FULL PAPER

BJC

British Journal of Cancer (2014) 111, 2351–2360 | doi: 10.1038/bjc.2014.511

Keywords: male breast cancer; *BRCA1*; *BRCA2*; BRCAX; *TP53*; *PIK3CA*; MiSeq

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

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Received 26 April 2014; revised 10 August 2014; accepted 18 August 2014

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 (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).

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

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 °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 ng μl^{-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.

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[a])	*BRCA2* 9161 C>A (S2978X)	58.7	22[a] (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.
[a]Multifocal MBCs with contralateral carcinoma diagnosed subsequently.

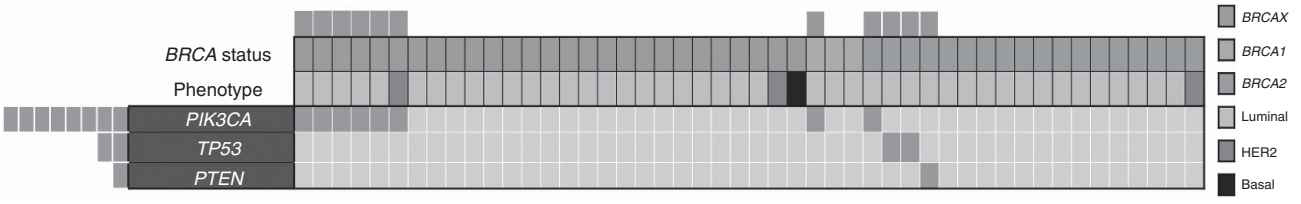

Figure 1. Mutations (red squares) present in MBC. Tumours are classified by *BRCA* status (orange = BRCAX; yellow = *BRCA1*; green = *BRCA2*) and phenotype (light grey = luminal; dark grey = HER2; black = basal). A full color version of this figure is available at *British Journal of Cancer* journal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

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 clinicopathologic 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	Rs 1800056	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
	Rs 3822214	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	Rs 33917957	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
	Rs 56391007	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	Rs 2228230	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	Rs 77711105	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	Rs 59912467	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.

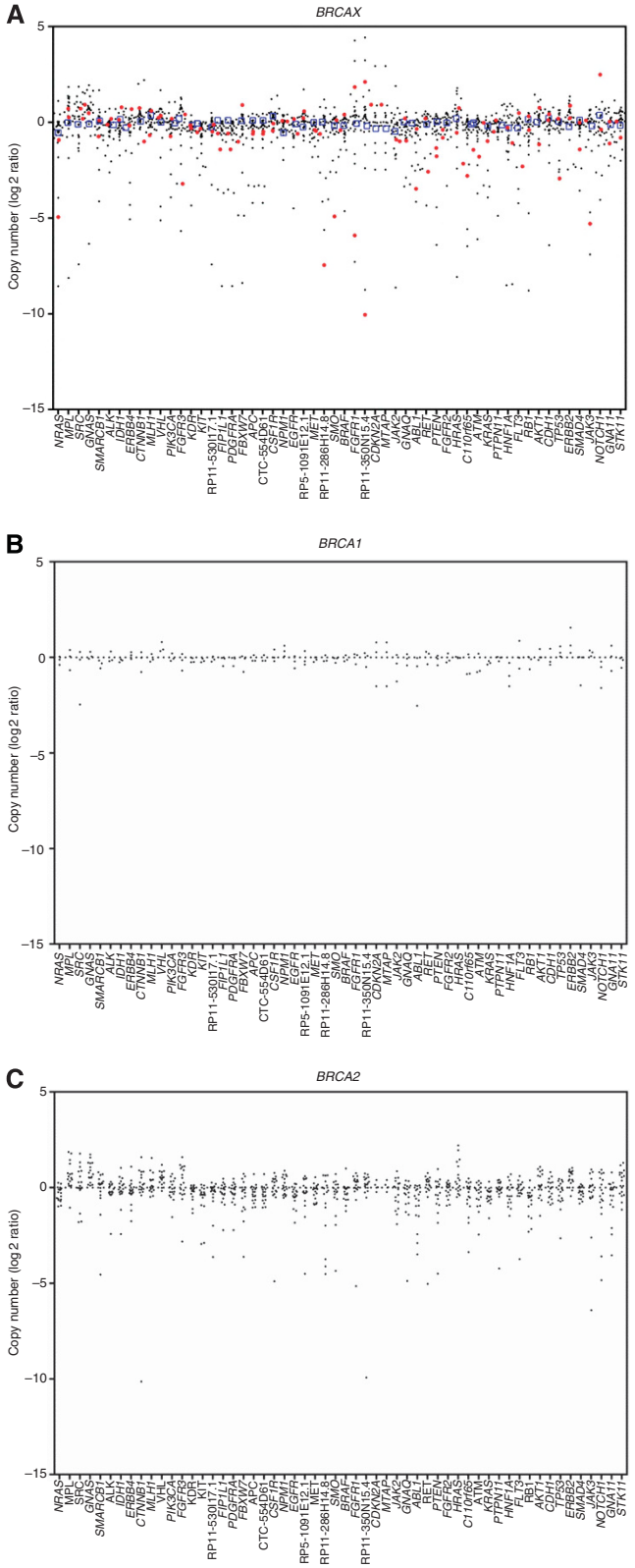

Figure 2. (**A–C**) Copy number changes in BRCAX, *BRCA1* and two cohorts (black circle – luminal phenotype; blue square – HER2 phenotype; red circle – basal phenotype).

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).

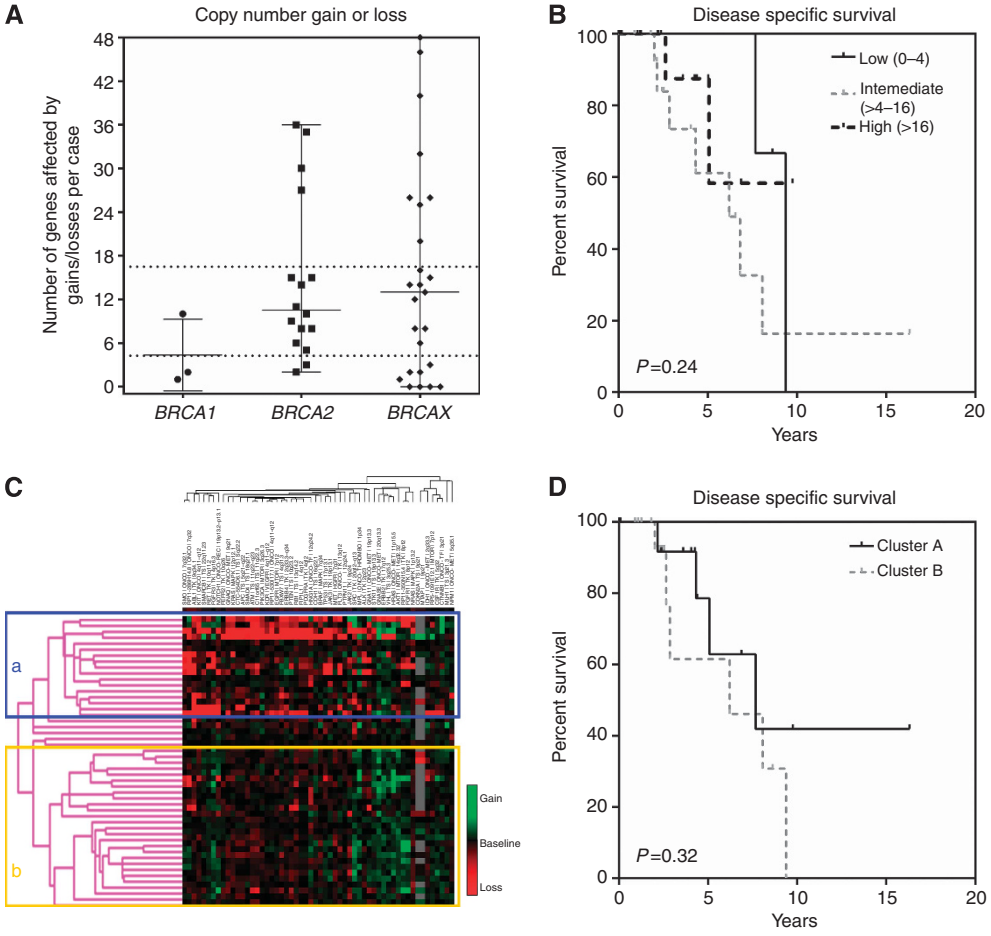

Figure 3. (**A**) Number of genes gained or lost per case, stratified by *BRCA* status, (**B**) disease-specific survival stratified by volume of copy number changes per case (0–4 = low, >4–16 = intermediate, >16 = high), (**C**) unsupervised cluster analysis of MBCs showing a loss predominant cluster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

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

		Gains				Losses			
Gene	Chromosome position	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

Table 4. (Continued)

		Gains				Losses			
Gene	Chromosome position	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.

ACKNOWLEDGEMENTS

We 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 No. 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. Siddhartha Deb received a postgraduate scholarship from the NHMRCS. Stephen Fox and Alexander Dobrovic received funding from the National Breast Cancer Foundation.

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