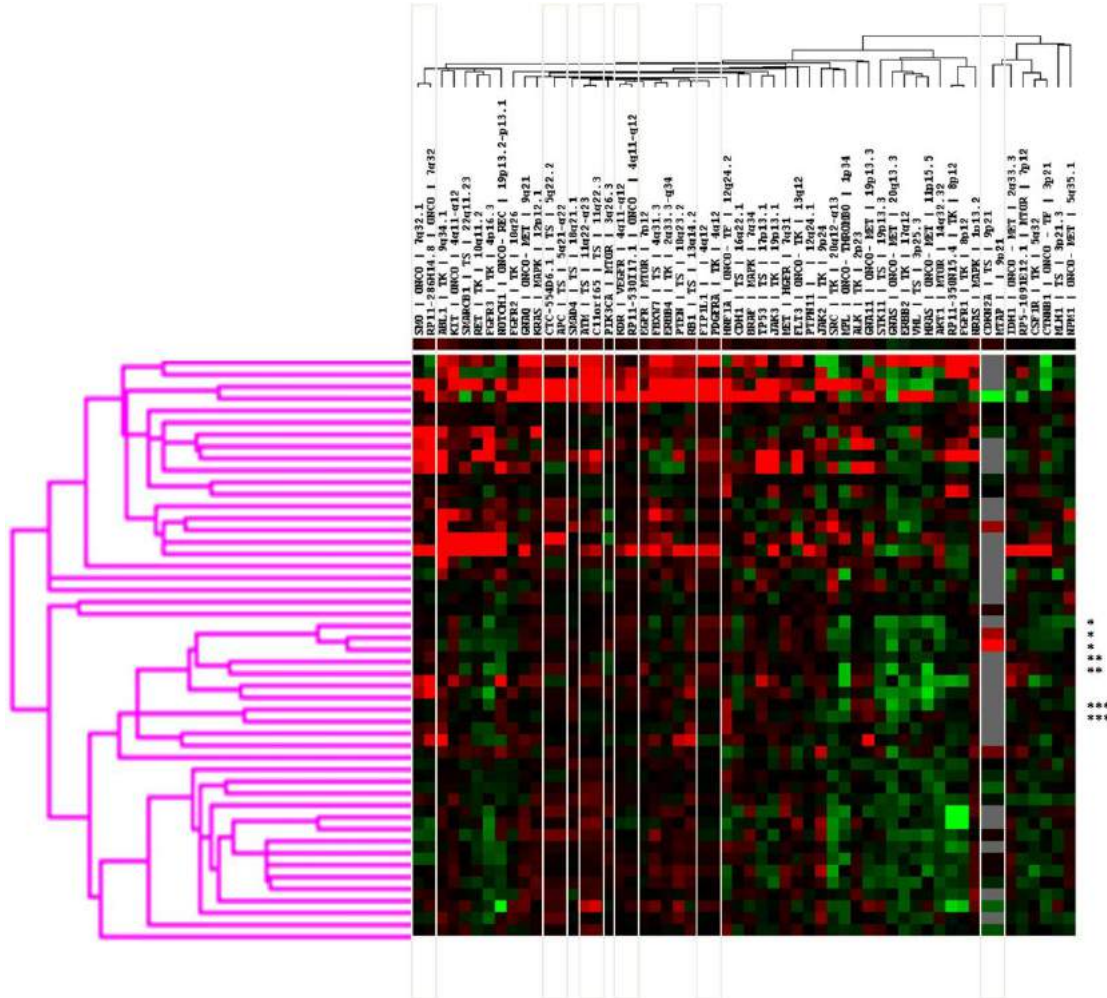
Bar – median).
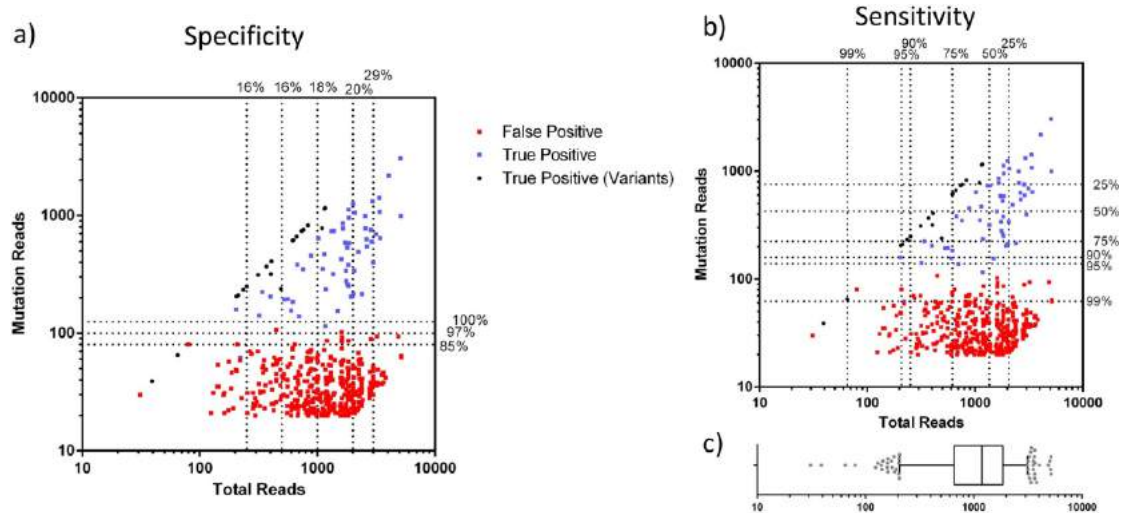
5.3.3 Additional File - Supplementary Figure 2 – Correlation between

ERBB2/HER2 gene copy numbers by HER2 SISH and MiSeq.
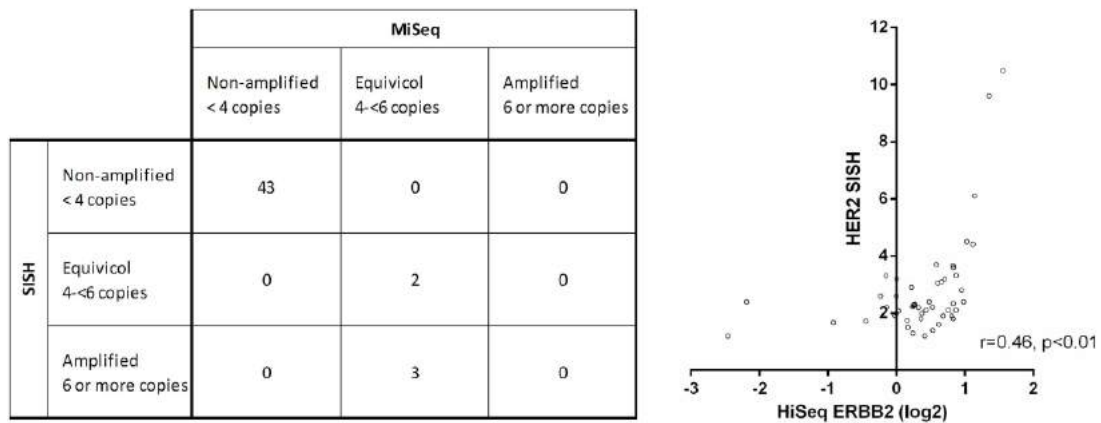
5.3.4 Additional File - Supplementary Figure 3 – Unsupervised cluster analysis - *,
** and *** are different extractions and TruSeq runs from the same tumour, grey
boxes highlight genes from adjacent chromosomal loci.

BMC Cancer

BRCA2 carriers with male breast cancer show elevated tumour methylation

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Abstract

Background: Male breast cancer (MBC) represents a poorly characterised group of tumours, the management of which is largely based on practices established for female breast cancer. However, recent studies demonstrate biological and molecular differences likely to impact on tumour behaviour and therefore patient outcome. The aim of this study was to investigate methylation of a panel of commonly methylated breast cancer genes in familial MBCs.

Methods: 60 tumours from 3 BRCA1 and 25 BRCA2 male mutation carriers and 32 males from BRCAX families were assessed for promoter methylation by methylation-sensitive high resolution melting in a panel of 10 genes (RASSF1A, TWIST1, APC, WIF1, MAL, RARβ, CDH1, RUNX3, FOXC1 and GSTP1). An average methylation index (AMI) was calculated for each case comprising the average of the methylation of the 10 genes tested as an indicator of overall tumour promoter region methylation. Promoter hypermethylation and AMI were correlated with BRCA carrier mutation status and clinicopathological parameters including tumour stage, grade, histological subtype and disease specific survival.

Results: Tumours arising in BRCA2 mutation carriers showed significantly higher methylation of candidate genes, than those arising in non-BRCA2 familial MBCs (average AMI 23.6 vs 16.6, $p = 0.01$, 45% of genes hypermethylated vs 34%, $p < 0.01$). RARβ methylation and AMI-high status were significantly associated with tumour size ($p = 0.01$ and $p = 0.02$ respectively), RUNX3 methylation with invasive carcinoma of no special type (94% vs 69%, $p = 0.046$) and RASSF1A methylation with coexistence of high grade ductal carcinoma in situ (33% vs 6%, $p = 0.02$). Cluster analysis showed MBCs arising in BRCA2 mutation carriers were characterised by RASSF1A, WIF1, RARβ and GSTP1 methylation ($p = 0.02$) whereas methylation in BRCAX tumours showed no clear clustering to particular genes. TWIST1 methylation ($p = 0.001$) and AMI ($p = 0.01$) were prognostic for disease specific survival.

Conclusions: Increased methylation defines a subset of familial MBC and with AMI may be a useful prognostic marker. Methylation might be predictive of response to novel therapeutics that are currently under investigation in other cancer types.

Keywords: Male breast cancer, Familial breast cancer, Methylation, BRCA1, BRCA2, Promoter methylation

Background

Male breast cancer (MBC) is a poorly studied disease. Indeed, MBC accounts for ~1% of all breast cancers but it contributes to a higher proportion of breast cancer-related deaths [1, 2]. As a significant proportion of MBCs arise within breast/ovarian families, the majority of MBC research has focused on cancer predisposition.

However, differences in genotype-phenotype between female and male breast cancers suggest that MBCs have alternate and novel drivers [3–5].

It is now well recognised that aberrant modification of gene expression by promoter methylation is often pathogenic and not an inconsequential contributor to oncogenesis: indeed epigenomic changes are often more commonly observed than gene mutations and chromosomal instability in many cancers [6]. In cancer, aberrant methylation is frequently seen within CpG islands in promoter regions often resulting in transcriptional silencing [7] often occurring early in cancer development.

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162

Deb *et al. BMC Cancer* (2017) 17:641

Page 2 of 11

From a clinical perspective, gene methylation may not only contribute to the biological understanding of cancer subsets, but may also be utilised in screening, staging and monitoring of disease activity, as methylation is stable in formalin-fixed paraffin-embedded pathology material and in plasma. Methylated genes may also be attractive treatment targets in MBC using therapies in trials in other tumour types [8].

To date only three MBC studies, composed of a total of 182 male breast cancers, have evaluated methylation in MBCs, which showed that promoter gene methylation in MBC, as compared to normal male breast tissue, is a common event and associated with a more aggressive phenotype [9–11]. However, the methodologies used are prone to give false positive results and/or are non-quantitative. To address the paucity of data we have performed methylation profiling in a well-characterised series of MBC. Our aims were to 1) determine the frequency and level of methylation of important breast cancer genes in a large cohort of familial MBCs, 2) identify clinicopathological associations, including patient outcome, that may define a biological effect of gene methylation and 3) identify and characterise potential molecular subgroups defined by their methylation patterns with clinicopathological correlation.

Methods

Patient samples

Primary male breast cancers examined in this study were obtained from the Kathleen Cunningham Foundation Consortium (kConFab) breast/ovarian familial cancer repository (Table 1). Cases are accepted into the registry based on a strong family history of breast and ovarian cancer with criteria for admission to the kConFab study as outlined previously [12], with all participants providing informed consent to participate in research studies. Patients were from Australia and New Zealand and diagnosed between 1980 and 2009.

The flow of patients through the study was according to the REMARK criteria outlined in Additional file 1 [13]. Of the 118 cases within the kConFab registry, 58 cases were excluded due to unavailability of tissue. Sixty cases had sufficient material at an appropriate DNA concentration for methylation testing as outlined below. These cases belonged to three groups: 3 MBCs that arose in *BRCA1* mutation carriers, 25 that arose in *BRCA2* mutation carriers and 32 that occurred in males from BRCAX families (i.e. where an underlying germline mutation had not been identified).

Clinical parameters, including disease specific survival (DSS) were obtained from referring clinical centres, kConFab questionnaires and state death registries [14, 15]. Information on pedigrees, mutational status and testing were available from the kConFab central registry.

Table 1 Clinicopathological description of male breast cancers in this study

Feature		
Age (years)	Median = 62.5	Range: 30–85
Mutation carrier status		
BRCA1	3	5.0%
BRCA2	25	41.7%
BRCAX	32	53.3%
Size (mm)	Median = 17	Range: 2–50
Histological subtype		
Invasive carcinoma - no special type (IC-NST)	46	76.7%
Invasive papillary carcinoma	8	13.3%
IC-NST with areas of micropapillary	4	6.7%
Invasive lobular carcinoma	2	3.3%
Grade		
1	2	3.3%
2	30	50.0%
3	28	46.7%
DCIS		
Present	41	68.3%
Absent	15	25.0%
Unknown	4	6.7%
Nodal Status		
N0	28	46.7%
N1	20	33.3%
Nx	12	20.0%
Paget's Disease		
Present	8	13.3%
Absent	44	73.3%
Unknown	8	13.3%
ER status (Allred score)		
Negative (0–4/8)	2	3.3%
Positive (5–8/8)	58	96.7%
PgR status (allred score)		
Negative (0–4/8)	8	13.3%
Positive (5–8/8)	52	86.7%
HER2 (SISH)		
Amplified	5	8.3%
Non-amplified	55	91.7%
Phenotype		
Luminal	54	90.0%
HER2	5	8.3%
Basal	1	1.7%

163

Histological classification was based on criteria set by the World Health Organisation 2012 [16] and all slides and pathological records from all cases were reviewed centrally. Immunohistochemistry for estrogen receptor (ERα), progesterone receptor (PgR), basal markers (cytokeratin 5 (CK5), EGFR) and HER2 silver in-situ hybridisation (SISH) was performed as previously reported [4]. Stratification of intrinsic phenotypes was based on Nielsen et al. [17], and placed into luminal (ERα/PgR positive, HER2 negative, CK5 and/or EGFR negative), basal (ER α/PgR and HER2 negative; CK5 and/or EGFR positive), HER2 (HER2 positive) and null/negative (HER2, ERα, PgR, CK5 and EGFR negative) phenotypes. Permission to access the kConFab samples and data was granted by the kConFab Executive Committee (Project #115/07−17). This work was carried out with approval from the Peter MacCallum Cancer Centre Ethics Committee (Project No: 11/61).

Germline *BRCA1/2* testing

Mutation testing for *BRCA1* and *BRCA2* mutations was performed as previously reported [18, 19]. Once the family mutation had been identified, all pathogenic (including splice site) variants of *BRCA1* and *BRCA2* were genotyped by kConFab in all available family members' DNA.

DNA extraction

Genomic DNA was extracted from formalin-fixed, paraffin embedded (FFPE) samples. A 3 μM haematoxylin and eosin (H&E) stained slide was cut from FFPE blocks and stained to identify for tumour enriched areas showing >80% tumour purity. From the relevant area on the FFPE block, one to two 2 mm punch biopsy cores were taken. The cores were then dewaxed and hydrated through a decreasing alcohol series. Genomic DNA was then extracted using the DNeasy Tissue kit (Qiagen, Hilden, Germany) following proteinase K digestion at 56 °C for 3 days.

Bisulfite modification

Genomic DNA (600 ng) was bisulfite modified using the MethylEasy™ Xceed kit (Genetic Signatures, North Ryde, Australia) according to the manufacturer's instructions. The bisulfite modified DNA was eluted into 50 μL of EB buffer. CpGenome™ Universal Methylated DNA (Chemicon/Millipore, Billerica, MA) and whole-genome amplified DNA [20] were used as the fully methylated and unmethylated controls, respectively. DNA methylation standards (10, 25 and 50%) were made by mixing the fully methylated control with the unmethylated DNA control.

Methylation-sensitive high resolution melting (MS-HRM)

Methylation screening was performed using MS-HRM to quantitate methylation in bisulfite-modified samples according to the sequence-dependent thermostability in which the level and presence of homogenous and heterogeneous methylation can be detected [21, 22]. MS-HRM primers were specifically designed to generate short amplicons enabling use in formalin-fixed paraffin embedded (FFPE) samples and are summarised in Additional file 2.
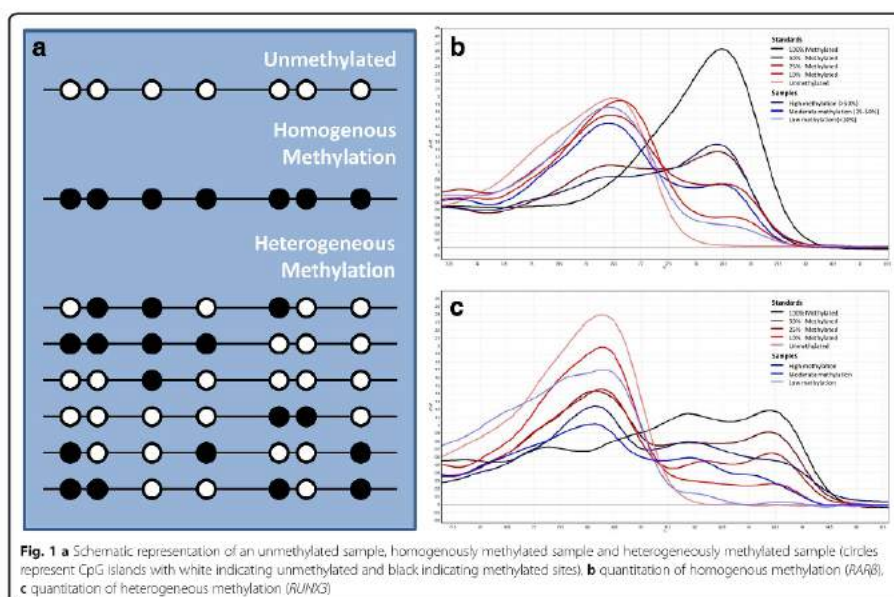
PCR amplification and HRM analysis were performed on the Rotor-Gene 6000 (Corbett, Sydney). Samples were run in duplicate. Conditions for each gene are described in Additional file 2. The reaction was performed using a final volume of 20 μL and the mixture consisted of 1 × PCR buffer (Qiagen, Hilden, Germany), 2.5–4.0 mmol/L of MgCl₂, 200 μmol/L of each dNTP, forward and reverse primers, 5 μmol/L of SYTO9 intercalating dye (Invitrogen, Carlsbad, CA), 0.5 U of HotStarTaq DNA polymerase (Qiagen, Hilden, Germany) and 10 ng of bisulfite modified DNA. The methylation level of each DNA sample was determined visually by comparing it against the standard curves. Heterogeneous DNA methylation was defined by melting profiles that did not directly conform to any of the methylation controls due to the formation of heteroduplexes between closely but not identically related single complementary DNA strands. Complexes that complete melting slightly after the unmethylated controls were indicative of low levels of DNA methylation. In contrast, complexes with a late melting profile typically contained more heavily methylated epialleles (Fig. 1).

Methylation scoring

A cut-off of 10% methylation was used to primarily exclude low level methylation of uncertain biological significance. The remaining samples were further grouped into moderate methylation (10–50% fully methylated, or moderate heterogenous methylation) and high methylation (>50% fully methylated, or high-level heterogenous methylation) (Fig. 1). Positive methylation (hypermethylation) for each gene was thus considered when duplicate samples showed >10% or moderate to high heterogenous methylation The samples were also given a percentage methylation for each gene by comparing the methylation to the curves of the standard, which was then averaged across all the genes to give a average methylation index (AMI) scored between 0 and 100% for each tumour sample [23]. The AMI measurement is based on the cumulative methylation index [24], which is the sum of the percentages of methylation of the individual genes, but corrects for the number of genes tested. Using the AMI scores, groups were dichotomised into low and high based on the median AMI as a cut-off point. This analysis does not make assumptions as to the effect of any particular level of methylation.

Cluster analysis

Unsupervised complete linkage clustering was performed with Euclidean metric distance. Unsupervised hierarchical

Deb et al. BMC Cancer (2017) 17:641

Page 4 of 11

Fig. 1 a Schematic representation of an unmethylated sample, homogenously methylated sample and heterogeneously methylated sample (circles represent CpG islands with white indicating unmethylated and black indicating methylated sites). **b** quantitation of homogenous methylation (*RARβ*). **c** quantitation of heterogeneous methylation (*RUNX3*)

cluster analysis of methylation at each gene was used to detect possible distinct molecular signatures. Analysis was performed using Cluster and Tree View software written by Michael Eisen (Stanford University) as previously published [25–27].

Statistical analysis

Comparison of groups was made with using Mann-Whitney U for non-parametric continuous distributions and Fisher's exact test for threshold data. Kaplan-Meier survival curves were plotted using breast cancer related death as the endpoint and compared using a log rank test. Pearson's correlation coefficient was measured for the cluster analysis. Analysis was performed with Graph-Pad Prism 5 software (GraphPad Prism version 5.04 for Windows, GraphPad Software, La Jolla California USA). A two-tailed P-value test was used in all analyses and a p-value or less than 0.05 was considered statistically significant.

Results

Methylation analysis of MBCs finds associations with genotype and clinico-pathological characteristics

We performed methylation analysis on 60 MBC (25 *BRCA2*, 3 *BRCA1* and 32 BRCAX), whose clinical and pathological features are summarised in Table 1. The

features of these cases are consistent with familial male breast cancers in the literature [28], primarily being invasive carcinomas of no special type (76%), ER and PR positive (97% and 87% respectively) and HER2 unamplified (92%). Fifty four (90%), five (8%) and one (2%) tumour(s) were luminal, HER2 and basal phenotypes respectively.

We selected 10 genes for analysis based on their frequency of methylation and/or association with prognosis in previous studies of breast cancer, as follows. Methylation of *GSTP1 and RASSF1A* is common in MBC [10, 11]. Methylation of *WIF1*, *TWIST*, *FOXC1*, *APC*, *RARb* and *MAL* have also been associated with patient outcome in FBC [29–33]. *CDH1*, *RARB* and *RUNX3* are frequently methylated in 22–72% [34–36], 20–45% [35, 37, 38] and 50–90% of FBC respectively [39, 40].

GSTP1 was the most commonly methylated gene (82%), followed by *RASSF1A* (68%), with both showing a pattern of predominantly high level methylation (Table 2). Other genes were more varied: *RARβ*, *APC* and *RUNX3* had moderate levels of methylation, while heterogeneous methylation was observed in *TWIST1*, *MAL* and *WIF1*, with a mix of moderate and high heterogeneous methylation. Only low level methylation was observed in *CDH1* with no cases showing hypermethylation. There were no statistically significant associations of specific gene methylation with patient genotype, however, there were trends

165

Deb *et al. BMC Cancer* (2017) 17:641

Page 5 of 11

Table 2 Percentage of cases with hypermethylation

	GSTP1	RASSF1A	MAL	TWIST	RUNX3	RARβ	APC	FOXC1	CDH1	WIF1	TOTAL HYPERMETHYLATED GENES	AMI (mean)
BRCA1 (n = 3)	2 (66%)	1 (33%)	1 (33%)	0	1 (33%)	1 (33%)	1 (33%)	1 (33%)	0	1 (33%)	9 (30%)	13.4
BRCA2 (n = 25)	22 (88%)	20 (80%)	14 (56%)	13 (52%)	8 (32%)	11 (44%)	8 (32%)	6 (24%)	0	11 (44%)	113 (45%)	23.6
BRCAX (n = 32)	25 (78%)	20 (63%)	12 (38%)	9 (28%)	9 (28%)	6 (19%)	7 (22%)	8 (25%)	0	14 (56%)	110 (34%)	17.0
All (n = 60)	49 (82%)	41 (68%)	27 (45%)	22 (37%)	18 (30%)	18 (30%)	16 (27%)	15 (25%)	0	26 (43%)	232 (39%)	14.0
				$p = 0.06$		$p = 0.08$					$P < 0.01$	$p = 0.01$

Deb *et al. BMC Cancer* (2017) 17:641

Page 6 of 11

for higher methylation frequency of *RARβ* (44% vs 20%, *p* = 0.08) and *TWIST1* (52% vs 26%, *p* = 0.06) in *BRCA2* carriers. Overall, the *BRCA2* group also showed a higher rate of gene hypermethylation (45% vs 34%, *p* < 0.01) in our target suppressor gene panel than the other groups.

We examined the association of specific gene methylation with patient and tumour characteristics (Table 3). *APC* hypermethylation was significantly associated with older age (69.1 years vs 60.4 years, *p* = 0.01, Table 2) whereas *MAL* hypermethylation was significantly inversely associated with age (59.1 years vs 65.7 years, *p* = 0.04). Significantly larger tumour size was noted for cases with *RARβ* hypermethylation (median 22.3 mm vs 16.5 mm; *p* = 0.01). *RARβ* hypermethylation was also associated with a higher percentage of Paget's disease (31% vs 8%, *p* = 0.04). *RUNX3* hypermethylation was associated with increased frequency of IC-NST histological type (94% vs 69%, *p* = 0.046) and *RASSF1A* hypermethylation associated with the coexistence of high grade DCIS (33% vs 6% (*p* = 0.02).

High overall levels of methylation have been associated with aggressive tumour features such as mitotic count, grade and poor patient outcome in MBC [10] and FBC [30, 41]. Therefore, we calculated a measure of overall methylation for each sample, the AMI. There was a significant increase in AMI in *BRCA2* mutation carriers compared with other MBCs (23.6 vs 16.6, *p* = 0.01, Fig. 2). In addition, the AMI was positively correlated with tumour size (median 22.4 mm vs 15.4 mm, *p* = 0.02).

Cluster analysis identifies subgroups of MBC

In order to evaluate whether methylation profiles could discover novel subgroups in MBC, as has been seen for FBC [42, 43] and colorectal cancer [44], we performed an unsupervised clustering analysis. Four main clusters with at least 7 samples in each group were identified (Fig. 3). MBCs arising in *BRCA2* carriers showed a significantly greater frequency (6/7 vs 19/53, *p* = 0.02) of Cluster 3 membership (characterised by *RASSF1A*, *WIF1*, *GSTP1* and *RARβ* methylation). No other clinicopathological association or prognostic differences were seen between the clusters.

Analysis of methylation patterns within the *BRCA2* subgroup of tumours showed two clusters with correlation coefficients >0.8 (Additional file 3). Cluster A contained 12 tumours and was characterised by high *GSTP1* methylation and *MAL* methylation and relatively lower *RASSF1A* methylation. Cluster B contained 8 tumours and showed primarily high *RASSF1A* methylation. Cluster A tumours showed an earlier age at diagnosis than

Table 3 Correlation of hypermethylation with clinicopathological variables (associations approaching significance, *p* < 0.05 in bold)

	GSTP1		RASSF1A		MAL		RUNX3		RARβ		APC		FOXC1		AMI (median)	
Hypermethylation	+	-	+	-	+	-	+	-	+	-	+	-	+	-	>	<
Age (years)					59.1	65.7			67.2	60.9	69.1	60.4				
p-value				**0.04**						0.07		**0.01**				
Tumour size (mm)									22.3	16.5	21.4	17.1			20.8	15.8
p-value										**0.01**		0.08				**0.02**
IC-NST Histology							94%	69%								
p-value								**0.046**								
Grade 3	51%	18%														
p-value		0.09														
Paget's Disease									31%	8%						
p-value										**0.04**						
DCIS present			33%	6%												
p-value				**0.02**												
Lymphovascular invasion	49%	18%									20%	51%	53%	40%		
p-value		0.09										0.07		0.09		
Perineural invasion									63%	36%						
p-value										0.07						
Node positive			52%	24%					62%	34%						
p-value				0.08						0.07						
HER2 positive															13%	0
p-value																0.11

Deb et al. BMC Cancer (2017) 17:641

Page 7 of 11

Fig. 2 Average methylation index (AMI) for samples stratified by BRCA status (Central bar – median, error bars = 1 standard deviation)
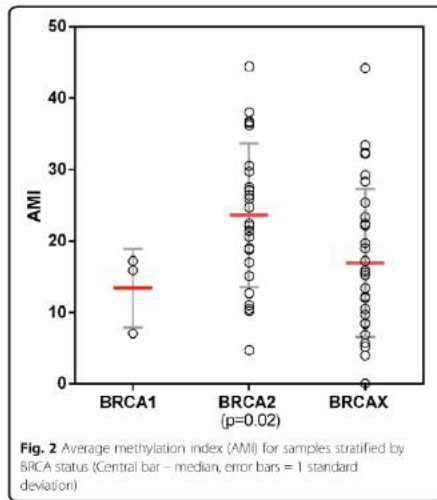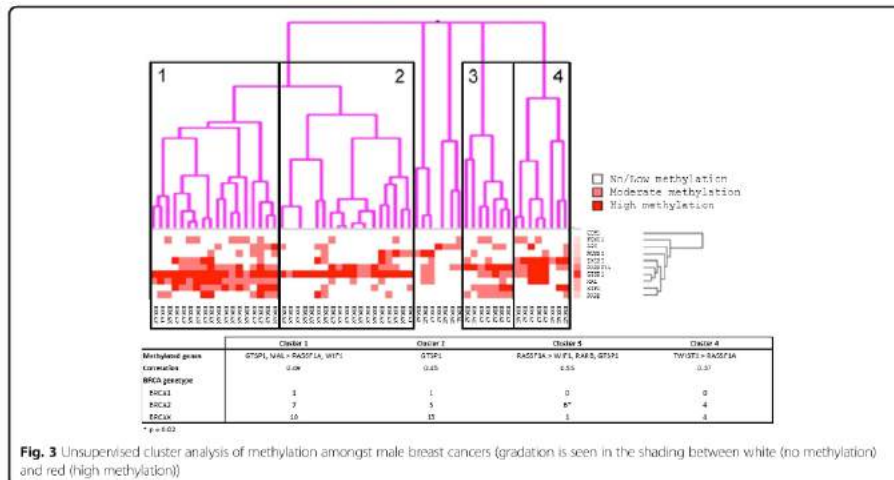
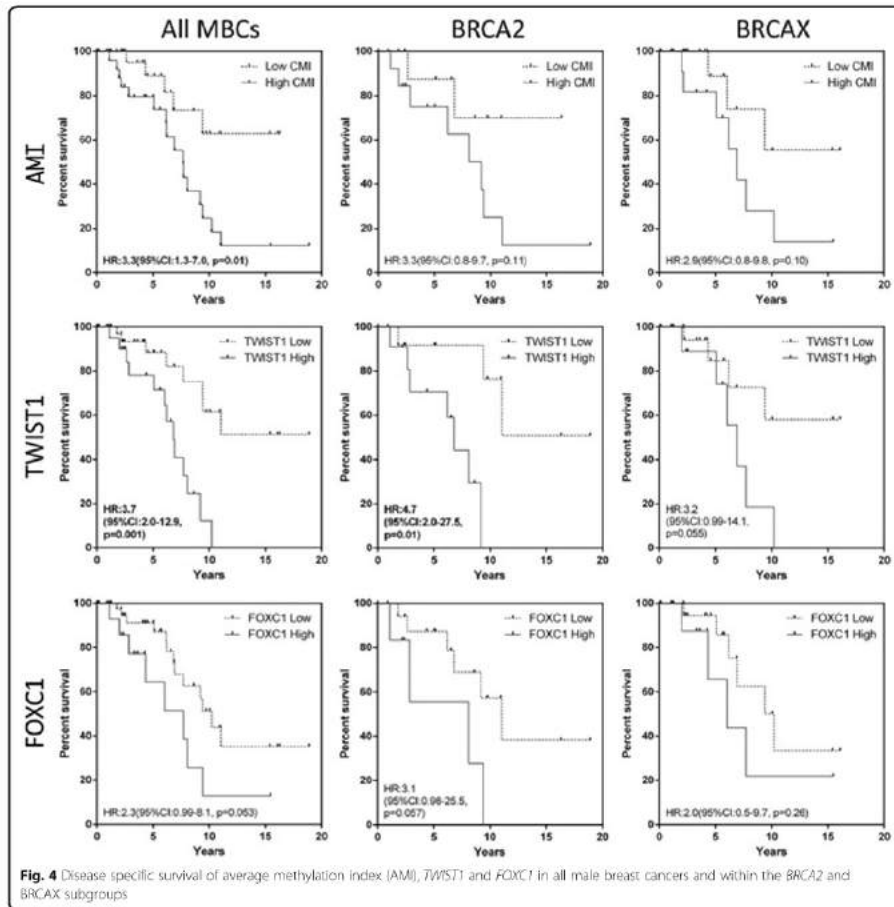### A high average methylation index and *TWIST1* hypermethylation associated with worse disease specific survival

Both a high AMI (HR:3.3, 95% CI:1.3–7.0, $p = 0.01$) and hypermethylation of *TWIST1* (HR:3.7, 95% CI:2.0–12.9, $p = 0.001$) were adverse features for disease specific survival (Fig. 4) with *TWIST1* methylation (HR:4.7, 95% CI:2.0–27.5, $p = 0.01$) also being associated with a significantly shorter survival in the *BRCA2* MBC sub-group. Because *BRCA2* tumours have higher methylation overall and also worse survival than other MBC cohorts [45, 46], we also evaluated survival within the *BRCA2* carriers, and observed a trend towards worse outcome with higher AMI in this sub-group (HR:3.3, 95% CI: 0.8–9.7, $p = 0.1$). Hypermethylation of *FOXC1* (HR:2.3, 95% CI:0.99–8.1, $p = 0.053$) showed a strong trend towards worse DSS; hypermethylation of other genes showed no prognostic information. No significant association with progression-free survival was detected for any gene or AMI. Multivariate analysis was not performed due to inadequate numbers of cases.

other *BRCA2* tumours. Other variables did not align to one or the other cluster. Analysis of BRCAX tumours by cluster analysis showed only very small clusters of 6 or less patients with a correlation coefficient above 0.8 (Additional file 3).

Discussion

Aberrant methylation of promoter regions of tumour suppressor genes has been shown to be a frequent mechanism of gene silencing in most cancers, including breast cancers [47–49]. In many instances, this is observed in adjacent normal tissues or in pre-invasive lesions [50]. Perhaps best seen in colorectal cancer [51],

Fig. 3 Unsupervised cluster analysis of methylation amongst male breast cancers (gradation is seen in the shading between white (no methylation) and red (high methylation))

Deb et al. BMC Cancer (2017) 17:641

Page 8 of 11

Fig. 4 Disease specific survival of average methylation index (AMI), *TWIST1* and *FOXC1* in all male breast cancers and within the *BRCA2* and *BRCAX* subgroups

subsets may demonstrate methylation patterns with clinical relevance.

We have used methylation sensitive high-resolution melting analysis of methylation as it has been demonstrated to be highly sensitive, robust and effective in evaluating FFPE tissue, able to differentiate and semi-quantitate homogenous and heterogeneous methylation [22, 52]. This current comparative study is the largest to examine methylation using a robust technology of well characterised and acknowledged tumour suppressor genes shown to be methylated and important in the pathogenesis FBC, in a clinically well annotated cohort of familial

male breast cancers with known mutation status. We have identified frequent promoter hypermethylation (≥30%) in *GSTP1, RASSF1A, MAL, TWIST, RUNX3,* and *RARβ,* and identified significant associations with clinico-pathological features in five of the genes assayed. One caveat to some of these associations is that the small sample size and their level of statistical significance close to the $p < 0.05$ threshold may mean that false positive results are included due to the multiple tests performed.

Currently there are only three published methylation studies in a total of 182 male breast cancers. Of the genes we investigated only methylation at *GSTP1, RARβ*

169

Deb *et al. BMC Cancer* (2017) 17:641

Page 9 of 11

and *RASSF1A* have been individually assessed, The largest study by Kornegoor et al. [10] examined candidate methylation of 25 genes in 108 MBCs by methylation specific multiplex ligation dependent probe amplification (MS-MLPA), detecting methylation in *RARβ* (5%) and *GSTP1* (44%), somewhat lower than our results. This study did not segregate MBC into sporadic and familial groups, which have been shown to contain distinct geno-phenotypic characteristics and may explain the difference in frequency observed. The second study by Pinto et al. [11] evaluated *RASSF1A* (76%) and *RARβ* (8%) in 27 familial MBCs using quantitative methyl-specific PCR. The lower frequency of *RARβ* hypermethylation observed may be explained by the lower proportion of *BRCA2* cases included (3/27 compared to 25/60 in our cohort). Consistent with this possibility we observed a trend for *RARβ* methylation to be higher in *BRCA2* cases. Finally, Johanssen et al. [9] performed genome-wide methylation profiling in 47 MBCs, and identified two clusters of cases; unfortunately germline mutation status was only available for 8 cases.

One of the most striking findings in this study is the high frequency of *GSTP1* methylation (82%), which has not been noted before. *GSTP1* encodes for glutathionine S transferase P [53] and may be a critical gene in the development of familial MBCs. Very high levels of *GSTP1* methylation are also seen in prostate cancer, which is another male cancer that can be associated with *BRCA2* mutation [54, 55]. We noted high levels of *GSTP1* methylation in both *BRCA2* (88%) and BRCAX tumours (78%), well above that noted by Kornegoor et al. (44%) and that reported in FBCs (generally <60%) [56, 57]. The reason for this result is unlikely to be assay related, as using the same methodology we have shown similar levels of methylation in FBC to that reported in the literature. There are two other possibilities. Firstly, *GSTP1* methylation may be ERβ mediated as studies of prostate cancer lines show that the ERβ/eNOS complex causes *GSTP1* repression by local chromatin remodelling following recruitment to estrogen responsive elements [58]. Secondly, *GSTP1* functions as a caretaker gene [53, 58, 59] with its loss resulting in increased oxidative DNA damage and mutagenesis, thus, in *BRCA2* deficient cancers already sensitive to oxidative stress [60], any loss of *GSTP1* may have a more pronounced effect and be integral in tumour development.

We also noted overall methylation differences between the *BRCA2* and BRCAX subgroups further supporting previous studies showing a possible *BRCA2* MBC subset. In female *BRCA2* carriers, promoter hypermethylation has also been shown to be elevated compared to non-familial and *BRCA1* carriers [49, 61]. Methylation profiling of FBC was able to discriminate *BRCA1*, *BRCA2* and two subsets of BRCAX tumours

[61]. This study is the first to report on methylation of male breast cancers arising in *BRCA1* mutation carriers. These tumours are rare, and while we only have three cases within our cohort, this is a novel group. We were unable to see a significant correlation between gene hypermethylation and *BRCA1* status but did observe the lowest levels of methylation of all the groups, mirroring the findings seen in *BRCA1* associated female breast cancer. Further investigation of this rare subgroup is warranted.

This high level of methylation could potentially be used for screening in *BRCA2* male carriers as methylation is not seen in normal tissues, serum or plasma of normal individuals but can be detected in blood. *GSTP1* may be the prime candidate as studies evaluating its use as a biomarker for prostate cancer are well advanced.

To aid the above possible screening strategies we have developed an index of methylation (AMI) to investigate the quanta of methylation. We observed that AMI correlated with larger tumour size and shorter disease specific survival suggesting that either a stochastic accumulation of methylation and/or a methylator phenotype leads to a more aggressive tumour, as observed in the study of Kornegoor et al. [10]. Similarly, Johansson et al. [9] found that a highly methylated MBC subgroup was more proliferative and showed a trend towards worse patient outcome. In sporadic FBC conflicting results regarding methylation and survival have been found, with higher methylation subgroups showing either improved prognosis [43] or poor overall survival [62]. These differences are perhaps explained by the influence of the intrinsic subtypes, which show distinct methylation patterns and patient outcome [49]. The association between multi-gene hypermethylation and outcome in familial FBC does not appear to have been evaluated. Notably, in our cohort a high AMI maintained a trend towards prognostic significance in *BRCA2* tumours further suggesting that as above, methylation has particular biological importance in this subset of tumours.

Conclusions

We have shown that tumour promoter methylation within our target suppressor gene panel is commonly observed in familial and particularly *BRCA2* male breast cancers suggesting aberrant hypermethylation may be a significant driver in MBCs carrying prognostic information. In addition, the presence of specific methylation patterns particular to MBC subtypes such as *BRCA2* carriers further supports emerging evidence suggesting the presence of unique and distinct MBC subsets that differ from other MBC subgroups and from FBC.

Deb *et al. BMC Cancer* (2017) 17:641

Page 10 of 11

Additional files

Additional file 1: Table S1. REMARK patient flow through study (XLSX 34 kb)

Additional file 2: Table S2. Methylation specific high resolution melting condition and primers (XLSX 36 kb)

Additional file 3: Figure S1. a) BRCA2 subgroup cluster analysis, b) BRCAX subgroup cluster analysis, c) Numbers and sizes of clusters within BRCA2 and BRCAX subgroups using various correlation coefficient cut-offs (listed on the x-axis), d) age of diagnosis of patient within Cluster A, B and other BRCA2 tumours (DOCX 234 kb)

Abbreviations
AMI: Average methylation index; CK5: Cytokeratin 5; DCIS: Ductal carcinoma in situ; DSS: Disease specific survival; ERα: Estrogen receptor; FBC: Female breast cancer; FFPE: Formalin-fixed, paraffin embedded; H&E: Haematoxylin and eosin; IC-NST: Invasive carcinomas of no special type; kConFab: Kathleen Cuningham Foundation Consortium; MBC: Male breast cancer; MS-HRM: Methylation-sensitive high resolution melting; MS-MLPA: Methylation-specific multiplex ligation dependent probe amplification; PgR: Progesterone receptor; QMSP: Quantitative methyl-specific PCR

Authors' contributions
SD – Project conceptualization, DNA extraction and performing methylation assays, data analysis, preparation of manuscript, KLG – data interpretation, preparation of manuscript, JMP - Preparation of standards and performing methylation assays, ET – Performing methylation assay, kConFab Investigators – preparation of clinical data, AD – Project and assay design, technical supervision, manuscript review, SBF – Project conceptualization, manuscript preparation and review. All authors read and approved the final manuscript.

Ethics approval and consent to participate
This work was carried out with approval from the Peter MacCallum Cancer Centre Ethics Committee (Project No: 11/61). All patients provided written informed consent for the use of their tissue and data.

Consent for publication
not applicable.

Competing interests
The authors declare that they have no competing interests.

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171

Deb *et al. BMC Cancer* (2017) 17:641

Page 11 of 11

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172

5.4.2 Additional file 1: REMARK patient flow through study

	MALE BREAST CANCERS
Total patients in kConFab repository	118
FFPE blocks available for DNA extraction	60
Methylation data	
APC	60
CDH1	60
FOXC1	60
GSTP1	60
MAL	60
RARB	60
RASSF1A	60
RUNX3	60
TWIST1	60
WIF1	60

5.4.3 Additional file 2: Methylation specific high resolution melting condition and primers

GENE	FORWARD	REVERSE	MgCl2 concentration (mmol/L)	Forward primer concentration (nmol/L)	Reverse primer concentration (mmol/L)	DNA amount per reaction (ng)	95C hold time (min)	Number of cycles	95C cycling time (sec)	Annealing temperature (celsius)	Annealing temp cycling time (sec)	72C cycling time (time)	97C hold time (min)	Temp range (celsius), 0.2C/step
			REACTION MIXTURE				ACTIVATION	AMPLIFICATION					INACTIVATION	MELT	
APC	CGGGGTTTTGTGTTTTATTG	TCCAACGAATTACACAACTAC	2.5	200	300	20	15	50	10	58.8	15	20	1	70-95
CDH1	GAGTTTGCGGAAGTTAGTTTAGATTTTAG	CGACTCCAAAAACCCATAACTAACC	2.5	200	200	10	15	55	10	61	10	20	1	69-90
FOXC1	CGGGATAATAAGTAGGGTTGGTAGAATAG	GTCCAATAACTACCCTTACCCNACTTC	2.5	200	200	20	15	55	10	58	10	30	1	63-87
GSTP1	GGGGCGGGATTATTTTTATAAGGTT	CGTACTCACTAATAACANAAACTAC	2.5	200	200	10	15	50	10	64.5	10	20	1	70-93
MAL	GCGGAGTTAGCGAGAGGTTTG	AACCACTAAACAAAATACTACCCCCC	2.5	200	200	10	15	45	10	60	10	20	1	72-95
RARB	CGAGTTGTTTGAGGATTGGGATGT	ACGATACCCAAACAAACCCTACTC	3	200	300	20	15	50	10	67	10	20	1	70-92
RASSF1A	TCGGGTTTTATAGTTTTTGTATTTAGGTTTT	CCTCCCCCAAAATCCAAACTAA	3	300	200	10	15	45	15	65	25	20	1	65-88
RUNX3	GTTTCGGGTTTCGTATTTATTTTGAAGG	GACAACCCCAACTTCCTCTACTC	3	200	200	10	15	50	10	58	20	25	1	70-90
TWIST1	AGTTTTTTTCGATCGTTTTTTGGGTTG	CGAAACGATTTCCTTCCCC	2.5	200	300	10	15	50	10	52	10	20	1	70-92
WIF1	TTAAGTGGCGGTCGTTTAGGTTT	ACGAAAACAAAAAAACGAAAAAAACTAA	3	400	400	10	15	50	10	54	20	25	1	70-90

5.4.4 Additional file 3: Supplementary figure 1: a) *BRCA2* subgroup cluster analysis, b) BRCAX subgroup cluster analysis, c) Numbers and sizes of clusters within *BRCA2* and BRCAX subgroups using various correlation coefficient cut-offs (listed on the x-axis), d) age of diagnosis of patient within Cluster A, B and other *BRCA2* tumours.
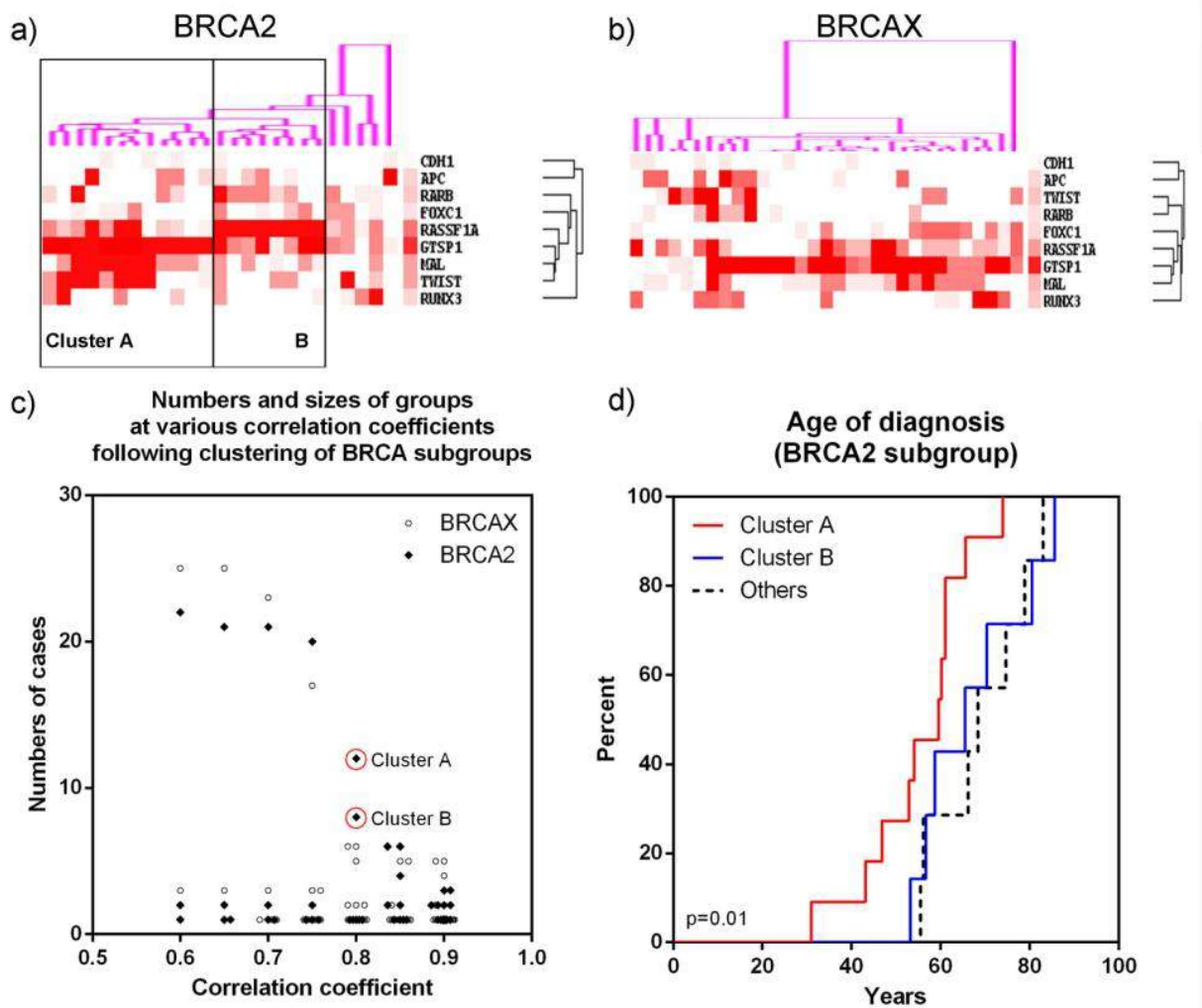
Chapter 6 - Concluding remarks:

Male breast cancers (MBCs) account for less than 1% of all breast cancers and less than 1% of all cancers in men. Their rarity has resulted in a paucity of large male breast cancer specific studies in comparison to females. This thesis has examined genotypic and phenotypic correlation in a subset of familial and sporadic male breast cancers. The hypothesis was that: 1) male and female breast cancer is different, 2) familial male and familial female breast cancer is different and 3) familial and sporadic male breast cancers are different with possible differences between familial male breast cancer subgroups.

1) Differences and similarities between male and female breast cancer:

Several novel observations were made alluding to differences between male and female breast cancers. Converse to female breast disease, and by a large majority, most male breast cancers studied were histologically invasive ductal carcinomas of no special type, with a paucity of lobular and medullary carcinomas. Compared to females, there was also a higher proportion of cancers with an invasive micropapillary component and also invasive papillary carcinoma. Accordingly, these MBCs were also more frequently oestrogen receptor (ER) and progesterone receptor (PgR) positive and less frequently HER amplified than female breast cancers (FBCs). Similar prognostic markers to those described in female breast cancer were noted, including primary tumor size and lymphovascular invasion. However, an older age at diagnosis

also resulted in a worse outcome and interestingly, the presence of perineural invasion (seen more commonly than in female breast cancer) was also prognostically detrimental, and had not been described before in MBC.

Several well described female breast cancer associated pathways and genes were examined within a cohort of male patients. The mutation profile was similar to that seen in Luminal A female breast cancers, with *PIK3CA* mutations most frequently seen, albeit only half as commonly seen as female breast cancer. The E547K mutation, only described in one female breast cancer, was surprisingly seen in two cases of male breast cancer suggesting a possible gender bias. Both this study and others(146, 147) demonstrated higher rates of dual *PIK3CA* mutations occurring in MBCs. An absence of somatic CDH1 mutations was seen corresponding with less frequent lobular MBCs.

Hypoxic effect, in the form of the expression of Hypoxia inducible factors such as HIF1, CA9 and GLUT showed some expression in MBC but again less frequently than seen in FBC, and corresponded with the known association of hypoxia with basal FBC phenotypes and loss of ER expression, both infrequently seen in MBC. Nevertheless, as with FBC, HIF1a expression was prognostically detrimental for disease-specific survival. An interesting observation was the association between HIF1a expression and the possibility of a male cancer phenotype, with increased incidence of second malignancy in both familial and sporadic male breast cancers overexpressing this protein. This is also novel and not previously described in female breast cancer.

Interrogation of several genes well known to be hypermethylated in FBC- showed several differences to MBC: notably, *GSTP1* methylation in both *BRCA2* (88%) and BRCAX tumours was well above that seen in FBC and also sporadic MBCs. This is not surprising when considering its mediation via the ERβ/eNOS complex, its function as a caretaker gene, and with high levels of the gene methylation seen in *BRCA2* associated prostate cancers. Similar to FBC, overall high levels of methylation were associated with increased tumour size and were also prognostically significant.

2) Differences and similarities between male and female familial breast cancers:

The penetrance of familial MBCs is different to that of familial FBC, showing an increased proportion of *BRCA2* male carriers and underrepresentation of *BRCA1* male tumours. A *BRCA1* associated medullary phenotype is not seen in MBC. A possible *BRCA2* associated phenotype was observed, with these tumours overrepresenting the invasive micropapillary carcinoma histological subtype.

In contrast to familial FBCs (Greenblatt *et al*, 2001), in the three *BRCA1* MBCs, no TP53 mutation was seen. Whiles these numbers are low, the low penetrance of MBCs in male *BRCA1* mutation carriers and a lack of tumours with basal cell phenotype suggest that the germline mutation may not be acting as a tumour driver and emphasises the difference of the *BRCA1* effect in MBCs compared with FBCs. The association between hypoxic drive and

overexpression of HIF1a, CA9 and GLUT1 that is seen in *BRCA1* associated female breast cancers, was not seen in this immunohistochemical based study of MBCs.

GWAS data in FBC shows that methylation profiles for familial breast cancers may be defined by the mutation status and are distinct from the intrinsic subtypes. Similar to this, some clustering of MBCs by methylation patterns into different BRCA subgroups was observed, albeit with small numbers.

3) Difference and similarities between male sporadic and male familial breast cancers.

Like familial FBC, the incidence of MBC in *BRCA2*, *BRCA1* and BRCAX males is significantly higher than the lifetime cumulative incidence of 0.1% in the general population [17,48] confirming this group as a high risk for MBC. Comparing studies of sporadic MBC, the median and mean age of onset in familial MBCs is also younger, with more frequent multifocality or bilateral disease. The familial MBCs studies also have a higher proportion of high grade tumours and invasive papillary carcinomas. The histopathological tumour characteristics of this group otherwise is comparable to that seen in previous studies of sporadic MBC, with the majority of cancers being invasive ductal carcinoma. As seen in several sporadic and familial MBC studies, this study also demonstrated a heightened risk of developing second non-mammary malignancies, with a possible suggestion of increased predisposition in familial MBC patients.

The effect of hypoxia and gene induction appears different between sporadic and familial MBCs. In sporadic male breast cancer, expression of any hypoxic marker correlated with a basal cell phenotype with no correlation with clinicopathological factors seen in familial MBCs. HIF1a expression was only prognostic in sporadic MBCs and CA9 only in BRCAX familial MBCs.

Overall, the common genes mutated and their frequency was relatively similar between the cohort studied and more recent sporadic MBC studies, with no specific somatic mutation clusters occurring in familial MBCs as a group. This study, however, noted more gene losses than other previous MBC studies, suggesting that familial MBCs may be unique in this respect, with differences suggested between *BRCA2* and BRCAX cancers. Comparing methylation of specific candidate genes between the group studied and a MBC cohort with more sporadic MBCs, showed consistently higher levels of methylation within familial MBCs. Similar to Johansson *et al*. (162), this study found that a highly methylated MBC subgroup was more proliferative and showed a trend towards worse patient outcome.

4) Subgroups of male breast cancer:

Several differences were noted when comparing familial MBCs according to their germline mutation carrier status. A correlation was seen between *BRCA2* associated cancers and the presence of an invasive micropapillary histological subtype. Analysis of somatic mutations showed TP53 loss was only seen in

BRCA2 carriers and *PIK3CA* mutation were more commonly seen in BRCAX MBCs, suggesting different drivers.   Furthermore, the *BRCA2* MBCs, compared to other MBCs, also showed novel *STK11* amplification and RB1 loss, suggesting enhanced ERα response in this subset. The RB1 loss was thought to reflect its chromosomal proximity to the *BRCA2* gene. Analysis of gene copy number showed differences between *BRCA2* and BRCAX cohorts. Comparison of co-expressed genes also demonstrated differences between *BRCA2* and BRCAX cases with a distinct concordance of tumour suppressor genes with BRCAX patients and more heterogeneity in *BRCA2* cases. Gene methylation was also varied with *BRCA2* tumours showing higher rates of candidate gene methylation. *BRCA1* associated male breast cancers are extremely rare and this course of study represents one of the largest cohorts examined, albeit with only 3 cases present. Unlike female breast cancers, a correlation with medullary type cancers, basal cell phenotype and *TP53* mutation was not seen.

The clinical and therapeutic utility of male breast cancers studies will be to develop gender-specific screening and management of male breast cancers. As these tumours are somewhat infrequent with potentially small centres only seeing these cancers every few months or years, collaborative and multicentric/multinational studies and trials are essential. Consortia such as kConFab, Hereditary Breast and Ovarian Cancer Research Group Netherlands (HEBON), **E**pide**mi**ological Study of Familial **Br**east **Cancers (**EMBRACE), Consortium of Investigators of Modifiers of *BRCA1*/2 (CIMBA) have been invaluable in collecting MBCs, especially within ovarian and

breast cancer families. The possibilities of further high powered observational studies and male specific trials will not be possible without these collaborations.

The next major area for emphasis is in the development of guidelines specific for MBCs. This should focus on three key aspects:

1) Screening – As yet, there is no MBC screening. There are, however, subgroups such as BRCA2 mutation carriers and patients with Klinefelter's syndrome where the lifetime incidence approaches that of FBCs. Screening protocols may be established in these groups, and other high-risk populations as they are identified, to improve detection and awareness of MBCs.

2) Treatment – As yet, there are no MBC specific treatment guidelines. Recommendations are required specifically for surgery, and in particular axillary node dissection given the higher rates of node positive disease when compared to FBC. Further utility of hormone-based therapies and AIs is also required to develop MBC specific regimens. This may be most effectively achieved through multi-centre and probable multi-national prospective trials.

3) Ongoing studies into the biology of MBCs is also critical in further establishing differences to FBCs, and in an era of personalized medicine, to define potential MBC specific targets for future therapies. The development of preclinical models is also integral to furthering the scientific understanding of

male breast cancer, with no current, robust, well described cell lines or animal

models currently available.

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8. Appendices – Author declarations.

Declaration for a thesis with publication

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PhD and MPhil students may include a primary research publication in their thesis in lieu of a chapter if:

- The student contributed greater than 50% of the content in the publication and is the "primary author", ie. the student was responsible primarily for the planning, execution and preparation of the work for publication
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A. PUBLICATION DETAILS (to be completed by the student)

Full title	Genotypic and phenotypic analysis of familial male breast cancer shows under representation of the HER2 and basal subtypes in BRCA-associated carcinomas.
Authors	Siddhartha Deb, Nicholas Jene, kConFab Investigators, Stephen B Fox.
Student's contribution (%)	90%
Journal or book name	*BMC Cancer.*
Volume/page numbers	*2012 Nov 9;12;510. doi: 10.1186/1471-2407-12-510.*
Status	Published

Date accepted/ published

9th November 2012

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Siddhartha Deb		5/4/18

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- All of the co-authors of the publication have reviewed the above information and have agreed to its veracity
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Co-author's name

Stephen B Fox

Co-author's signature

Date (dd/mm/yy)

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Nicholas Jene	*[signature]*	21/1/2011

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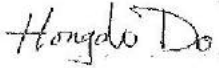*gradresearch.unimelb.edu.au/preparing-my-thesis/thesis-with-publication*

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kConFab Investigators (Heather Thorne)	Heather Thorne	27.03.18

203

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A. PUBLICATION DETAILS *(to be completed by the student)*

Full title	PIK3CA mutations are frequently observed in BRCAX but not BRCA2 –associated male breast cancer.
Authors	Siddhartha Deb, David Byrne, Nicolas Jene, kConFab Investigators, Alexander Dobrovic, Stephen B Fox.
Student's contribution (%)	90%
Journal or book name	*Breast Cancer Research.*
Volume/page numbers	*2013 August 23; 15(4); R69.*
Status	Published	Date accepted/ published — 23rd August 2013

B. STUDENT'S DECLARATION

I declare that the publication above meets the requirements to be included in the thesis

Student's name	Student's signature	Date (dd/mm/yy)
Siddhartha Deb		5/4/18

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Stephen B Fox		

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A. PUBLICATION DETAILS *(to be completed by the student)*

Full title	*PIK3CA* mutations are frequently observed in BRCAX but not BRCA2 associated male breast cancers.
Authors	Siddhartha Deb, Hongdo Do, David Byrne, Nicholas Jene, kConFab Investigators, Alexander Dobrovic, and Stephen B Fox.
Student's contribution (%)	90%
Journal or book name	Breast Cancer Research
Volume/page numbers	2013; 15(4):R69.
Status	Published	Date accepted/published August 23rd 2013.

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Hongdo DO | *Hongdo Do* | 29/03/2018

Co-author's name | Co-author's signature | Date (dd/mm/yy)

David J. Byrne | *DJB* | 27.03.2018

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Nicholas Jene | | 29 | 1 | 2014

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Authors	Siddhartha Deb, Hongdo Do, David Byrne, Nicholas Jene, kConFab Investigators, Alexander Dobrovic, and Stephen B Fox.
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Journal or book name	Breast Cancer Research
Volume/page numbers	2013; 15(4):R69.
Status	Published

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kConFab Investigators (Heather Thorne)	Heather Thorne .	27·03·18

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A. PUBLICATION DETAILS *(to be completed by the student)*

Full title	Nuclear HIF1A expression is strongly prognostic in sporadic but not familial male breast cancer.
Authors	Siddhartha Deb, Ida Johansson, David Byrne, Cecilia Nilsson, kConFab Investigators, Leonie Constable, Marie-Louise Fjällskog, Alexander Dobrovic, Ingrid Hedenfalk, Stephen B. Fox.
Student's contribution (%)	70%
Journal or book name	*Modern Pathology.*
Volume/page numbers	*2014 Sep;27(9):1223-30. doi: 10.1038/modpathol.2013.231. Epub 2014 Jan 24.*
Status	Published
Date accepted/ published	24th January 2014

B. STUDENT'S DECLARATION

I declare that the publication above meets the requirements to be included in the thesis

Student's name	Student's signature	Date *(dd/mm/yy)*
Siddhartha Deb		5/4/18

C. PRINCIPAL SUPERVISOR'S DECLARATION

209

I declare that:

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- The advisory committee has met and agreed to the inclusion of this publication in the student's thesis
- All of the co-authors of the publication have reviewed the above information and have agreed to its veracity
- 'Co-Author Authorisation' forms for each co-author are attached.

Co-author's name	Co-author's signature	Date (dd/mm/yy)
Stephen B Fox		4/4/18

Co-author authorisation form

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Student's contribution (%)	70%
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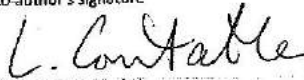### B. CO-AUTHOR'S DECLARATION *(to be completed by the collaborator)*

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211

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Co-author's name	Co-author's signature	Date (dd/mm/yy)
Stephen B Fox		4/4/18

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Co-author's name	Co-author's signature	Date (dd/mm/yy)
Ida Johansson (now Ida Marixholm) due to marriage.	*Ida Malu*	02/04/18

213

Co-author's name	Co-author's signature	Date (dd/mm/yy)
David J. Byrne		27.03.2018

Co-author's name	Co-author's signature	Date (dd/mm/yy)
Cecilia Nilsson		27 mar 2018

Co-author's name	Co-author's signature	Date (dd/mm/yy)
Leonie Constable	L. Constable	29/03/2018

Co-author's name	Co-author's signature	Date (dd/mm/yy)
Marie-Louise Fjällskog		28/3/18

Co-author's name	Co-author's signature	Date (dd/mm/yy)
Alexander Dobrovic		29/3/2018

Co-author's name	Co-author's signature	Date (dd/mm/yy)
Ingrid Hedenfalk		27/03/18

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Co-author's name	Co-author's signature	Date (dd/mm/yy)
kConFab Investigators (Heather Thorne)	Heather Thorne	27·03·18

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A. PUBLICATION DETAILS *(to be completed by the student)*

Full title	**Mutational profiling of familial male breast cancers reveals similarities with luminal A female breast cancer with rare TP53 mutations.**
Authors	Siddhartha Deb, Stephen Q Wong, Jason Li, Hongdo Do, Jonathan Weiss, David Byrne, Anannya Chakrabarti, Trent Bosma, kConFab Investigators, Andrew Fellowes, Alexander Dobrovic, Stephen B Fox.
Student's contribution (%)	75%
Journal or book name	*Br J Cancer.*
Volume/page numbers	*2014 Dec 9;111(12):2351-60. doi: 10.1038/bjc.2014.511*
Status	Published

Status	Date accepted/ published
Published	9th December 2014

B. STUDENT'S DECLARATION

I declare that the publication above meets the requirements to be included in the thesis

Student's name	Student's signature	Date (dd/mm/yy)
Siddhartha Deb		5/4/18

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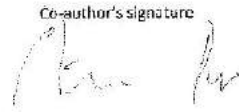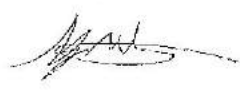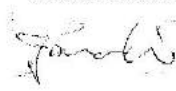
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Stephen B Fox		4/4/18

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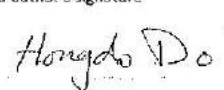|	
Full title	Mutational profiling of familial male breast cancers reveals similarities with luminal A female breast cancer with rare *TP53* mutations.
Authors	S Deb, S Q Wong, J Li, H Do, J Weiss, D Byrne, A Chakrabarti, T Bosma, kConFab Investigators, A Fellowes, A Dobrovic, and S B Fox
Student's contribution (%)	75%
Journal or book name	British Journal of Cancer
Volume/page numbers	2014; 111(12): 2351-2360.
Status	Published
Date accepted/published	Dec 9 2014

B. CO-AUTHOR'S DECLARATION *(to be completed by the collaborator)*
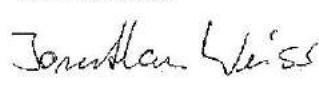
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218

Co-author's name	Co-author's signature	Date (dd/mm/yy)
S Q Wong		26.03.18

Co-author's name	Co-author's signature	Date (dd/mm/yy)
J Li		7/11/2018

Co-author's name	Co-author's signature	Date (dd/mm/yy)
Hongdo DO	Hongdo Do	29/-3/18

Co-author's name	Co-author's signature	Date (dd/mm/yy)
Jonathan Weiss	Jonathan Weiss	27/03/18

Co-author's name	Co-author's signature	Date (dd/mm/yy)
David J. Byrne		27.03.2018

Co-author's name	Co-author's signature	Date (dd/mm/yy)
Anannya Chakrabarti		04/04/18

219

Co-author's name	Co-author's signature	Date (dd/mm/yy)
Trent Bosma		2-/04/18

Co-author's name	Co-author's signature	Date (dd/mm/yy)
Andrew Fellowes	A.P. Fellowes.	27/03/18

Co-author's name	Co-author's signature	Date (dd/mm/yy)
Alexander Dobrovic		29/3/2018

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Co-author's name	Co-author's signature	Date *(dd/mm/yy)*
kConFab Investigators (Heather Thorne)	*Heather Thorne.*	27·03·18

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Authors	Siddhartha Deb, Kylie L Gorringe, Jai-Min B Pang, David J Byrne, Elena A Takano, kConFab Investigators, Alexander Dobrovic, Stephen B Fox.
Student's contribution (%)	75%
Journal or book name	*BMC Cancer*
Volume/page numbers	*2017 Sep 11;17(1):641. doi: 10.1186/s12885-017-3632-7.*
Status	Published

Date accepted/ published: 11th September 2017

B. STUDENT'S DECLARATION

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Student's name	Student's signature	Date *(dd/mm/yy)*
Siddhartha Deb		5/4/18

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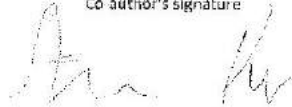## C. PRINCIPAL SUPERVISOR'S DECLARATION

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Stephen B Fox		4/4/18

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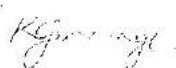*gradresearch.unimelb.edu.au/preparing-my-thesis/thesis-with-publication*

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Student's contribution (%)	75%
Journal or book name	BMC Cancer
Volume/page numbers	2017; 17:641.
Status	Published	Date accepted/published: Sep 11 2017

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Co-author's name

Kylie L Gorringe

Co-author's signature

Date (dd/mm/yy)

27-3-18

Co-author's name

Jia-Min B. Pang

Co-author's signature

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29/03/18

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Date (dd/mm/yy)

27.03.2018

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Elena A. Takano

Co-author's signature

Date (dd/mm/yy)

27/03/18

Co-author's name

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Deb, Siddhartha

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File Description:
Genotypic phenotypic correlation in male breast cancer