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Copy number profiling of oncogenes in ductal carcinoma *in situ* of the male breast

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Abstract

Characterizing male breast cancer (BC) and unraveling male breast carcinogenesis is challenging because of the rarity of this disease. We investigated copy number status of 22 BC-related genes in 18 cases of pure ductal carcinoma *in situ* (DCIS) and in 49 cases of invasive carcinoma (IC) with adjacent DCIS (DCIS-AIC) in males using multiplex ligation-dependent probe amplification (MLPA). Results were compared to female BC and correlated with survival. Overall, copy number ratio and aberration frequency including all 22 genes showed no significant difference between the 3 groups. Individual unpaired analysis revealed a significantly higher *MTDH* copy number ratio in IC compared to DCIS-AIC and pure DCIS ($P=0.009$ and $P=0.038$, respectively). *ADAM9* showed a significantly lower copy number aberration frequency in male BC, compared to female BC ($P=0.020$). In DCIS-AIC, *MTDH*, *CPD*, *CDC6* and *TOP2A* showed a lower frequency of copy number increase in males compared to females ($P<0.001$ for all 4 genes). In IC, *CPD* gain and *CCNE1* gain were independent predictors of poor overall survival. In conclusion, male DCIS and IC showed a similar copy number profile for 21 out of 22 interrogated BC-related genes, illustrating their clonal relation and the genetically advanced state of male DCIS. *MTDH* showed a higher copy number ratio in IC compared to adjacent and pure DCIS and may therefore play a role in male breast carcinogenesis. Differences were detected between male and female DCIS for 4 genes pointing to differences in breast carcinogenesis between the sexes.

Key Words

- breast cancer
- DCIS
- male
- MLPA
- copy number

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Introduction

Breast carcinogenesis is a multi-step process involving accumulation of DNA alterations and epigenetic changes. An important event during cancer development is oncogene amplification. Several genes have been described to be frequently amplified in female breast cancer (BC), of which the best-known example is the human epidermal growth factor receptor 2 (*HER2*). *HER2* is amplified in 10–20% of female BC and is correlated to overall survival, time to relapse and response to trastuzumab, a humanized monoclonal anti-*HER2* antibody (Slamon *et al.* 1987, Hudis 2007, Moelans *et al.* 2009). Other oncogenes that have been described to have clinical implications in

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female BC include the estrogen receptor (*ESR1*), epidermal growth factor receptor 1 (*EGFR*), *MYC*, topoisomerase IIa (*TOP2A*), fibroblast growth factor receptor 1 (*FGFR1*), cyclin E (*CCNE1*) and cyclin D1 (*CCND1*) (Holst *et al.* 2007, Rodriguez-Pinilla *et al.* 2007, Turner *et al.* 2010, Holm *et al.* 2012, Masuda *et al.* 2012, Almeida *et al.* 2014, Lundgren *et al.* 2015).

Invasive ductal type cancers (IDC) of the breast are thought to arise from ductal carcinoma *in situ* (DCIS) via parallel breast cancer progression pathways in which low-grade DCIS progresses to low-grade IDC and high-grade DCIS to high-grade IDC. These parallel pathways have been postulated to have distinct genomic aberrations (Hwang *et al.* 2004, Moelans *et al.* 2010*a*, Burger *et al.* 2013). Progression through grade is a phenomenon that has been rarely observed in BC (Schymik *et al.* 2012).

The final step in breast carcinogenesis, where the basement membrane of the ducts is breeched and the malignant epithelial cells infiltrate the surrounding stroma, is poorly understood. Several female BC studies have shown similar levels of gene amplification in DCIS and adjacent IC, indicating that these genes play an early role in breast carcinogenesis, but not in the progression from DCIS to invasive carcinoma (Aubele *et al.* 2000, Burkhardt *et al.* 2010, Moelans *et al.* 2010*a*). Furthermore, not all patients diagnosed with pure DCIS show progression to IC when left untreated. A previous study showed progression from low-grade DCIS to IC in 11/28 cases, the remaining cases showing an indolent course (Sanders *et al.* 2005). Therefore, unraveling the drivers that control the progression of DCIS to IC has proved to be challenging in female BC, let alone in male BC, where the rarity of the disease hampers thorough investigation. This knowledge is however needed to understand the biological course of male DCIS, to predict patients' outcome and to optimize DCIS treatment strategies. In this study, we compare pure DCIS, DCIS adjacent to IC (DCIS-AIC) and IC, as differences at molecular level have been described between these two types of DCIS in females, using RT-PCR (Doebar *et al.* 2017).

Male BC is a rare disease, accounting for approximately 1% of all BC (Siegel *et al.* 2015). Pure DCIS represents approximately 5% (range 1–17%) of all cancers in the male breast (Pappo *et al.* 2005). In female BC, the diagnosis of pure DCIS is made in approximately 20% of all BC, and this difference in DCIS frequency between male and female BC can perhaps be explained by the participation of women in BC screening programs (Leonard & Swain 2004).

There are many similarities but also important differences between male and female BC. There are differences in distribution of histologic subtypes as well as molecular subtypes; men tend to be older at the time of diagnosis and have more advanced disease at presentation compared to women (Giordano *et al.* 2004, Ge *et al.* 2009, Anderson *et al.* 2010, Kornegoor *et al.* 2012*b*). Also, there is some evidence suggesting differences in gene amplification frequencies (Kornegoor *et al.* 2012*a*). In a previous male BC study, gain of *CCND1* and *EGFR* was more frequent in male BC compared to female BC, and amplification of *TRAF4* and *EMSY* was more often observed in female BC in comparison to male BC (Kornegoor *et al.* 2012*a*).

In the present study, we used multiplex ligation-dependent probe amplification (MLPA) to investigate DNA copy number changes of 22 breast cancer-related genes in a group of male IC with adjacent DCIS and in a group of male pure DCIS. We correlated these copy number aberrations with clinicopathologic features and 10-year survival data and compared our results to a previous female BC study using a similar MLPA kit (Moelans *et al.* 2010*a*).

Materials and methods

Patient material

Patients with DCIS and adjacent IC or pure DCIS were enrolled from a previously selected large male BC cohort (Cardoso *et al.* 2015, Vermeulen *et al.* 2017). A subgroup of this initial population was selected based on availability of a tumor tissue block for central pathology review and sufficient tissue for DNA isolation. This resulted in a total of 51 cases with IC and adjacent DCIS and 20 cases of pure DCIS. Patient and tumor characteristics including age at diagnosis and 10-year overall survival status (defined as death due to any cause) were recorded. Data concerning BRCA1/2 testing was not available. Hematoxylin and eosin (H&E) slides were reviewed by an experienced pathologist to confirm the diagnosis and to type and grade the IC according to the World Health Organization and modified Bloom and Richardson score (Elston & Ellis 1991). DCIS was graded according to the classification by Holland and coworkers (1994). ER, PgR and HER2 were evaluated using immunohistochemistry and scored according to the Allred score (Allred *et al.* 1998) and ASCO-CAP guidelines (Wolff *et al.* 2013). The areas of interest (pure DCIS, DCIS-AIC and IC) were dissected either manually with a sterile scalpel when big enough or by laser capture microdissection using a Zeiss PALM

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MD3 laser microdissection system, from 5 sections (4 µm) of formalin-fixed paraffin-embedded (FFPE) tissue blocks. Laser capture microdissection was done in cases with only small areas of DCIS or with abundant inflammatory cells surrounding the area of interest. The DNA was extracted by overnight incubation in proteinase K (10 mg/mL; Roche) at 56°C, followed by boiling for 10 min and centrifugation. Normal male breast tissue was taken along as control. Results from a previous female BC study comparing DCIS and adjacent IC ($N=39$) using a similar MLPA kit were used to compare copy number status in female and male BC (Moelans *et al.* 2010*a*).

Clinicopathological data are shown in Table 1. Hormone receptor status showed a high concordance (100%) between DCIS and adjacent IC.

Multiplex ligation-dependent probe amplification (MLPA)

MLPA analysis was performed on all isolated DNA using the P078-C1 kit (MRC Holland, Amsterdam, The Netherlands), containing 41 probes targeting 22 breast cancer-related genes (Supplementary Table 1, see section on supplementary data given at the end of this article). MLPA was performed according to the manufacturer's instructions (MRC Holland), using an ABI 9700 PCR machine (Applied Biosystems). All tests were done in duplicate, and each MLPA run included 7 negative reference samples (3 healthy blood samples, 3 normal male breast FFPE samples and 1 normal female breast FFPE sample). The PCR products were separated by capillary electrophoresis on a 3730 DNA analyzer (Applied Biosystems). Gene copy numbers were analyzed using GeneScan analysis (Applied Biosystems) and Coffalyser.net software (MRC-Holland). For genes targeted by more than one probe, the mean of all probe ratios was calculated. Four of the 12 reference probes showed above average copy number variations and were excluded from further analyses (NRAP located at 10q25.3, TGIF1 located at 18p11.31, CETN3 located at 05q14.3 and SNCA located at 04q22.1).

Cut-off values were set as described previously with a copy number ratio of <0.7 for gene loss, 1.3–2.0 for copy number gain and >2.0 for amplification (Moelans *et al.* 2010*a*, Kornegoor *et al.* 2012*a*). Values between 0.7 and 1.3 were considered copy number neutral.

Statistics

Statistical calculations were done using SPSS, version 21.0. The Kruskal–Wallis test was used to compare the overall copy number ratio including all 22 genes between the 3

Table 1 Clinicopathological data of all male breast cancer cases (invasive carcinoma (IC), male pure ductal carcinoma *in situ* (pure DCIS) and DCIS adjacent to invasive carcinoma).

	Invasive carcinoma	Adjacent DCIS	*Pure* DCIS
Age (years)			
Mean (range)	63.2 (37–85)	63.2 (37–85)	62.3 (37–76)
Histologic subtype IC			
Ductal type carcinoma	46 (90.2%)		
Mucinous carcinoma	1 (2%)		
Micropapillary carcinoma	1 (2%)		
Encapsulated papillary carcinoma	1 (2%)		
Mixed type			
Ductal/micropapillary	1 (2%)		
Ductal/mucinous	1 (2%)		
Grade			
1	14 (27.5%)	11 (21.6%)	3 (15%)
2	22 (43.1%)	32 (62.7%)	16 (80%)
3	15 (29.4%)	8 (15.7%)	1 (5%)
ER			
Positive	51 (100%)	51 (100%)	20 (100%)
Negative	0 (0%)	0 (0%)	0 (0%)
PR			
Positive	49 (96.1%)	49 (96.1%)	20 (100%)
Negative	2 (3.9%)	2 (3.9%)	0 (0%)
HER2			
Positive	2 (3.9%)	2 (3.9%)	1 (5.3%)
Negative	49 (96.1%)	49 (96.1%)	18 (94.7%)
Missing	0	0	1

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Table 2 Frequencies of losses, gains and amplifications in 22 genes for male pure ductal carcinoma *in situ* (pure DCIS), DCIS adjacent to invasive carcinoma (DCIS-AIC) and invasive carcinoma (IC) including the *P* value for gain/amplification (copy number ratio >1.3), amplification (copy number ratio >2.0) and the average copy number aberration frequency for all 22 genes.

		Frequencies (%)										
		Pure DCIS (*N* = 18)			DCIS-AIC (*N* = 49)			IC (*N* = 49)			***P*-value** (gain/ amplification, >1.3) Chi-square	***P*-value** (amplification, >2.0) Chi-square
Gene	**Chromosome**	Loss (<0.7)	Gain (1.3–2.0)	Amplification (>2.0)	Loss (<0.7)	Gain (1.3–2.0)	Amplification (>2.0)	Loss (<0.7)	Gain (1.3–2.0)	Amplification (>2.0)		
ESR1	6q25.1	0	0	0	1 (2%)	5 (10%)	0	0	5 (10%)	0	0.362	–
EGFR	7p11.2	0	2 (11%)	1 (6%)	0	5 (10%)	1 (2%)	0	8 (16%)	1 (2%)	0.697	0.701
ZNF703	8p11.23	0	11 (61%)	5 (28%)	0	22 (45%)	12 (24%)	1 (2%)	25 (51%)	10 (20%)	0.267	0.395
FGFR1	8p11.22	0	2 (11%)	1 (6%)	1 (2%)	11 (22%)	7 (14%)	1 (2%)	13 (27%)	7 (14%)	0.162	0.443
ADAM9	8p11.22	5 (28%)	0	1 (6%)	8 (16%)	7 (14%)	3 (6%)	9 (18%)	5 (10%)	4 (8%)	0.426	0.901
IKBKB	8p11.21	0	4 (22%)	0	0	7 (14%)	2 (4%)	0	15 (31%)	1 (2%)	0.252	0.682
PRMD14	8p13.3	0	4 (22%)	0	0	7 (14%)	1 (2%)	3 (6%)	12 (24%)	1 (2%)	0.375	0.817
MTDH	8q22.1	0	3 (17%)	2 (11%)	0	9 (18%)	1 (2%)	0	22 (45%)	1 (2%)	**0.018**	0.237
MYC	8q24.21	0	4 (22%)	2 (11%)	0	17 (35%)	3 (6%)	3 (6%)	14 (29%)	9 (18%)	0.429	0.137
CCND1	11q13.3	0	7 (39%)	1 (6%)	0	18 (37%)	8 (16%)	0	24 (49%)	9 (18%)	0.166	0.241
C11ORF30	11q13.5	0	1 (6%)	0	2 (4%)	3 (6%)	1 (2%)	5 (10%)	3 (6%)	1 (2%)	0.897	0.814
CDH1	16q22.1	0	4 (22%)	0	1 (2%)	9 (18%)	1 (2%)	2 (4%)	8 (16%)	2 (4%)	0.992	0.618
CPD	17q11.2	1 (6%)	0	0	7 (14%)	0	0	5 (10%)	4 (8%)	0	0.061	–
MED1	17q12	0	2 (11%)	1 (6%)	0	11 (22%)	2 (4%)	1 (2%)	10 (20%)	7 (14%)	0.295	0.148
ERBB2	17q12	0	5 (27%)	1 (6%)	0	7 (14%)	2 (4%)	0	9 (18%)	3 (6%)	0.423	0.857
CDC6	17q21.2	0	1 (6%)	0	1 (2%)	2 (4%)	1 (2%)	6 (12%)	4 (8%)	2 (4%)	0.376	0.532
TOP2A	17q21.2	0	0	0	1 (2%)	2 (4%)	2 (4%)	2 (4%)	5 (10%)	3 (6%)	0.109	0.483
MAPT	17q21.31	0	2 (11%)	0	1 (2%)	4 (8%)	0	1 (2%)	6 (12%)	1 (2%)	0.628	0.486
PPM1D	17q23.2	0	1 (6%)	0	1 (2%)	3 (6%)	2 (4%)	1 (2%)	4 (8%)	2 (4%)	0.715	0.672
BIRC5	17q25.3	0	3 (17%)	0	0	8 (16%)	0	1 (2%)	9 (18%)	2 (4%)	0.682	0.231
CCNE1	19q12	0	0	0	1 (2%)	1 (2%)	0	1 (2%)	3 (6%)	0	0.366	–
AURKA	20q13.2	1 (6%)	0	0	2 (4%)	8 (16%)	1 (2%)	3 (6%)	8 (16%)	2 (4%)	0.166	0.566
Total		**7**	**56**	**15**	**27**	**166**	**50**	**45**	**216**	**68**	***0.133***	***0.012***

groups and to compare copy number ratios between the 3 groups for the 22 individual genes. After dichotomization, the chi-square test was used to compare the frequency of gains, amplifications or losses between groups. Mean copy number aberration frequency, for gains, amplifications and losses, including all genes was analyzed using the Kruskal–Wallis test. Individual genes in pure DCIS and DCIS-AIC were compared using Mann–Whitney test for copy number ratio and chi-square for dichotomized results.

For paired data (IC and DCIS-AIC) the Wilcoxon signed-rank test was used to compare mean copy number ratio, and McNemar's test was used to compare copy number aberration frequency for the 22 individual genes.

The overall copy number ratios between low/intermediate-grade and high-grade DCIS, as well as between low/intermediate-grade and high-grade IC were compared by Mann–Whitney test. Dichotomized data per grade category were evaluated by chi-square. *P* values less than 0.05 were considered significant and correction for multiple comparisons was done using the Holm–Bonferroni method. Survival data were available for all IC and DCIS-AIC cases with a median follow-up of 8.1 years (range 0.86–19.56 years). For univariate survival analysis, Kaplan–Meier curves were plotted and analyzed with the log-rank test. Multivariate survival analysis was done with Cox regression (backward LR) and included age, mitosis and grade.

Finally, unsupervised hierarchical clustering (Euclidian distance method) of copy number ratios was performed using the statistical program R (www.r-project.org).

Results

Copy number ratio and aberration frequencies in DCIS and invasive carcinoma

One case of invasive carcinoma, one case of DCIS-AIC and two cases of pure DCIS had an insufficient DNA yield and were excluded from further analysis, leaving 49 cases of DCIS with adjacent IC and 18 cases of pure DCIS suitable for copy number analysis. Supplementary Table 2 shows raw MLPA copy number data.

Table 2 summarizes copy number status for all 22 analyzed genes in each subgroup and Fig. 1 illustrates the copy number aberration frequency for each studied gene. The frequencies of losses, gains and amplifications were similar between the three groups ($P=0.167$, $P=0.132$ and $P=0.361$, respectively). Copy number gain/amplification (cut-off >1.3) was most frequently observed for *ZNF703*, *CCND1* and *MYC*, but none of these genes showed a significant difference between the groups.

Overall, the copy number ratio including all 22 genes showed no significant difference between pure DCIS, DCIS-AIC and IC. At the individual gene level, *MTDH* showed a significantly higher copy number ratio in IC as compared to DCIS-AIC and pure DCIS ($P=0.009$ and $P=0.038$, respectively). Using a cut-off of >1.3, *MTDH* showed a significantly higher aberration frequency in IC (46.9%) as compared to DCIS-AIC (20.4%) ($P=0.005$).

The copy number ratio for *PRDM14*, *C11ORF30* and *FGFR1* was higher in DCIS-AIC compared to pure DCIS ($P=0.007$, $P=0.027$ and $P=0.042$, respectively). However, these genes lost their significance after dichotomization.

No significant differences were found when comparing copy number aberration frequency (gain and amplification) with histologic subtype in IC, although these results should be interpreted with caution due to small sample sizes.

Paired comparison of DCIS-AIC and adjacent IC showed a high concordance of copy number status for all interrogated genes, with no significant differences present. The highest concordance rates were seen for the genes *CCNE1* (95.9%) and *CDC6* (93.9%). Copy number ratio was significantly higher in IC compared to the paired DCIS-AIC for *MTDH* ($P<0.001$), *MYC* ($P=0.039$),

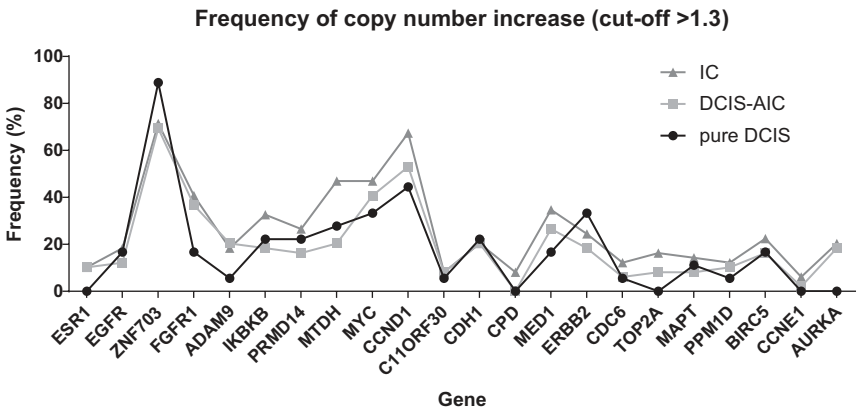

Figure 1
Frequencies of gain/amplification by MLPA for all 22 analyzed genes in male pure ductal carcinoma *in situ* (pure DCIS), DCIS adjacent to invasive carcinoma (DCIS-AIC) and invasive carcinoma (IC).

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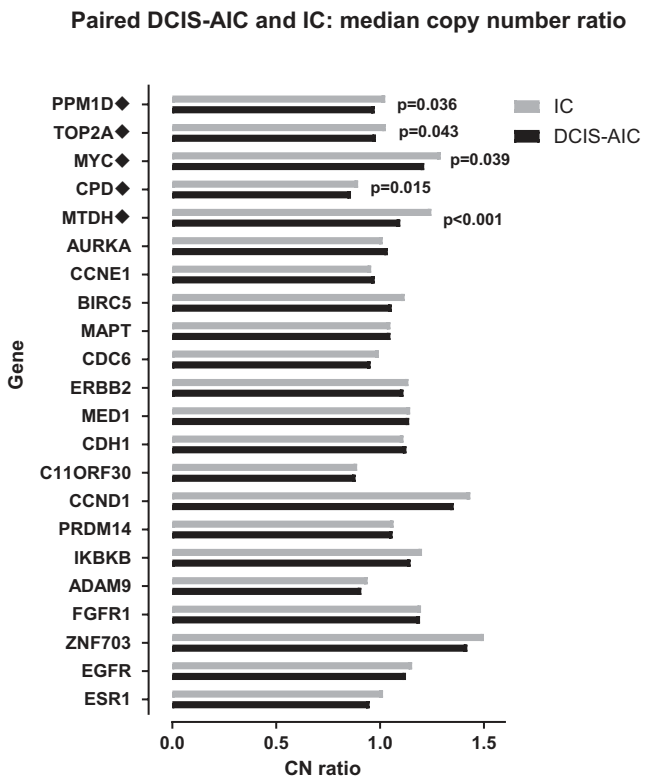

Figure 2
Median copy number ratio for all 22 analyzed genes in male invasive carcinoma (IC) and adjacent ductal carcinoma *in situ* (DCIS-AIC). Genes with a diamond show a significantly higher copy number ratio in IC.

CPD ($P=0.015$), *TOP2A* ($P=0.043$) and *PPM1D* ($P=0.036$). Figure 2 shows the median copy number ratio for the 22 analyzed genes and Fig. 3 the copy number ratio for *MTDH*, *MYC*, *CPD*, *TOP2A* and *PPM1D* in paired IC and DCIS-AIC.

Correlation between copy number and grade in DCIS-AIC and invasive carcinoma

Copy number ratios and aberration frequencies were compared for DCIS-AIC and IC between low/intermediate-grade and high-grade lesions. The mean copy number ratio was 1.17±0.22 vs 1.32±0.25 for low/intermediate-grade vs high-grade DCIS-AIC ($P=0.165$), and 1.15±0.16 vs 1.42±0.44 for low/intermediate grade vs high grade IC ($P=0.040$). The average number of gains/amplifications in the 22 analyzed genes was 3.7 vs 8.4 for low/intermediate-grade vs high-grade DCIS-AIC ($P=0.019$) and 4.8 vs 8.3 for low/intermediate-grade vs high-grade IC ($P=0.037$).

DCIS-AIC showed a significantly higher copy number ratio in high-grade lesions for the genes *ESR1*

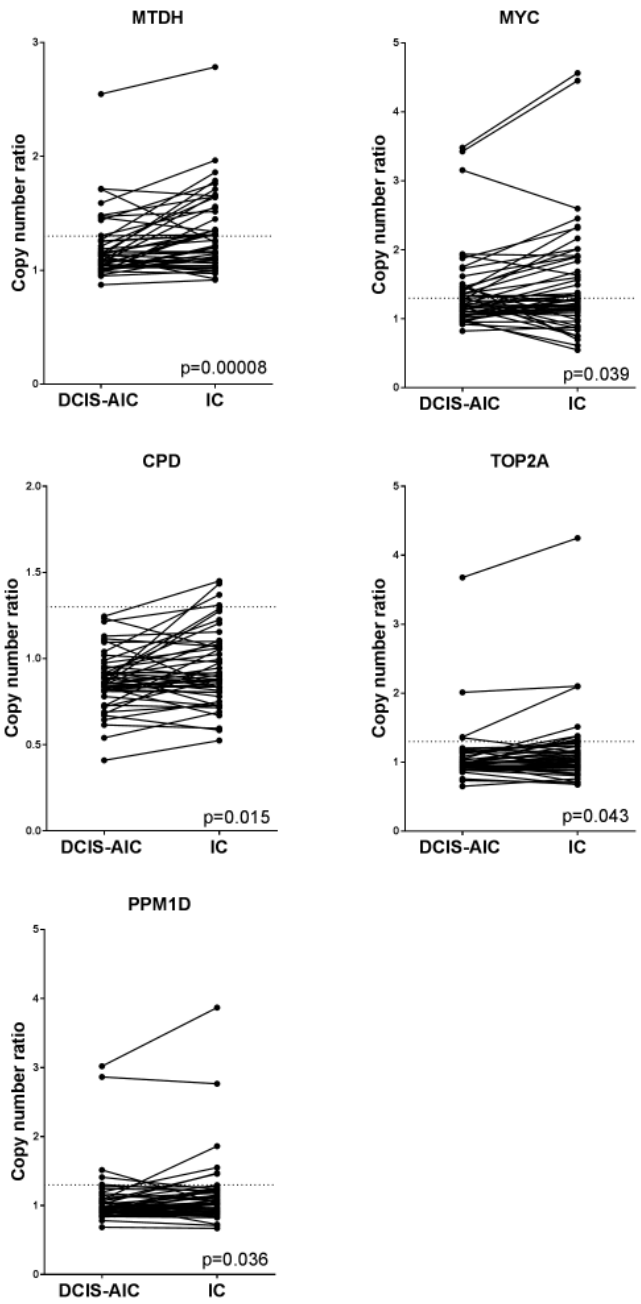

Figure 3
Copy number ratios for *MTDH*, *MYC*, *CPD*, *TOP2A* and *PPM1D* in male invasive carcinoma (IC) and adjacent ductal carcinoma *in situ* (DCIS-AIC).

($P=0.047$), *PPM1D* ($P=0.004$), *BIRC5* ($P=0.002$) and *CCNE1* ($P=0.005$). After dichotomization (cut-off >1.3), these differences remained significant ($P<0.001$, $P=0.002$, $P=0.040$ and $P=0.014$, respectively). In addition, *PRDM14* ($P=0.040$), *CDC6* ($P=0.003$), *TOP2A* ($P=0.018$) and *AURKA* ($P=0.006$) showed a significantly higher copy number aberration frequency in high-grade DCIS-AIC lesions. Only *MTDH* showed a significantly

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higher frequency of amplification in high-grade DCIS-AIC ($P=0.007$).

IC showed a significantly higher copy number ratio in high-grade lesions for the genes *EGFR* ($P=0.005$) and *CCND1* ($P=0.005$). Dichotomized data (cut-off >1.3) showed a significantly higher aberration frequency for *ESR1* ($P=0.007$), *EGFR* ($P=0.047$), *C11ORF30* ($P=0.001$), *CDC6* ($P=0.022$) and *PPM1D* ($P=0.020$) in high-grade lesions. *ADAM9* ($P=0.029$), *MYC* ($P=0.031$), *CCND1* ($P=0.005$), *CDH1* ($P=0.029$), *CDC6* ($P=0.013$), *TOP2A* ($P=0.004$) and *PPM1D* ($P=0.012$) showed significant amplification more often in high-grade lesions.

After correction for multiple comparisons, only *BIRC5* in DCIS-AIC remained significant with regard to copy number ratio difference (1.068 in low/intermediate grade vs 1.353 in high grade). For the dichotomized data, *C11ORF30* in IC (17.1% in low/intermediate grade vs 35.7% in high grade) and *ESR1* in DCIS-AIC (2.4% vs 57.1%) remained significant.

Comparison of DCIS-AIC and IC copy number status between male and female breast cancer

Results from a previous female BC study including 39 patients (IC and adjacent DCIS) were used to compare copy number status between female and male BC (Moelans *et al.* 2010*a*). This previous study used a prior version of the MLPA kit used here. Twenty genes were similar in both MLPA kits, with some differences in the probes used for the genes and were used for analysis.

In IC, *ADAM9* showed a significantly lower copy number aberration frequency (cut-off >1.3) in male BC (22.5%) compared to female BC (56.4%) ($P=0.020$). In DCIS, *MTDH*, *CPD*, *CDC6* and *TOP2A* showed a lower frequency of copy number increase in male compared to female BC ($P<0.001$ for all 4 genes) (Fig. 4). The frequencies of amplifications (cut-off >2.0) and losses were similar between female and male BC.

In addition, we compared copy number aberration frequencies of 21/22 interrogated genes (EMSY data not available) with a large public female breast cancer cohort (METABRIC, www.cbioportal.org, (Curtis *et al.* 2012, Pereira *et al.* 2016)). Supplementary Table 3 shows a high amplification frequency similarity for all genes except for *PRDM14* and *MTDH*, which both showed a difference of at least 10% in amplification frequency, with a higher amplification percentage in the METABRIC population ($N=2173$).

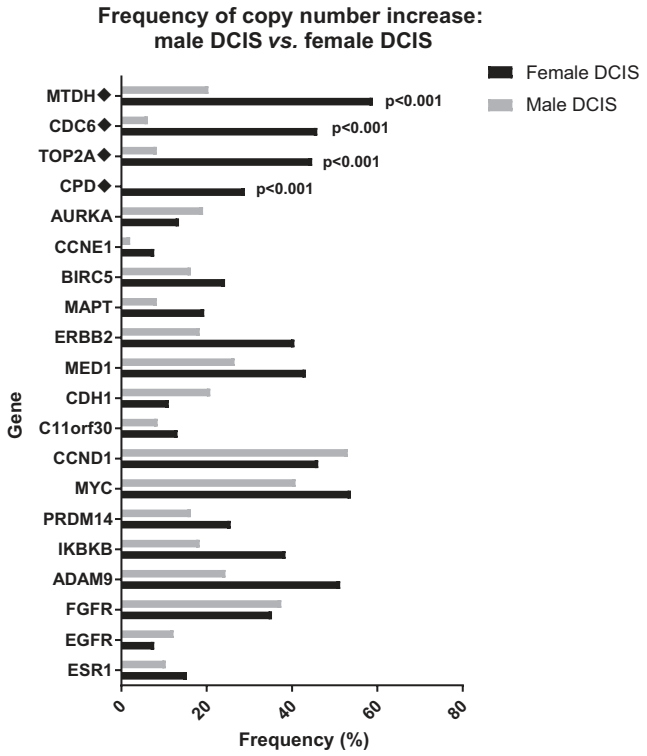

Figure 4
Frequency of copy number increase (cut-off >1.3) in female and male ductal carcinoma *in situ* (DCIS). Genes with a diamond show a significantly higher frequency of copy number gain in female BC.

Correlation between copy number alterations and survival

CPD and *CCNE1* gain (no amplifications were observed) in IC were predictors of poor 10-year overall survival ($P=0.050$ and $P=0.001$) and remained independent prognosticators when grade, mitoses and age were included in multivariable analysis ($P=0.017$ (HR 5.1) and $P=0.003$ (HR 6.9)). Kaplan–Meier curves are presented in Fig. 5. None of the other interrogated genes were associated with survival.

Cluster analysis of all male pure DCIS, DCIS-AIC and IC lesions

Unsupervised hierarchical cluster analysis of all pure DCIS, DCIS-AIC and IC showed 2 main clusters that differed significantly according to grade (grade 1/2 vs grade 3) with more high-grade lesions in cluster B ($n=29$) compared to cluster A ($n=20$) ($P=0.001$) (Fig. 6). In addition, all genes showed a higher copy number ratio in cluster B. Of the 49 paired DCIS-AIC

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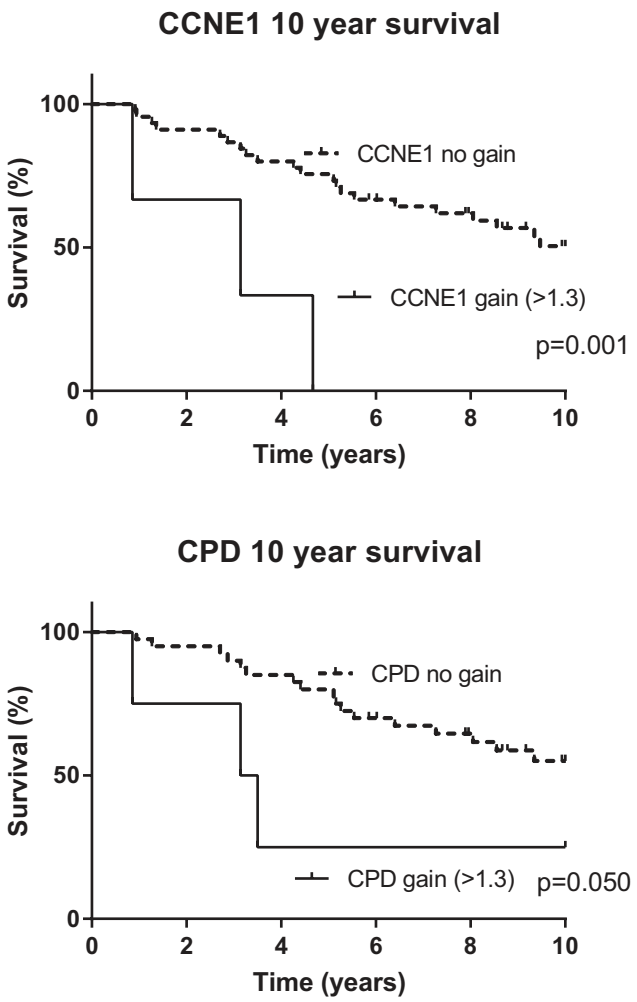

Figure 5
K Kaplan–Meier 10-year overall survival plots for *CCNE1* gain and *CPD* gain.

and IC samples in the cluster analysis, 40 samples (81.6%) were in the same cluster, and of these, 17 pairs (34.7%) clustered closely together indicating that these adjacent *in situ* and invasive components share many genetic alterations.

Discussion

To discover drivers that may control the progression of DCIS to IC and to establish the precursor role of DCIS in male breast carcinogenesis, we studied copy number status of 22 breast cancer-related genes in IC, DCIS-AIC and pure DCIS of the male breast by MLPA. Overall, in line with previous studies on their female counterparts, there were only few copy number differences between male DCIS and IC (Aubele *et al.* 2000, Burkhardt *et al.* 2010, Moelans *et al.* 2010*a*).

Copy number ratios were similar in pure DCIS, DCIS-AIC and IC for most of the studied genes, indicating that copy number gain of the majority of these genes does not seem to play a significant role in the transition from male DCIS to IC. This finding is in line with a previous copy number and gene expression study in female BC (Moelans *et al.* 2010*a*). There was however one gene, *MTDH*, that showed a significantly higher copy number ratio and frequency of gain in IC as compared to DCIS-AIC. This implies that gain of *MTDH* could play a role in the progression of DCIS to IC. In a previous MLPA-based male BC study, *MTDH* showed gain/amplification in 46% of the IC samples, similar to our results (Kornegoor *et al.* 2012*a*). *MTDH* is located on chromosome 8 and encodes Metadherin, a transmembrane protein that plays a key role in the activation of several signaling pathways including PI3K/Akt, NFκβ, Wnt/βcatenin and the MAPK pathways (Shi & Wang 2015). These pathways play a role in cell proliferation, apoptosis, invasion, angiogenesis and metastasis. Metadherin is frequently overexpressed in female BC and overexpression correlates with advanced clinical stage, distant metastasis and an aggressive phenotype (Tokunaga *et al.* 2014). Moelans and coworkers compared *MTDH* copy number in 39 paired cases of female DCIS-AIC and IC but found no significant differences in copy number ratio, suggesting that this event may be specific for male breast carcinogenesis (Moelans *et al.* 2010*a*).

Interestingly, almost all of the analyzed genes showed copy number changes in DCIS, indicating that copy number gain is a relatively early event in male breast carcinogenesis. Paired analysis of IC and DCIS-AIC samples showed a high concordance of gain/amplification status between individual patients, supported by cluster analysis. This confirms the clonal relation between male DCIS and IC, as has also been accepted in female breast carcinogenesis (Moelans *et al.* 2010*a*). CCND1, a cell cycle regulatory protein, showed a high copy number aberration frequency in all three groups with 49% *CCND1* gain and 18% *CCND1* amplification in IC. *CCND1* amplification is more frequent in ER-positive and PR tumors, so these high frequencies can be explained by the high rate of ER positivity (all cases being ER positive) and PR positivity (96% of DCIS-AIC/IC cases and 100% of pure DCIS cases being positive) in our male BC cohort (Reis-Filho *et al.* 2006).

Several genes showed a higher aberration frequency in high-grade lesions compared to low-grade lesions (*ESR1*, *PPM1D*, *BIRC5*, *CCNE1*, *PRDM14*, *CDC6*, *TOP2A* and *AURKA* for DCIS-AIC and *ESR1*, *EGFR*, *C11ORF30*,

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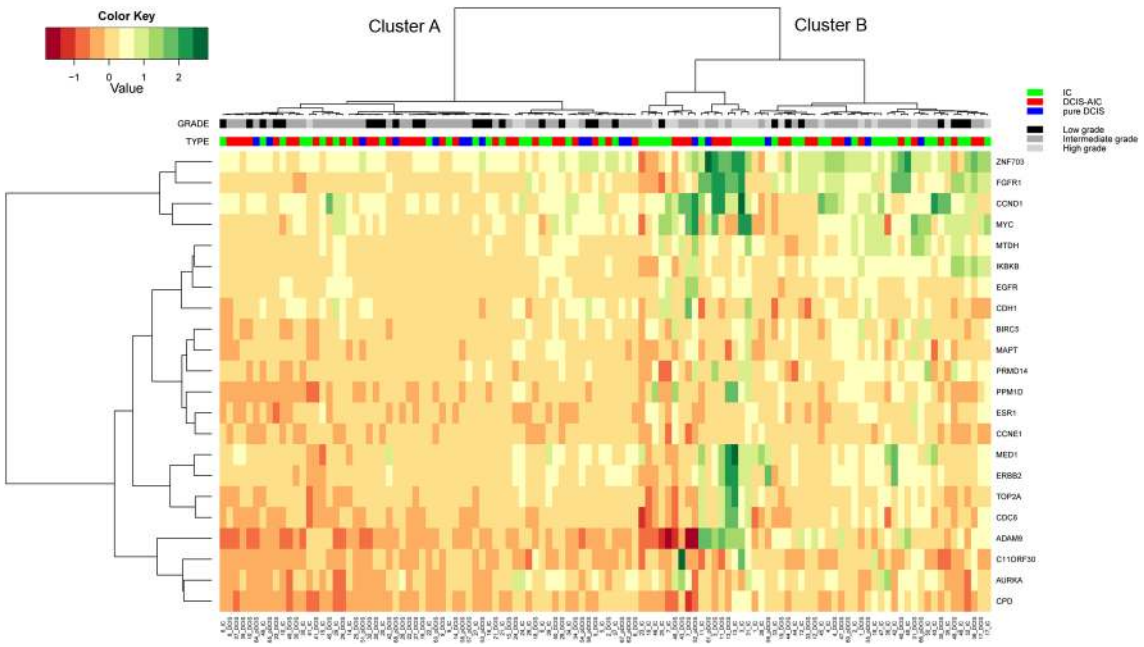

Figure 6
Unsupervised hierarchical cluster analysis of 22 genes in male breast cancer lesions, including pure ductal carcinoma *in situ* (DCIS), DCIS adjacent to invasive carcinoma (DCIS-AIC) and invasive carcinoma (IC).

CDC6 and *PPM1D* for IC). Also, the average copy number ratio was higher in high grade IC compared to low/intermediate-grade IC. After correction for multiple comparisons, *BIRC5* copy number ratio and *ESR1* gain in DCIS-AIC and *C11ORF30* gain in IC were significantly higher/more frequent in high-grade lesions. Although the sample sizes of high-grade DCIS-AIC and high-grade IC were small ($n=7$ and $n=14$, respectively), this does suggest that tumors with a higher copy number gain have a tendency to have higher histological grade, as previously demonstrated in male BC (Kornegoor *et al.* 2012*a*). *BIRC5* codes for the protein Survivin, a regulatory protein involved in cell proliferation and apoptosis. It has been extensively studied in female BC where an increased expression of Survivin was correlated with a higher risk of recurrence and with a decreased overall survival rate (Davis *et al.* 2007, Li *et al.* 2014). *ESR1* codes for estrogen receptor alpha, a transcription factor located on chromosome 6q25 and an important therapeutic target in female BC with tamoxifen being the standard endocrine therapy for ER-positive breast cancers (Holst *et al.* 2007). In a previous study using MLPA, *ESR1* amplification and gain were shown in 2% and 6% of 135 female breast tumors, respectively (Moelans *et al.* 2011). *C11ORF30* (also known as *EMSY)* is a transcription regulatory protein that can compromise BRCA2 function in sporadic breast cancer and ovarian cancer (Hughes-Davies *et al.* 2003). In female BC, it has been associated with a reduced overall survival in ER-positive patients (Kirkegaard *et al.* 2008).

Upon comparison of our findings with female BC, a high concordance was evident, especially for IC. For DCIS, 4 genes (*MTDH*, *CPD*, *CDC6* and *TOP2A*) showed a higher frequency of gain in female BC, although no differences in amplification frequency were observed. Copy number aberration frequencies for 21 genes were also compared with a large female breast cancer cohort (METABRIC, www.cbioportal.org, (Curtis *et al.* 2012, Pereira *et al.* 2016)), showing a high amplification frequency similarity.

Two of the 22 studied genes showed a correlation with overall survival. *CCNE1* and *CPD* gain were both indicative of a decreased 10-year overall survival; however, the number of cases showing gain of these genes ($n=3$ and $n=4$, respectively) were small and none of the cases showed amplification. Also, treatment regimens and lymph node status were not known so could not be included in the survival analysis. Therefore, results should be interpreted with caution. High levels of Cyclin E have been described to have prognostic value in female breast cancer, especially as a predictor of endocrine therapy failure (Keyomarsi *et al.* 2002, Span *et al.* 2003).

CPD has been investigated in breast cancer cell lines (MCF-7 cells), where prolactin/17β-estradiol-induced

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cell surface *CPD* increased intracellular NO production, which increased the survival and inhibited apoptosis (Abdelmagid & Too 2008).

Although a limitation of this study is the relatively small study population, it should be noted that male BC is rare, male DCIS is even rarer, and our DCIS samples have been extracted from a large cohort study and were enriched for tumor cells by scalpel or laser microdissection. We used MLPA for copy number analysis, a multiplex PCR-based method that simultaneously assesses relative copy numbers of a variety of genes in a quantitative way. The major advantage of this technique is that it requires only minimal amounts of small DNA fragments, which makes it very suitable to study small lesions in paraffin-embedded tissue, such as DCIS (Moelans *et al.* 2009). The MLPA kit used was pre-designed by the manufacturer and contains 22 cancer-related genes that often show copy number aberrations in female BC (Moelans *et al.* 2010*a*,*b*). Although there are some genetic differences between male and female BC, we expected the bigger part of these genes to play a role in male breast carcinogenesis as well (Moelans *et al.* 2010*b*). We did not include *PIK3CA*, *TP53* and *GATA3*, possible important genomic drivers in female BC and described to be frequently mutated in female BC (2012). In this study, we only focused on copy number variations and not on specific mutations.

In conclusion, this MLPA-based study showed a similar copy number status for 21 out of 22 studied breast cancer-related genes in male DCIS and IC, illustrating the clonal relation between male DCIS and adjacent IC, and the genetically advanced state of male DCIS. *MTDH* showed a higher copy number ratio and aberration frequency in IC compared to DCIS and could therefore play a role in the transition of male DCIS to IC.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/ERC-17-0338.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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