Male versus Female Breast Cancer differences hidden behind similarities

Miangela Marie Lacle

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Male versus Female Breast Cancer differences hidden behind similarities

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Chapter 1

General introduction and outline of the thesis

General introduction to male breast cancer

Breast cancer in male is a rare disease. In The Netherlands an average of 91 men were diagnosed with breast cancer per year between 2007 and 2011 with a 5-years survival rate of 82% [1]. Morbidity and mortality of this disease is significant and its incidence is rising [2]. Related to its rarity, limited research has been conducted on male breast cancer, especially when compared to its female counterpart. Knowledge on male breast cancer is therefore based on small single institutional studies, as large series are lacking. Therefore, the optimal clinical management of male breast cancer may not be known and most treatment algorithms are yet derived from studies on its female counterpart. An expert panel recently emphasized that, rather than being considered analogous to (postmenopausal) female breast cancer, male breast cancer should be considered a unique disease [3].

Pathology

Male breast tissue lies on the chest wall posterior to the nipple and it is composed of fat, connective tissue and sparse rudimentary ducts ending in terminal buds. In contrast to the female breast there is usually no lobule formation unless the male breast is exposed to high levels of endogenous or exogenous estrogens [4].

It is general knowledge that invasive ductal carcinoma is the most common type of breast cancer in males and that invasive lobular carcinoma is a very rare event in this group. Men are older, more likely to have lymph node involvement and higher stage at time of diagnosis than females [5,6].

The majority of tumors is ER positive and of luminal phenotype. A high percentage of male breast cancer is PR positive and AR positivity is also common. HER2 driven and triple negative cases are rare in the male breast [7-10].

In our institution we collaborated with different institutes to collect a large group of male breast cancer (MBC) cases. Our aim was to better identify differences between male and female breast cancer (FBC), find better prognosticators, novel targets for therapy and thereby a further understanding of the carcinogenesis of breast cancer for male patients.

Prognostic factors

Bcl2 and mitotic index

Bcl2 is a proto-oncogen initially identified in non-Hodgkin's B-cell follicular lymphomas [11]. Its protein product is mainly localised in the mitochondrial membrane [12]. It promotes

tumorigenesis by preventing cell death and by inhibiting cell proliferation arresting cells in the Go/G1 phase of the cell cycle [13]. In the normal breast Bcl2 is characteristically expressed in the non-pregnant and early pregnancy mammary glands [14]. The anti-apoptotic and anti-proliferative effects of Bcl2 has been demonstrated in mouse models of breast cancer progression [15,16]. Bcl2 expression in breast cancer is sensitive to oestrogen and its presence is correlated with oestrogen receptor positivity [17,18]. The association of Bcl2 expression and good prognosis in breast cancer has been proposed to be linked to the effects of down regulation of its expression by ER antagonists thereby promoting apoptosis of malignant cells [18]. It is however also speculated that the inhibitory effect of Bcl2 on cell cycle progression may also be directly associated with good prognosis [16,19]. Counting mitoses is probably the most widely studied and widely applied way of assessing cellular proliferation [20,21], and is the main prognostic constituent of histological grading in breast cancer [22]. In a recent study on FBC, a combination of mitotic index and Bcl2 expression proved to be of value in stratifying patients into low risk vs. high risk groups regarding mortality and recurrence [23]. Although there are some data published regarding Bcl2 expression and its prognostic value in MBC, the prognostic value of Bcl2 combined with mitotic index had not been studied in MBC. Therefore we set out to investigate the prognostic value of Bcl2 in combination with mitotic index in MBC and to evaluate whether its prognostic value equals that observed in FBC.

CTGF

Connective tissue growth factor (CTGF/CCN2) is a member of the CCN family of six secreted proteins consisting of four conserved subdomains: an insulin-like growth factor-binding protein (IGFBP) domain, a von Willebrand factor type C (VWC) domain, a thrombospondin type I (TSP) repeat and a C-terminal cysteine knot (CT) domain [24], which is expressed in several cell types, such as endothelial cells, fibroblasts and leukocytes but especially in osteoblasts and chondrocytes. It functions as a multi-functional signalling modulator involved in a wide variety of biologic and pathologic processes including control of proliferation, migration and adhesion, regulation of growth, development and differentiation, wound healing, regeneration and cell death [25-27]. CTGF/CCN2 has been reported to play an important role in mammary tumor genesis and progression of FBC [25,28]. In the current study, we set out to investigate the expression, clinicopathologic correlations and prognostic value of CTGF in a relatively large group of MBC.

Prognostic index

Accurate prognostication is essential in advising on adjuvant systemic treatment following surgery for early breast cancer. A number of predictive models have been

developed to assess prognosis in female breast cancer. One of the first described models is the Nottingham Prognostic Index, first described in 1982, based on tumor size, tumor grade and lymph node status [29]. In 1985 the Morphometric Prognostic Index was described based on mitotic index, tumor size and lymph node status [30]. Both models have been validated thereafter in many further studies.
More recently online prognostication tools such as Adjuvant! (www.adjuvantonline.com) and Predict (www.predict.nhs.uk) were developed to provide survival estimates and absolute individual adjuvant treatment benefits predictions for FBC patients [31,32].
All these prognostication tools are based on FBC data and it is unknown whether these outcome predictions are applicable to MBC. We aimed to evaluate the prognostic performance of these models in a relatively large group of MBC.

Molecular changes
More recently published studies have revealed molecular differences hidden behind similarities between MBC and FBC [7-10,33]. On a protein level there is a much lower percentage of HER2 overexpression and p53 accumulation in MBC compared to FBC [10]. On a genetic level MBC has fewer gains and losses than FBC [33]. In our study we focused on copy number changes in MBC.

Chromosome 16
Copy number changes mapping to chromosome 16 have been reported to be the most frequent alteration in FBC. In FBC, loss of chromosome 16 is associated with low-grade ductal and lobular cancer, and favorable prognosis. High grade ductal cancer often has complex changes on chr16, typically small regions of gain together with larger regions of loss [34-38]. Genetic alterations on chromosome 16 in MBC are poorly characterized compared with FBC and only a few studies have been performed [33,39,40]. These studies report frequent chromosomal imbalances on both 16p and 16q. We therefore set out to further characterize copy number changes on chr16 in MBC using a novel multiplex ligation-dependent probe amplification (MLPA) kit with multiple genes on chromosome 16, and to correlate these genomic anomalies with clinicopathological features and patients' outcome.

Chromosome 17
Copy number changes on chromosome 17q have been extensively studied in different cancer types including FBC. This is firstly related to the presence of the ERBB2 oncogene (HER2) on chromosome 17q. Amplification of HER2 is present in about 10-20% of FBC, usually leads to overexpression of the protein and correlates with high grade, high

mitotic index, worse prognosis and response to targeted therapy with trastuzumab [41-43]. Further, there are several other important oncogenes on chr17 such as TOP2A and PPM1D [44-46]. Lastly, to assess HER2 amplification status by *in situ* hybridization, correction for polysomy of chr17 is still widely applied, although several studies have shown that polysomy of chr17 is at best very rare in FBC, and that copy number status of the centromere does not represent the number of chr17. Rather, chr17 shows very complex rearrangements in FBC [47-50]. In MBC, HER2 amplification occurs at much lower frequency [39,41,49,51-53]. Only a few (mainly CGH) studies have been published discussing genetic alterations on chr17 in small cohorts of MBC [33,40], but their association with outcome and polysomy 17 has not previously been studied in MBC. We therefore aimed to characterize copy number changes on chr17 in a large group of MBC using a dedicated chr17 MLPA kit that was previously used to study polysomy 17 in FBC [49]. In addition, HER2 chromogenic *in situ* hybridization (CISH) was done and correlated with clinicopathologic features and patients' outcome.

Copy number profiling

Optimal treatment is best based on the biology of the cancer, so it is important to further molecularly characterize MBC to better define biologically and clinically relevant subgroups to catch up with FBC where many new treatment options and biomarkers are becoming available [54-57].

We therefore analyzed a group of MBC by array Comparative Genomic Hybridization (aCGH) in search of gains and losses specific for MBC, while paying special attention to the prevalence of BRCA1-like and BRCA2-like copy number profiles [58,59]. These profiles have been associated with sporadic or hereditary defects in respectively *BRCA1* and *BRCA2* and predict good response to high dose platinum-based chemotherapy and probably to poly(ADP)ribose polymerase (PARP) inhibitors, agents that induce DNA double-strand breaks[60]. In view of the assumed high prevalence of *BRCA2* mutations in MBC, a fairly high proportion of MBC could qualify for such treatment.

Outline of the thesis

In the first part of the thesis we focus on immunohistochemical techniques in search for prognostic markers in male breast cancer and its difference compared with its female counterpart. In *Chapter 2* Bcl2 expression and mitotic index are combined to assess its prognostic value in MBC. In *Chapter 3* we assess the prognostic value of CTGF expression in our MBC series.

In *Chapter 4* we evaluate the prognostic performance of several prognostic models developed in FBC in our MBC series.

In the second part of the thesis we focus on molecular techniques to investigate genetic alterations and their prognostic value in MBC compared to FBC. In *Chapter 5* we look into Chr16q copy number changes and in *Chapter 6* into Chr17 copy number changes in MBC in comparison with a previously studied FBC group. In *Chapter 7* we analyzed a group of MBC by array Comparative Genomic Hybridization (aCGH) in search of gains and losses specific for MBC, while paying special attention to the prevalence of BRCA1-like and BRCA2-like copy number profiles.

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chapter

1

Chapter 2

Prognostic value of mitotic index and Bcl2 expression in male breast cancer

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Abstract

The incidence of male breast cancer (MBC) is rising. Current treatment regimens for MBC are extrapolated from female breast cancer (FBC), based on the assumption that FBC prognostic features and therapeutic targets can be extrapolated to MBC. However, there is yet little evidence that prognostic features that have been developed and established in FBC are applicable to MBC as well. In a recent study on FBC, a combination of mitotic index and Bcl2 expression proved to be of strong prognostic value. Previous papers on Bcl2 expression in MBC were equivocal, and the prognostic value of Bcl2 combined with mitotic index has not been studied in MBC. The aim of the present study was therefore to investigate the prognostic value of Bcl2 in combination with mitotic index in MBC.

Immunohistochemical staining for Bcl2 was performed on tissue microarrays of a total of 151 male breast cancer cases. Mitotic index was scored. The prognostic value of Bcl2 expression and Bcl2/mitotic index combinations was evaluated studying their correlations with clinicopathologic features and their prediction of survival.

The vast majority of MBC (94%) showed Bcl2 expression, more frequently than previously described for FBC. Bcl2 expression had no significant associations with clinicopathologic features such as tumor size, mitotic count and grade. In univariate survival analysis, Bcl2 had no prognostic value, and showed no additional prognostic value to tumor size and histological grade in Cox regression. In addition, the Bcl2/mitotic index combination as opposed to FBC did not predict survival in MBC.

In conclusion, Bcl2 expression is common in MBC, but is not associated with major clinicopathologic features and, in contrast to FBC, does not seem to have prognostic value, also when combined with mitotic index.

Introduction

The incidence of male breast cancer (MBC) is relatively low compared to female breast cancer (FBC) but is rising [1]. Currently, treatment regimens for MBC are based on the assumption that it is similar to its female counterpart, and that FBC prognostic features and therapeutic targets can be extrapolated to MBC. Although there are indeed similarities between MBC and FBC, there is also mounting evidence that they are in fact biologically quite different [2-7]. At the same time, there is yet little evidence that prognostic features that have been developed and established in FBC work on MBC as well.

Cellular proliferation is a strong traditional prognostic feature in FBC [8,9] and was shown to have important prognostic value in MBC as well already years ago [10]. Cellular proliferation can be assessed in different ways, but counting mitoses is probably the most widely studied and widely applied way of assessing cellular proliferation [8,9], and is the main prognostic constituent of histological grading [11].

In a recent study on FBC, a combination of mitotic index and Bcl2 expression proved to be of value in stratifying patients into low risk vs. high risk groups regarding mortality and recurrence [12]. Bcl2 is an anti-apoptotic protein which is significantly more often expressed in male breast cancer compared to FBC [13-15] . Although there are some data published regarding Bcl2 expression and its prognostic value in MBC, the prognostic value of Bcl2 combined with mitotic index has not been studied in MBC. The aim of the present study was therefore to investigate the prognostic value of Bcl2 in combination with mitotic index in MBC and evaluate whether its prognostic value equals that observed in FBC.

Materials and Methods

Patients and specimens

All consecutive cases of surgical breast specimens of invasive male breast cancer from 1986 - 2011 were collected from 7 different pathology laboratories in The Netherlands (St. Antonius Hospital Nieuwegein, Diakonessenhuis Utrecht, University Medical Center Utrecht, Laboratory for Pathology East Netherlands) and in Germany (Paderborn, Koeln, Kassel) as described before [4-7]. Hematoxylin and eosin (HE) slides were reviewed by three experienced observers (PJvD, RK, MML) to confirm the diagnosis and assess tumor characteristics. The tumors were categorized by histological type according to the WHO [16]. Pathology reports were used to retrieve information on age, tumor size and lymph node status. A total of 151 cases were included from which there was enough material left in the paraffin blocks to perform immunohistochemistry. Table 1 shows baseline clinicopathological data.

Table 1 Baseline clinicopathologic features of 151 male breast cancers

Characteristics	All cases (n=151)	Characteristics	All cases (n=151)
Age (mean), years	66 (n=150)	Histological grade	
</= 50	15 (10%)	I	38 (25.2%)
>50	135 (90%)	II	66 (43.7%)
		III	47 (31.1%)
Histological type			
Ductal	136 (90%)	Lymph node metastasis	n= 124
Lobular	3 (2%)	Absent	56 (45.2%)
Invasive cribriform	3 (2%)	Present	68 (54.8%)
Mixed (ductal/lobular)	3 (2%)		
Mucinous	2 (1.3%)	Immunohistochemistry	
Papillary	2 (1.3%)	ER	n=150
Invasive micropapillary	1 (0.7%)	(+)	135 (90%)
Adenoid cystic	1 (0.7%)	(-)	15 (10%)
Tumor size (mean), cm	2.235 (n=147)	PR	n=150
T1	79 (54.1)	(+)	99 (66%)
T2	64 (43.5%)	(-)	51 (34%)
T3	4 (2.7%)		
		AR	n=150
Tubule formation		(+)	120 (80%)
>75%	14 (9.3%)	(-)	30 (20%)
10–75%	59 (39.1%)		
<10%	78 (51.7%)	HER2	n=150
		(+)	5 (3.3%)
Nuclear atypia		(-)	145 (96.7%)
Mild	13 (8.6%)		
Moderate	89 (58.9%)	Bcl2 (10%)	n=149
Severe	49 (32.5%)	(+)	140 (94%)
		(-)	9 (6%)
Mitotic activity index/2 mm^2			
< 8 mitoses	60 (49.7%)	Bcl2 (30%)	n=149
8-14 mitoses	40 (26.5%)	(+)	137 (92%)
15 or > mitoses	51 (33.8%)	(-)	12 (8%)

Mitosis counting

Mitotic activity was assessed on regular sections according to the protocol described earlier[17,18] and expressed per 2 mm^2. Mitotic index was scored as follows: M1= low if < 8 mitoses/2 mm^2; M2= medium if 8-14 mitoses/2 mm^2; and M3= high if 15 or more mitoses/2 mm^2 [11].

Immunohistochemistry

Tissue microarrays (TMAs) were constructed using three replicate 0.6 mm cores from different invasive regions for each tumor, and four µm thick sections were cut from the TMA blocks. Immunohistochemical staining for Bcl2 (dilution 1:200, code MO887, DAKO, clone 124) was performed using the Bond automated staining machine (Leica, Germany) with the Bond polymer refine detection kit (Leica , cat. no DS9800): peroxidase block 5 min, antigen retrieval with epitope retrieval 1, 20 min 99°, primary antibody 15 min RT, Bond polymer 8 min RT, DAB 10 min RT. Lymphoid tissue was used as positive control and kidney tissue as negative control throughout the immunohistochemical stainings. Immunohistochemical data of two patients was lost in the process due to poor core morphology.

Bcl2 was scored using TMAs by two experienced observers as negative when less than 10% of tumor cells showed expression, otherwise Bcl2 was scored as positive (fig. 1) [12,19]. No difference in score was noticed when using the highest score between cores versus the mean of the three cores for each case. The mitotic index/Bcl2 profiles were defined as M1/Bcl2+, M1/Bcl2-, M2/Bcl2+, M2/Bcl2-, M3/Bcl2+ and M3/Bcl2-.

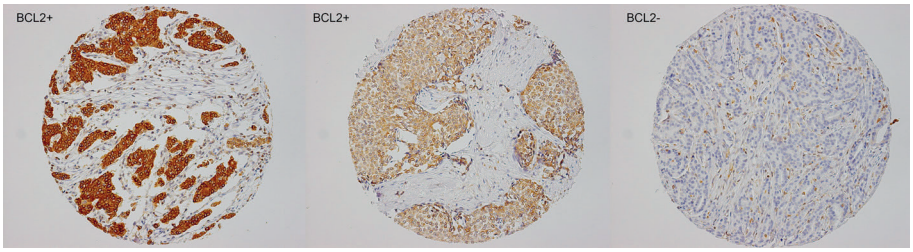

Figure 1 Expression of Bcl2 in male breast cancer

Statistics

Information regarding prognosis and therapy was requested from the Integral Cancer Registration of The Netherlands (IKNL). Survival data was available for 103 (68%) cases. The mean follow up was 5.7 years (range 0.1 – 20.3 years). Therefore, survival analysis was based on 5 years survival rates.

Data analysis was performed using IBM SPSS statistics (version 20). Pearson's chi-square or Fisher exact tests were used when appropriate. Survival rates were estimated using the Kaplan–Meier method and differences between curves were tested for significance using the log-rank test. Cox regression analysis was used for multivariate survival analysis. Hazard ratios (HRs) and 95% confidence intervals (95% CIs) were estimated for each variable. All tests were two-sided with a 95% CI and *p*-values less than 0.05 were considered significant.

Results

Patients and tumor characteristics

The age of these patients ranged from 32 to 89 years (average: 66 years). Tumor size ranged from 0.2 to 7.2 cm (average: 2.235 cm). In 82.1% of patients their lymph node status was known by axillary lymph node dissection or sentinel node procedure and in 54.8% of these patients lymph node metastases were found. The majority of cases were diagnosed as invasive ductal carcinoma (90.1%). The remaining cases were lobular (n=3), mixed type (ductal/lobular) (n=3), invasive cribriform (n=3), papillary (n=2), mucinous (n=.2), invasive micropapillary (n=1) or adenoid cystic carcinomas (n=1).

Most tumors were ER positive (135/150; 90%) while PR and AR positivity was also common; this was 66% and 80% respectively. HER2 amplification was rare (5/150; 3.3%).

According to the modified Bloom and Richardson score 25.2 % of the tumors were grade 1, 43.7% were grade 2 and 31.1% were grade 3.

Clinicopathological significance of Bcl2 expression

The majority of the tumors (140/149, 94%) showed positive Bcl2 expression using the 10% threshold, and 92% of the tumors (137/149) showed positive Bcl2 expression when applying a 30% threshold. Bcl2 expression had no significant association with favorable clinicopathologic features such as small tumor size, low mitotic count and low grade (Table 2), irrespective of these thresholds.

There was a positive correlation between Bcl2 and ER status (p=0.04). No significant correlation was found between Bcl2 expression and p53 or HER2 expression.

Table 2 Associations between Bcl-2 expression and clinicopathologic features in male breast cancer

	n (% of total)	Bcl2 -	Bcl2 +	P-value
Size T				
T1	77 ; 53.8%	5 ; 7.7%	72 ; 92.3%	0.232
T2	64 ; 44.1%	3 ; 4.7%	61 ; 95.3%	
T3	4 ; 2.8%	1 ; 25.0%	3 ; 75.0%	
Grade				
G1	38 ; 25.3%	1 ; 2.6%	37 ; 97.4%	0.618
G2	65 ; 44.0%	5 ; 9.1%	60 ; 90.9%	
G3	46 ; 30.7%	3 ; 6.5%	43 ; 93.5%	
Mitosis				
M1	60 ; 40.0%	3 ; 5.0%	57 ; 95.0%	0.908
M2	38 ; 26.0%	3 ; 10.3%	35 ; 89.7%	
M3	51 ; 34.0%	3 ; 5.9%	48 ; 94.1%	

Survival analysis

Bcl2 expression was assessed in 101/103 cases with available survival data. In univariate survival analysis, Bcl2 expression was not associated with survival (p=0.180)(fig. 2), as was true for the Mitotic Index (M1-M2-M3) (p=0.144) (Table 3). In multivariate Cox regression only tumor size and histological grade emerged as independent prognostic factors. None of the other features had additional prognostic value. The combination of Bcl2 expression with mitotic index as described before in FBC did not predict survival in MBC (n=101) (Table 3) (fig. 3).

Table 3 Survival analysis results of 151 male breast cancers

Feature	Grouping	5-Year survival			
		N	Alive	Survival rate (%)	P-value
Tumor size	T1	54	44	81.5	0.007*
	T2	44	29	65.9	
	T3	2	0	0.0	
Mitoses/2 mm^2	0–7	43	36	83.7	0.144
	8-14	30	20	66.7	
	15>	30	19	63.3	
Grade	I	25	22	88.0	0.026*
	II	50	38	76.0	
	III	28	15	53.6	
Lymph node metastases	Absent	43	36	83.7	0.128
	Present	48	35	72.9	
Bcl2	Positive	96	69	71.9	0.180
	Negative	5	5	100	
M/Bcl2 profiles	M1/Bcl2+	42	35	83.3	0.262
	M1/Bcl2-	1	1	100	
	M2/Bcl2+	26	17	65.4	
	M2/Bcl2-	2	2	100	
	M3/Bcl2+	28	17	60.7	
	M3/Bcl2-	2	2	100	

The *asterisk* indicates a statistically significant association ($p < 0.05$) with survival

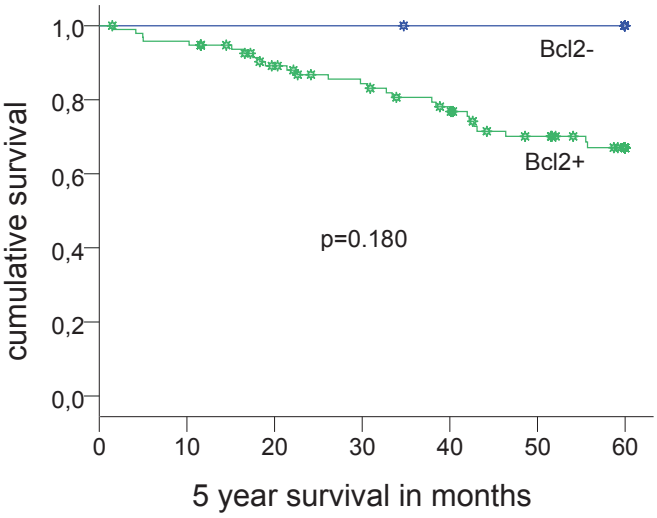

Figure 2 Survival curves for 103 male breast cancer cases with low or high Bcl2 expression

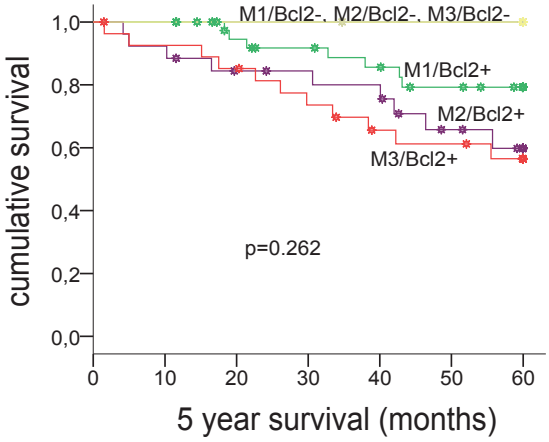

Figure 3 Survival curves for 103 male breast cancer cases according to different classes of the mitotic (M1-M2-M3)-Bcl2 expression grouping previously described in female breast cancer

Discussion

The aim of the present study was to investigate the prognostic value of Bcl2 in conjunction with mitotic index in MBC, a combination previously described to have strong prognostic value in FBC. The vast majority of MBC showed Bcl2 expression, but Bcl2 expression had no significant associations with clinicopathologic features such as tumor size, mitotic count and grade. Although Bcl2 negative cases appear to have a better prognosis than Bcl2 positive cases this finding was not significant as only a few (5/101) cases were Bcl2 negative. In univariate and multivariate survival analysis, Bcl2 had no prognostic value. The Bcl2/mitotic index combination as previously described in FBC as a useful prognosticator, did not predict survival of MBC either. The widespread Bcl2 expression as demonstrated in the present study is largely in accordance with the few previous studies on Bcl2 in MBC [2,13-15], although the percentages of positive cases vary somewhat in these studies due to variations in methodology and thresholds (Table 4).

The biological mechanisms of Bcl2 as a prognostic factor for breast cancer remain largely unclear. The Bcl2 (B-cell CLL/lymphoma 2) gene is located in 18q21.33 and encodes an integral outer mitochondrial membrane protein that blocks the apoptotic death of some cells. Bcl2 is well known as an anti-apoptotic oncogene in lymphoma [20],

Table 4 Overview of studies on Bcl2 in male breast cancer

Article	n	Cutoff	% positive	Associations
Weber Chapius et al. Eur J Cancer. 1996	66	30%	67%	inverse association with p53, no correlation with survival
Rayson et al. Cancer. 1998	77	20%	94%	unable to assess prognostic value
Pich et al. Virchows Arch. 1998	34	30%	82.3%	trend for association with low tumor stage, no association with survival
Pich et al. J Clin Oncol. 2000	50	30%	74%	no prognostic value
Temmim et al. The Breast. 2001	18	any +	78%	inverse association with p53 and Ki67
Present study	151	10%	94%	no prognostic value
		30%	92%	no prognostic value

however the paradoxical function of the tumor suppressor gene has been reported in many solid tumors, including breast cancer [21,22]. Bcl2 may be both oncogenic and tumor suppressive in specific cell types or under specific conditions, and it is postulated that the tumor suppressive effect is more prominent in breast cancer. Bcl2 expression has extensively been studied in FBC [12,19,23-28], rather consistently showing its prognostic value also independent of hormone receptor status. A correlation between Bcl2 expression and hormonal receptor status has been repetitively reported in FBC [29,30]. Bcl2 was supposed to be up-regulated by oestrogen, possibly as a result of direct transcriptional induction with negative regulation by p53-dependent mechanisms [19]. In the present study we find a positive correlation between Bcl2 and ER status (p=0.04), but not as strong as described in FBC (p< 0.00001) [29,30]. On the other hand we did not find a significant inverse relationship between Bcl2 expression and p53 accumulation. In literature data concerning the relationship between Bcl2 and ER/p53 in MBC are not consistent, making it difficult to draw conclusions from these findings [2,13,14].

Compared to FBC, there is a higher expression of Bcl2 (94% vs. 68.2%) in MBC [25]. Its expression was not associated with favorable clinicopathologic features such as small tumor size, low mitotic count and low grade in male breast cancer (Table 2), which is largely in line with previous studies (Table 4). None of the tumors expressing HER2, which has been previously reported to be associated with more aggressive phenotype and adverse prognosis in MBC [31], showed Bcl2 expression. However, we found no significant association between HER2 and Bcl2 expression, which can be due to the fact that a very low percentage (3.3%) of the tumors expressed HER2. Only a few Bcl2 studies have been performed in small cohorts of MBC [2,13-15], showing no relation with survival (Table 4). Also the present study (n=151) failed to show prognostic value of Bcl2 expression as a single feature, nor did it emerge in multivariate survival analysis. In FBC, a combination of Bcl2 expression and mitotic index (grouped according to the Nottingham grading classes) appeared to have very strong prognostic value in a large cohort of 1650 patients [12]. Other than in FBC, that very combination of mitotic index and Bcl2 expression had no prognostic value in MBC. This is not surprising given the fact that in our study we also found no significant associations between the M1-M2-M3 mitotic index groups and survival, although a mitotic index threshold of 8 per 2 mm^2 did have strong prognostic value in our previous study [32]. Apparently, MBC and FBC differ with regard to the clinical significance of Bcl2 expression and the prognostically optimal thresholds for mitotic index. The biological background for this is unclear until now.

In conclusion, Bcl2 expression is common in MBC, but is not associated with major clinicopathologic features and does not seem to have prognostic value, also not when combined with mitotic index, which previously did seem to work well in FBC.

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Chapter 3

Expression of Connective Tissue Growth Factor in male breast cancer: clinicopathologic correlations and prognostic value

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Submitted

Abstract

Connective tissue growth factor (CTGF/CCN2) is a member of the CCN family of secreted proteins that are believed to play an important role in the development of neoplasia.In particular, CTGF has been reported to play an important role in mammary tumorigenesis and to have prognostic value in female breast cancer (FBC). The aim of the present study was to investigate clinicopathologic correlations and prognostic value of CTGF in male breast cancer (MBC) and to compare these findings with FBC. For this, we studied CTGF protein expression by immunohistochemistry in 109 MBC cases and 75 FBC cases.

In MBC, stromal CTGF expression was seen in the majority of the cases 78% (85/109) with high expression in 31/109 cases (28.4%), but expression in tumor cells was only seen in 9.2% (10/109) of cases. High stromal CTGF expression correlated with high grade and high proliferation index (>15%) assessed by MIB-1 immunohistochemical staining. CTGF expression in tumor epithelial cells did not correlate with any of the clinicopathologic features. In FBC, stromal CTGF expression positively correlated with mitotic count and tumor CTGF expression was associated with triple negative status of the tumor (p=0.002). Neither stromal nor tumor epithelial cell CTGF expression had prognostic value in MBC and FBC.

In conclusion, stromal CTGF expression was seen in a high percentage of MBC and was correlated with high grade and high proliferation index. In view of the important role of the microenvironment in cancer progression, this might suggest that stromal CTGF could be an interesting target for novel therapies and molecular imaging. However, the lack of association with prognosis warrants caution. The potential role of CTGF as a therapeutic target for triple negative FBC deserves to be further studied.

Introduction

Connective tissue growth factor (CTGF/CCN2) is a member of the CCN family of six secreted matricellular proteins consisting of four conserved subdomains [1], and is expressed in several cell types, such as endothelial cells, fibroblasts and leukocytes but especially in osteoblasts and chondrocytes. It functions as a multi-functional signalling modulator involved in a wide variety of biologic and pathologic processes including control of proliferation, migration and adhesion, regulation of growth, development and differentiation, wound healing, regeneration and cell death [2-4]. CTGF has been reported to play an important role in mammary tumor genesis and its expression has been proven to be associated with increased migration and angiogenesis [2]. Significant associations have been found between CTGF overexpression and stage, tumor size, lymph node status, age and prognosis in female breast cancer (FBC) patients suggesting that CTGF may play a role in the progression of breast cancer [5].

Male breast cancer (MBC), contrary to FBC, is rare and not well characterized. Currently, treatment regimens for MBC are based on the assumption that it is largely similar to its female counterpart, and that prognostic features and therapeutic targets of FBC can be extrapolated to MBC. Although there are indeed similarities between MBC and FBC, there is also mounting evidence that they are in fact biologically quite different [6-12]. At the same time, there is yet little evidence that prognostic features that have been established in FBC are valid for MBC as well. In the current study, we set out to investigate the expression, clinicopathologic correlations and prognostic value of CTGF in a relatively large group of MBC.

Materials and Methods

Patients and specimens

All consecutive cases of surgical breast specimens of invasive MBC from 1986 - 2011 were collected from 7 different pathology laboratories in The Netherlands (St. Antonius Hospital Nieuwegein, Diakonessenhuis Utrecht, University Medical Center Utrecht, Laboratory for Pathology East Netherlands) as described before [7-10]. Hematoxylin and eosin (HE) slides were reviewed by three experienced observers (PJvD, RK, MML) to confirm the diagnosis and assess tumor characteristics. The tumors were categorized by histological type according to the WHO [13]. Pathology reports were used to retrieve information on age, tumor size and lymph node status. For the MBC cases immunohistochemical CTGF data of 3 patients was lost due to poor core morphology. A total of 109 cases were included

from which there was enough material left in the paraffin blocks to perform tissue microarrays and immunohistochemistry. For the FBC cases immunohistochemical CTGF data of 1 patient was lost due to poor core morphology. A total of 75 random FBC cases from one newly constructed TMA were used to compare the immunohistochemistry findings between MBC and FBC.

Since we used archival pathology material which does not interfere with patient care and does not imply the physical involvement of the patient, no ethical approval is required according to Dutch legislation [14]. Use of anonymous or coded left over material for scientific purposes is part of the standard treatment contract with patients and therefore informed consent procedure was not required according to our institutional medical ethical review board. This has also been described earlier by van Diest et al. [15].

Immunohistochemistry

MBC tissue microarrays (TMAs) were constructed as described before [16] and four µm thick sections were cut from the TMA blocks. For FBC a previously constructed TMA was used. Immunohistochemical staining for CTGF (dilution 1:5000, Santa Cruz, goat anti-CTGF (L-20), sc-14939) was performed as follow: 20 minutes antigen retrieval with citrate buffer (pH 6) method followed by 60 minutes primary antibody incubation at room temperature. CTGF in stromal and tumor cells was scored by three experienced observers (TN, RG, ML). The intensity was quantified as weak (1), moderate (2) or strong (3) (Figure 1). The percentage of positive stromal or tumor cells was given a score of 1 if less than a third were positive, 2 if between one and two thirds and 3 if more than two thirds were positive. For stromal cells, a combined score was calculated by adding intensity and percentage of positive cells which resulted in a score of 0 to 6. This score was then digotomized into low (1-3) and high expression (4-6). ER, PR, HER2, HIF1α and Ki67/MIB1 data were derived from our previous study [9].

Statistics

Information regarding prognosis and therapy was requested from the Integral Cancer Registration of The Netherlands (IKNL). Survival data was available for 100 (92%) cases. The mean follow up was 5.7 years (range 0.1 – 20.3 years). Therefore, survival analysis was based on 5 years survival rates.

Data analysis was performed using IBM SPSS statistics (version 20). Pearson's chi-square or Fisher's exact tests were used when appropriate. Survival rates were estimated using the Kaplan–Meier method and differences between curves were tested for significance using the log-rank test. All tests were two-sided and *p*-values less than 0.05 were considered significant.

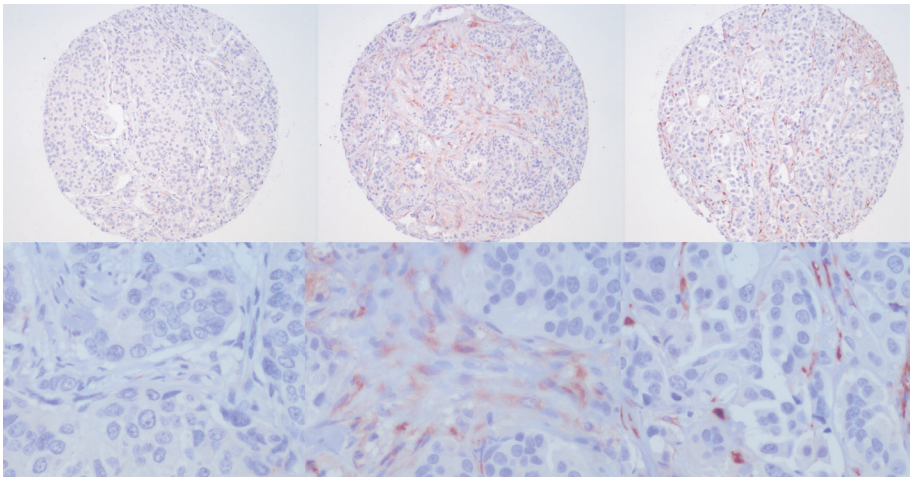

Figure 1 Representative low and high magnification examples of stromal CTGF expression in male breast cancer. Left: weak, middle: moderate, right: strong

Results

Patients and tumor characteristics

The clinicopathologic characteristics of the MBC and FBC patients are shown in Table 1. The age of the male patients ranged from 32 to 89 years (average: 66 years). Tumor size ranged from 0.4 to 5.5 cm (average: 2.1 cm). In 86.2% of patients the lymph node status was known by axillary lymph node dissection or sentinel node procedure and in 54.3% of these patients lymph node metastases were found. The majority of cases was diagnosed as invasive ductal carcinoma (90.8%). Most tumors were ER positive (92.7%) while PR and AR positivity was also common (65.1% and 77.8%, respectively). HER2 amplification was rare (3.7%). According to the modified Bloom and Richardson score 23.9% of the tumors were grade 1, 50.4% were grade 2 and 25.7% were grade 3.

CTGF expression and clinicopathologic correlations

In MBC, any CTGF expression (in stromal and/or tumor cells) was seen in 89/109 (81.7%) of cases. CTGF expression in tumor cells was seen in 10/109 (9.2%) of cases, and stromal cell expression in 85/109 (78%) of cases of which 31 (36.5%) showed high expression. Stromal cell expression of CTGF did not correlate with epithelial tumor cell CTGF expression.
Stromal CTGF expression correlated positively with grade (p=0.018) and high stromal expression even stronger with high grade (G3) versus grade 1/2 (p=0.007). Of the three

components of the Bloom and Richardson grading system, a high stromal CTGF expression positively correlated with gland formation and nuclear pleomorphism (p=0.005 and p=0.006 respectively) but not with mitotic index. High stromal CTGF expression was positively correlated with high MIB1 proliferation index (>15%) (p=0.034). Table 2.

Stromal CTGF expression did not correlate with other clinicopathologic features such as age, tumor type, tumor size, lymph node status or hormonal status, or HIF-1α expression. CTGF expression in tumor cells did not correlate with any of the clinicopathologic features mentioned above. In univariate survival analysis, stromal nor epithelial, nor combined stromal and epithelial CTGF expression was correlated with survival. Figure 2.
In FBC, any CTGF expression (in stromal and/or tumor cells) was seen in 71/75 (94.7%) of cases, stromal cell expression in 69/75 (82%) of cases of which 35 (50.7%) showed high expression, and CTGF expression in tumor cells in 18/75 (24%) of cases. Stromal CTGF expression was correlated with triple negative status of the tumor (p=0.002); all of the eight triple negative tumors showed stromal expression of CTGF, five of which had high stromal expression. Any stromal CTGF expression correlated positively with mitotic count (p=0.010).

Table 1 Baseline clinicopathologic features of 109 male breast cancers (MBC) and 75 female breast cancers (FBC)

Characteristics M	MBC (n=109)	FBC (n=75)
Age (mean), years	66	56
</= 50	12 (11%)	27 (36%)
>50	97 (89%)	48 (64%)
Histological type		
Ductal	99 (91%)	61 (81.3%)
Lobular	2 (1.8%)	7 (9.3%)
Invasive cribriform	1 (0.9%)	0 (0%)
Mixed (ductal/lobular)	2 (1.8%)	5 (6.7%)
Mucinous	2 (1.8%)	1 (1.3%)
Papillary	1 (0.9%)	1 (1.3%)
Invasive micropapillary	1 (0.9%)	0 (0%)
Adenoid cystic	1 (0.9%)	0 (0%)
Tumor size (mean), cm	2.2 (n=106)	2.8 (n=74)
T1	53 (50%)	32 (43.2%)
T2	50 (37.2%)	34 (46%)
T3	3 (2.8%)	8 (10.8%)
Histological grade		
I	26 (23.9%)	6 (8%)
II	55 (50.4%)	26 (34.7%)
III	28 (25.7%)	43 (57.3%)
Mitotic index		
M1	47 (43.1%)	14 (18.7%)
M2	30 (27.5%)	21 (28%)
M3	32 (29.4%)	40 (53.3%)
Lymph node metastasis	n= 94	n= 73
Absent	43 (45.7%)	49 (67.1%)
Present	51 (54.3%)	24 (32.9%)

Table 2 Associations between stromal CTGF expression and clinicopathologic features in male breast cancer

		CTGF expression		
Variable				
	n	**low**	**high**	**P-value**
Tumor size				1
T1	53	38	15	
T2/T3	53	38	15	
Grade				0.003
G1/G2	81	64	17	
G3	28	14	14	
Mitosis				0.176
M1/M2	77	58	19	
M3	32	20	12	
Gland formation				0.005
G1/G2	55	46	9	
G3	54	32	22	
Nuclear pleomorphism				0.006
G1/G2	74	59	15	
G3	35	19	16	
MIB				0.009
0-15	91	69	22	
>15	16	7	9	

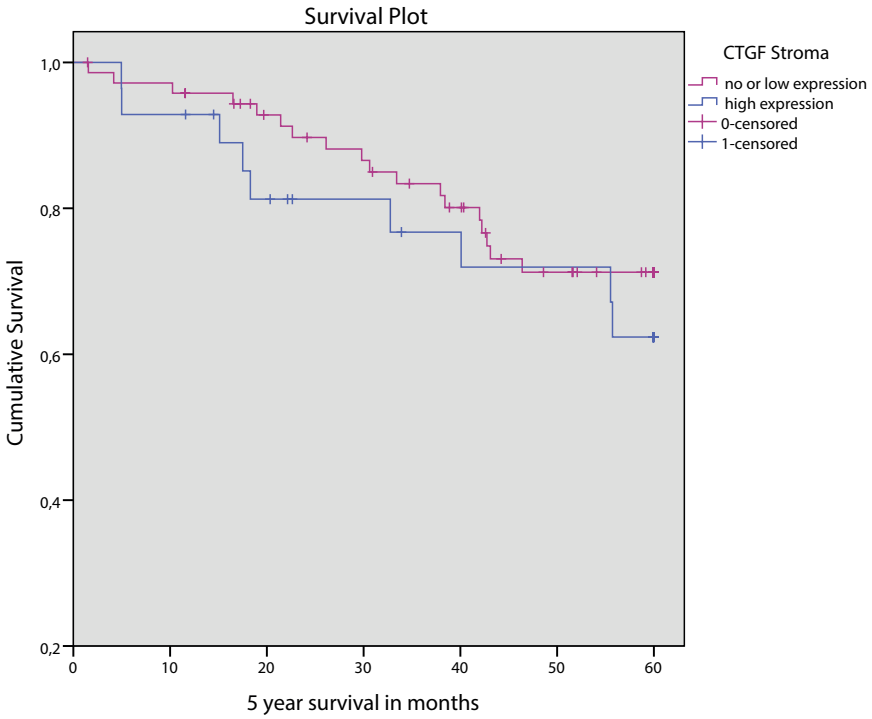

Figure 2 5 year survival curves for low and high stromal CTGF expression in male breast cancer

Discussion

The aim of the present study was to investigate the prognostic value of CTGF in MBC, as well as evaluate clinicopathologic correlations, since CTGF was previously described to be an important player in carcinogenesis and a biomarker of aggressive FBC. Although most previous studies on CTGF used molecular techniques we chose to evaluate CTGF expression on the protein level by a previously validated immunohistochemical protocol [17], and compared expression to FBC.

Stromal CTGF expression was seen in the majority of MBC cases 78% (85/109) with even high expression in 31 of the 109 cases (28.4%). However, CTGF expression in tumor cells was seen in only 9.2% (10/109) of the cases. This is in keeping with a previous report where CTGF expression was observed only in fibroblasts of mammary tumors and not

in tumor epithelial cells [18]. The latter report suggested that CTGF is produced in the stromal fibroblasts under influence of TGF-β1 which in turn is produced by mammary tumor epithelial cells. A more recent study by Caparelli et al. proposed a compartment specific role for CTGF for tumor formation in breast cancer showing that overexpression of CTGF by tumor epithelial cells leads to tumor cell digestion and inhibition of tumor growth. On the other hand, overexpression of CTGF in fibroblasts changes the tumor microenvironment and fuels anabolic tumor cells, but drives the induction of autophagy and a senescence phenotype in fibroblasts, which may further promote tumor growth [19]. Stromal CTGF correlated with high grade and high proliferation. This is in line with previous studies in hepatocellular carcinoma showing that CTGF contributed to dedifferentiation and growth [20,21]. Our results point to a similar mechanism, but further studies are necessary to unravel the mechanisms by which stromal CTGF expression affects tumorigenesis in MBC.

There was no significant correlation between CTGF stromal expression and HIF-1α expression, in contrast with Caparelli et al. who demonstrated that the metabolic promotion of tumor growth in breast cancer cells by CTGF is through activation of HIF-1α. These results may suggest that the mechanism by which CTGF promotes tumor growth in MBC is not HIF-1 mediated.

Stromal CTGF expression did not correlate with other studied clinicopathologic features such as age, tumor type, tumor size, lymph node status, hormone receptor or HER2 status. This is at variance with previous findings in FBC where overexpression of CTGF on a molecular level was reported to be significantly associated with tumor size, lymph node status and HER2 [5]. In our study expression of stromal CTGF on a protein level in FBC did correlate with mitotic count but not with other clinicopathologic features including grade, tumor size and lymph node status. Epithelial cell CTGF expression was not correlated with any of the studied clinicopathologic features, suggesting that the mechanisms that are important for CTGF mediated tumor growth do not depend on CTGF overexpression in tumor epithelial cells in MBC.

However, epithelial cell CTGF expression did correlate with a triple negative status of the tumor in FBC. All of the triple negative FBC also had stromal CTGF expression, most with high expression. This is an interesting finding and should be confirmed in a larger cohort. A compartment specific role for CTGF in tumor formation in FBC was proposed by Caparelli et al. as mentioned before. In their study using a triple negative breast cancer cell line, overexpression of CTGF by tumor epithelial cells lead to tumor cell digestion and inhibition of tumor growth, and overexpression of CTGF by fibroblast supports tumor growth. In the light of our results it would be interesting to consider and analyze the therapeutic potential of anti-CTGF therapy in triple negative FBC. In MBC epithelial cell

CTGF expression did not correlate with a triple negative status, which could be due to the low number of triple negative MBC (n=5).
CTGF expression in MBC was not associated with survival in univariate survival analysis, despite the correlation between high stromal expression and high grade and high proliferation. This is in line with the lack of prognostic of CTGF in FBC, although also in FBC some correlations with established prognostic variables have been described [5].
In conclusion, stromal CTGF expression was seen in a high percentage of MBC and was correlated with high grade and high proliferation index. Expression in tumor epithelial cells was seen in a much lower percentage of cases. In view of the important role of the microenvironment in cancer progression, this makes stromal CTGF an interesting target for novel therapies and molecular imaging, but as a prognosticator. The role of CTGF as a therapeutic target for triple negative FBC deserves to be further studied.

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Chapter 4

Prognostic models in male breast cancer

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Abstract

Male breast cancer (MBC) is a rare disease and its treatment is still largely extrapolated from its female counterpart. Accurate prognostication is essential for advising on adjuvant systemic treatment and informing patients. Several predictive models are available for female breast cancer (FBC) including, chronologically, the Morphometric Prognostic Index (MPI), Nottingham Prognostic Index (NPI), Adjuvant! and Predict. The aim of this study was therefore to compare the prognostic performance of these models in a group of 166 early MBC patients.

The MPI describes a "good"- (MPI<0.60) and a "poor" prognostic group (MPI>=0.60). The NPI divides patients in three groups; "good"-, "intermediate"- and "poor" prognosis. For the programs Adjuvant! and Predict, similar groups with "good-", "intermediate-" and "poor" prognosis were formed by using tertiles. The prognostic performance of each test was studied by using the LogRank test and comparison between the models by C-statistics.

Survival was highest for the "good" predicted groups and higher (MPI: 87%, NPI: 90%, Adjuvant!: 91%, Predict: 88%) than for the "moderate" groups (NPI: 76%, Adjuvant!: 77%, Predict: 75%) and lowest for the "poor" predicted groups (MPI: 51%, NPI: 43%, Adjuvant!: 45%, Predict: 42%). P-values were highly significant.

In terms of discrimination, all models were moderately able to discriminate between good and poor survivors (C-statistics; MPI; 0,674, NPI; 0,678, Adjuvant!; 0,717 and Predict; 0,711).

In conclusion, MPI, NPI, Adjuvant! and Predict, prognostic models, originally developed and validated for FBC, also perform quite well for MBC. These models may therefore help in MBC prognostication and decisions on adjuvant systemic therapy.

Introduction

Male breast cancer (MBC) is a rare disease [1]. Although more and more is known about this disease and its differences with female breast cancer (FBC) [2-5], the actual treatment is still largely extrapolated from the female counterpart. Accurate prognostication is essential in advising on adjuvant systemic treatment following surgery for early breast cancer. A number of predictive models have been developed to assess female breast cancer (FBC) prognosis including the Morphometric Prognostic Index (MPI) [6], Nottingham Prognostic Index (NPI) [7, 8-10], Adjuvant! and Predict [11, 12].

The MPI was first described in 1985 and validated in 8 further studies in over 3000 women with breast cancer. The MPI is based on the mitotic activity index, tumor size and lymph node status [6, 13, 14-16].

The NPI was first described in 1982 and validated in many further studies. The NPI is based on tumor size, tumor grade and lymph node status [7, 9, 10, 17-19].

Adjuvant! (www.adjuvantonline.com) and Predict (www.predict.nhs.uk) are online prognostication tools that provide survival estimates and absolute individual adjuvant treatment benefits predictions for FBC patients. Adjuvant! calculates 10 years survival data and Predict calculates 5- as well as 10 years survival data. Adjuvant! was first described in 2001 as a computer program calculating overall survival as well as absolute treatment benefits for hormone therapy and chemotherapy based on the SEER data (Surveillance Epidemiology and End Results program) for FBC patients [20]. This model has been validated in several European countries [21-23]. However, in the Asian population this model seems to provide overoptimistic results [24]. Also for older women very recent data show that it does not accurately predict overall survival and recurrence [25].

Predict was developed in the United Kingdom based on 5694 women diagnosed with breast cancer in East Anglia from 1999 to 2003 [11]. It was the first prognostication tool for early FBC patients including HER-2-status and mode of detection. This model was validated in 2011 in the British Colombia Dataset and compared with Adjuvant! [12, 26]. The conclusion was that both Adjuvant! and Predict provide accurate overall and breast cancer-specific survival (BCSS) estimates that were comparable, for FBC patients.

All these prognostication tools are based on FBC data and it is unknown whether these outcome predictions are applicable to MBC. The aim of this study was therefore to compare the prognostic performance of these models in a relatively large group of MBC.

Patients and methods

Study Population

All men operated for invasive breast cancer between 1976–2010 were collected from four hospitals in The Netherlands (St Antonius Hospital Nieuwegein, Diakonessenhuis Utrecht, University Medical Center Utrecht, Laboratory for Pathology East Netherlands), two hospitals in Germany (Paderborn and Koeln) and in Switzerland (Geneve). Clinicopathological characteristics, adjuvant treatment data and survival data were retrieved from hospital records and medical registries. Pathology reports were used to extract age, tumor size, and lymph node status, regarding cases with isolated tumor cells as lymph node negative. Also treatment and survival data were collected. The original study group comprised 199 MBC patients and follow-up data were available for 166 patients. For each patient, predicted survival time was calculated with the four prognostic models and compared with the actual survival time. 5 years survival data were analysed.

Model calculations

Morphometric Prognostic Index (MPI) was calculated as before using the formula: MPI=0.3341*√(MAI)+0.2342*(tumour size in cm)-0.7654*(lymph node status, pos=1, neg=2), where MAI is the mitotic activity index (number of mitoses per 1.6 mm^2) [6, 13]. According to the previously established threshold, prognosis was regarded as "good" if the MPI was smaller than 0.60, while a MPI of 0.60 and higher indicated "poor" survival [6, 13]. An "intermediate" group was not defined.

Notthingham Prognostic Index (NPI) was calculated using the formula: NPI = [0.2 x S] + N + G, where **S** = the size in cm, **N** = the number of lymph nodes involved: 0 =1, 1-3 = 2, >3 = 3, **G** = tumor grade: Grade I =1, Grade II =2, Grade III =3). The NPI defines three groups of patients; 'good' prognosis for index <=3.4, 'moderate' prognosis for 3.4<NPI<=5.4 and 'poor' prognosis for >5.4 [8, 9, 19].

Adjuvant! Online

The web based program www.adjuvantonline.com for Breast Cancer (Version 8.0) is used to calculate prognosis for each individual patient. Factors like age, comorbidity, ER-status, tumor grade, tumor size, number of positive ipsilateral axillary nodes, adjuvant antihormonal treatment and adjuvant chemotherapy are essential for this calculation. For each patient, it is possible to generate 10-year predictions of breast cancer specific survival (BCSS) and disease free survival (DFS), as well as the absolute benefit of adjuvant chemotherapy and hormonal therapy. Because it was not possible to retrieve reliable

data on comorbidity in our cohort, the comorbidity assumption 'average for age' was used for the entire cohort as a default setting. Tamoxifen was entered in 'Adjuvant Therapy Effectiveness', hormonal therapy. 'First generation regimens' was filled out as adjuvant chemotherapy. Tertiles were defined: 'good' prognosis if the predicted overall survival was greater than 70%, 'intermediate' if the predicted overall survival was between 45% and 70% and 'poor' predicted survival if equal or smaller than 45%.

Predict

The online Predict tool (www.predict.nhs.uk) uses age, mode of detection, tumor size, tumor grade, number of positive nodes, ER-status, HER2 status, Ki67 status, adjuvant antihormonal treatment and adjuvant chemotherapy. Breast cancer screening for men does not exist, therefore 'mode of detection' was coded as 'symptomatic' for every patient. As 'chemo regimen' 'second' was filled out. The threshold for Ki67 was defined by the program; Ki67 positivity was defined as greater than 10% of tumor cells staining positive. Tertiles were defined: equal or greater than 90% indicated 'good' 5 yrs-overall survival, between 80% and 90% was defined as 'moderate' and when equal to or less than 80%, prognosis was regarded as 'poor'.

Statistics

Kaplan-Meier curves were plotted and differences in survival were tested with the LogRank test. Discrimination of the different models was estimated by means of the concordance index (C-index). A C-index of 1 indicates a perfect match of predicted and observed outcome. If the C-index is 0.5 the test does not better than chance. Statistical analyses were performed by means of IBM SPSS (version 20.0) and R.

Results

Table 1 shows patient basic characteristics for the total group of 166 patients as well as for each predictive test. The mean age of the patients was 66.4 (32-92) years old. Most patients had T1 (55.4%) or T2 (41%) tumours, mostly ER positive (83.7% with 10.8% unknown) and HER2 negative (58.4% with 39.8% unknown). The axilla did not have metastases in 42.2% of the cases (N0), 1-3 lymph nodes with metastases in 19.9% of the cases, while 16.8% had more than three lymph nodes with metastases and 21.1 % was unknown. 39.2% of the patients underwent adjuvant radiotherapy and 41.6% received anti-hormonal treatment, while most of the patients did not receive adjuvant chemotherapy (77,7%). Median survival was 4.6 years.

Table 1 Baseline characteristics of male breast cancer patients for subgroups in which prognostic models were studied (MPI=Morphometric Prognostic Index, NPI= Notingham Prognostic Index). Varying numbers between the models are explained by missing data.

	N=166	MPI N=88 (%)	NPI N=124 (%)	Adjuvant! N=130 (%)	Predict N=158 (%)
Age:				*	*
mean	66,4	65,9	65,4	65,7	65,9
≥65	74 (44,6)	40 (45,5)	58 (46,8)	59 (45,4)	74 (46,8)
>65	92 (55,4)	48 (54,5)	66 (53,2)	71 (54,6)	84 (53,2)
T-status		*	*	*	*
T1=0-2cm	92 (55,4)	47 (53,4)	64 (51,6)	67 (51,5)	86 (54,4)
T2=2,1-5cm	68 (41,0)	40 (45,5)	58 (46,8)	61 (46,9)	66 (41,8)
T3>5cm	3 (1,8)	1 (1,1)	2 (1,6)	2 (1,5)	3 (1,9)
Unknown	3 (1,8)	0	0	0	3 (1,9)
N-status		*	*	*	*
N0=0	70 (42,2)	45 (51,1)	64 (51,6)	69 (53,1)	70 (44,3)
N1=1-3	33 (19,9)	25 (28,4)	33 (26,6)	33 (25,4)	32 (20,3)
N2=4-9	18 (10,8)	13 (14,8)	17 (13,7)	18 (13,8)	18 (11,4)
N3≥10	10 (6,0)	5 (5,7)	10 (8,1)	10 (7,7)	10 (6,3)
Unknown	35 (21,1)	0	0	0	28 (17,7)
ER-status				*	*
positive	139 (83,7)	83 (94,3)	117 (94,4)	123 (94,6)	136 (86,1)
negative	9 (5,4)	5 (5,7)	7 (5,6)	7 (5,4)	9 (5,7)
unknown	18 (10,9)	0	0	0	13 (8,2)
Grade			*	*	*
1	25 (15,1)	19 (21,6)	23 (18,5)	23 (17,7)	24 (15,2)
2	69 (41,6)	37 (42,0)	59 (47,6)	59 (45,4)	68 (43,0)
3	50 (30,1)	32 (36,4)	42 (33,9)	42 (32,3)	49 (31,0)
Unknown	22 (13,2)	0	0	6 (4,6)	17 (10,8)
HER2					*
negative	97 (58,4)	85 (96,6)	85 (68,6)	85 (65,4)	97 (61,4)
positive	3 (1,8)	2 (2,3)	2 (1,6)	2 (1,5)	3 (1,9)
unknown	66 (39,8)	1 (1,1)	37 (29,8)	43 (33,1)	58 (36,7)
MAI		*			
low <10	49 (29,5)	43 (48,9)	43 (34,7)	43 (33,1)	49 (31,0)
high ≥10	52 (31,3)	45 (51,1)	45 (36,3)	45 (34,6)	52 (32,9)
unknown	65 (39,2)	0	36 (29,0)	42 (67,7)	57 (36,1)

	N=166	MPI N=88 (%)	NPI N=124 (%)	Adjuvant! N=130 (%)	Predict N=158 (%)
Ki67					*
low <10	80 (48,2)	68 (77,3)	71 (57,3)	71 (54,6)	80 (50,6)
high ≥10	21 (12,6)	20 (22,7)	17 (13,7)	17 (13,1)	21 (13,3)
unknown	65 (39,2)	0	36 (29,0)	42 (32,3)	57 (36,1)
Radiotherapy					
No	94 (56,6)	54 (61,4)	71 (57,3)	76 (58,5)	94 (59,5)
Yes	65 (39,2)	34 (38,6)	53 (42,7)	54 (41,5)	64 (40,5)
Unknown	7 (4,2)	0	0	0	0
AHT[1]				*	*
No	90 (54,2)	50 (56,8)	62 (50,0)	68 (52,3)	90 (57,0)
Yes	69 (41,6)	38 (43,2)	62 (50,0)	62 (47,7)	68 (43,0)
Unknown	7 (4,2)	0	0	0	0
Chemotherapy				*	*
No	129 (77,7)	75 (85,2)	96 (77,4)	102 (78,5)	128 (81,0)
Yes	30 (18,1)	13 (14,8)	28 (22,6)	28 (21,5)	30 (19,0)
Unknown	7 (4,2)	0	0	0	0

* Factors needed for the respective predictive models

[1] Anti hormonal treatment

Due to missing data not all patients could be included in each predictive model. The MPI could be calculated for 88 patients, NPI for 124 patients, Adjuvant! for 130 and Predict for 158 patients. The subgroups were equally divided for the 88 patients and the 124, 130 and 158, respectively (table 2).

All four predictive models clearly and significantly separated MBC patients with a favourable and unfavourable outcome. MPI showed 87% (95% confidence interval (CI): 86.9-87.1) five years survival for the "good" prognostic group and 51% (95% CI: 50.8-51.2) for the "poor" prognostic group with p=0.001. For NPI this was 90% (95% CI: 89.9-90.1) for the "good-" and 43% (95%CI: 42.8-43.2) for the "poor" prognostic group with p=0.001. Using Adjuvant! this was 91% (95% CI: 90.9-91.1) and 45% (95% CI: 44.8-45.2) respectively (p=0.000) and according to Predict 88% (95% CI: 87.9-88.1) in the "good" prognostic group would be alive after 5 years and 42% (95% CI: 41.9-42.1) in the "poor" prognostic group with a p-value of 0.000 (table 2, figures 1-4). Tests recognizing an intermediate prognostic group did not well differentiate between good and intermediate prognostic groups (NPI: p= 0.112, Adjuvant! p= 0.130 and Predict: p=0.221), in contrast with differentiating between intermediate and poor prognosis (NPI p=0.014, Adjuvant p=0.003 and Predict p=0.001).

The C-index for MPI was 0.674 (95% CI 0.583 -0.765), for NPI 0.678 (95% CI 0.600 -0.756), for Adjuvant! 0.717 with 95% CI 0.647-0.786 and 0.711 for Predict with 95% CI 0.646 -0.777 (table 2).

Table 2 Composition of the male breast cancer prognostic subsets per model (MPI= Morphometric Prognostic Index, NPI= Notingham Prognostic Index), 5 years survival and C-index

	MPI	NPI		Adjuvant!		Predict	
	N=88 (%)	N=88 (%)	N=124 (%)	N=88 (%)	N=130 (%)	N=88 (%)	N=158 (%)
Good	53 (60,2)	27 (30,7)	37 (29,8)	29 (33)	45 (34,6)	26 (29,5)	50 (31,6)
5 yrs survival% (95% CI)	87% (86,90-7,10)	86% (85,84-86,16)	90% (89,90-90,10)	87% (86,86-87,14)	91% (90,90-91,10)	95% (94,90-95,10)	88% (87,90-88,10)
Moderate	-	45 (51,1)	63 (50,8)	31 (35,2)	44 (33,8)	35 (39,8)	56 (35,4)
5 yrs survival% (95% CI)	-	83% (82,88-83,12)	76% (75,88-76,12)	83% (82,16-83,14)	77% (76,86-77,14)	74% (73,84-74,16)	75% (74,88-75,12)
Poor	35 (39,8)	16 (18,2)	24 (19,4)	28 (31,8)	41 (31,5)	27 (30,7)	52 (32,9)
5 yrs survival% (95% CI)	51% (50,80-51,20)	22% (21,76-22,24)	43% (42,76-43,24)	48% (47,78-48,22)	45% (44,82-45,18)	54% (53,80-54,20)	42% (41,86-42,14)
C-index	0,674	0,680	0,678	0,690	0,717	0,693	0,711
(95% CI)	(0,583-0,765)	(0,584-0,776)	(0,600-0,756)	(0,603-0,777)	(0,647-0,786)	(0,600-0,785)	(0,646-0,777)

Figure 1

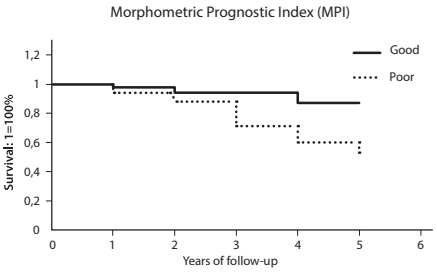


Figure 2

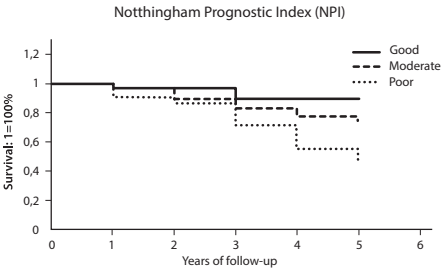


Figure 3

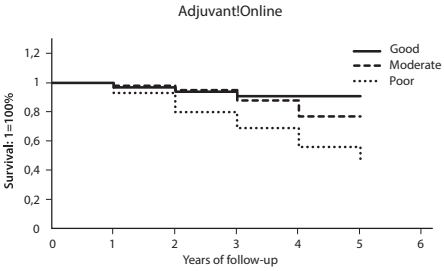
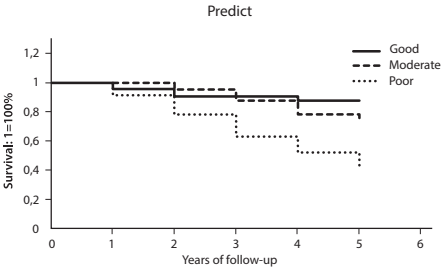


Figure 4



Discussion

A number of prognostic models have been developed to assess FBC prognosis, and the aim of the present study was to compare the performance of these models including the Morphometric Prognostic Index (MPI), Nottingham Prognostic Index (NPI), Adjuvant! and Predict in MBC.

The tumor features of this present MBC cohort is representative compared to literature: most of them are luminal-A (ER+ and HER2 negative) [3]. Age was around 65, which is about 10 years older than FBC patients which is also usual. This older age may be the reason why only 18.1% of the patients were treated with adjuvant chemotherapy, while 36.7% of the patients had one or more positive axillary lymph node(s). Changing indications for adjuvant chemotherapy over time and the wide time frame of this cohort could also partly declare this observation.

The MPI is the oldest model, followed by NPI, Adjuvant! and Predict. All these models performed well in survival analysis, with comparable C-indexes and confidence intervals, indicating that there are no major differences in the performance of these models in MBC. The most recently developed models use more features like HER2-status, Ki67 and mode of detection, that would be expected to allow better prognostication. However, since the vast majority of MBC is HER2 negative, Ki67 low and symptomatic (in absence of a screenings program for men), it is understandable that no big differences were found between the models [5]. The slightly better C-indexes of Adjuvant! and Predict are probably due to the higher number of patients included, since these tests allow some features to be scored as unknown.

It is interesting to see that an old model as the MPI did almost as good as Predict in prognostication of MBC, while it does not even use grade. However, it takes mitotic index into account that has been well established to be the most important constituent of grade [14, 16, 27]. The mitotic index as a prognosticator in MBC was previously validated [28].

The NPI also performed well, even though FBC validations were done with a group of patients aged <70 (8)operable breast cancer. This index was based on a retrospective analysis of 9 factors in 387 patients. Only 3 of the factors (tumour size, stage of disease, and tumour grade or with mean age of 54 [19], while the present MBC cohort was on average significantly older.

Since male breast cancer is morphologically most comparable with FBC in older women (high age, ER+ and HER2 negative) it is understandable that Adjuvant!Online survival prediction in this MBC cohort, where factor comorbidity was filled out as " average for age", was comparable to Adjuvant!Online validation in older women (n=2012) and input parameter "comorbidity" filled out as "average for age" (C-index of 0.72

vs 0.75, respectively) [25]. In the latter paper describing 10 years overall survival data, an overestimation was suggested for this group of patients. Disease specific survival data were not known. In the present paper, only 5 years survival data were studied because after 5 years many patients were lost to follow up.

Predict takes into account HER2 and mode of detection. The model is based on breast cancer specific mortality and competing mortality modelled separately. Comparison between prognostication by Adjuvant!Online and Predict showed similar results in FBC [26]. Even so, in the present MBC cohort with almost exclusively HER2-negative tumours, prognostication by Predict (C-index 0.711) was as good as by Adjuvant!Online (C-index; 0.717).

In conclusion, the MPI, NPI, Adjuvant! and Predict prognostic models that were originally validated for FBC also perform quite well for MBC. These models may therefore aid in MBC prognostication for decisions on adjuvant systemic therapy. Further improvements in MBC prognostication may be expected from molecular studies that have revealed prognostic features [2,4]. Gene expression studies in MBC are eagerly awaited.

chapter
4

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Chapter 5

Analysis of copy number changes on chromosome 16q in male breast cancer by multiplex ligation-dependent probe amplification

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Abstract

Gene copy number changes play an important role in carcinogenesis and could serve as potential biomarkers for prognosis and targets for therapy. Copy number changes mapping to chromosome 16 have been reported to be the most frequent alteration observed in female breast cancer and a loss 16q has been shown to be associated with low grade and better prognosis. In the present study we aimed to characterize copy number changes on 16q in a group of 135 male breast cancers using a novel multiplex ligation-dependent probe amplification kit.

One hundred and twelve out of 135 (83%) male breast cancer showed copy number changes of at least one gene on chromosome 16, with frequent loss of 16q (71/135; 53%), either partial (66/135; 49%), or whole arm loss (5/135; 4%). Losses on 16q were thereby less often seen in male breast cancer than previously described in female breast cancer. Loss on 16q was significantly correlated with favorable clinicopathological features such as negative lymph node status, small tumor size, and low grade. Copy number gain of almost all genes on the short arm was also significantly correlated with lymph node negative status. A combination of 16q loss and 16p gain correlated even stronger with negative lymph node status (n=112; p=0.012), which was also underlined by unsupervised clustering.

In conclusion, copy number loss on 16q is less frequent in male breast cancer than in female breast cancer, providing further evidence that male breast cancer and female breast cancer are genetically different. Gain on 16p and loss of 16q identify a group of male breast cancer with low propensity to develop lymph node metastases.

Introduction

Gene copy number changes play an important role in carcinogenesis and could serve as potential biomarkers for prognosis. In addition they could provide potential targets for molecular therapy.

Previous studies in male breast cancer showed clear differences in gene copy number changes when compared with female breast cancer, pointing towards differences in carcinogenesis between male and female breast cancer [1]. This emphasizes the importance of identifying biomarkers and therapeutic targets that could aid in clinical management of male breast cancer.

Copy number changes mapping to chromosome 16 have been reported to be the most frequent alteration in female breast cancer. In female breast cancer, aberrations on chromosome 16 have extensively been studied showing association between loss on the long arm of chromosome 16, low-grade ductal and lobular cancer, and favorable prognosis. High grade ductal cancer often has complex changes, typically small regions of gain together with larger regions of loss [2-6]. Genetic alterations on chromosome 16 in male breast cancer are poorly characterized compared with female breast cancer and only a few studies have been performed [1,7,8] of which the first analyzes only one gene on 16q and the latter studies analyze small series of male breast cancer. These studies report frequent chromosomal imbalances on both the short- and long arm of chromosome 16.

In the present study we aimed to further characterize copy number changes on the long arm of chromosome 16 in relation to the 16p copy number changes in a large group of male breast cancer using a novel multiplex ligation-dependent probe amplification (MLPA) kit with multiple genes on chromosome 16, and to correlate these genomic anomalies with clinicopathological features and patients' outcome.

Materials and methods

Patient material

All consecutive cases of surgical breast specimens of invasive male breast cancer from 1986 to 2011 were collected from four different pathology labs in The Netherlands (St. Antonius Hospital Nieuwegein, Diakonessenhuis Utrecht, University Medical Center Utrecht, and Laboratory for Pathology East Netherlands) as described in more detail previously [1,9] and from three pathology labs in Germany (Paderborn, Cologne, Kassel). Hematoxylin and eosin (HE) slides were reviewed by four experienced observers (PJvD, RK, AM, ML) to confirm the diagnosis and to type and grade according to current

standards. Pathology reports were used to retrieve information on age, tumor size, and lymph node status. A total of 145 cases from which the paraffin blocks contained enough tumor for DNA isolation were included. The age of these patients ranged from 32 to 89 years (average: 66 years). Tumor size ranged from 0.2 to 7.2 cm (average: 2.3 cm). In 120 cases (82%) the lymph node status was known by axillary lymph node dissection or sentinel node procedure and 55% of these patients had lymph node metastases. The majority of cases were diagnosed (according to the WHO) as invasive ductal carcinoma (130/145; 90%). The remaining cases were lobular (n = 3), mixed type (ductal/lobular) (n = 3), invasive cribriform (n = 3), papillary (n = 2), mucinous (n = 2), invasive micropapillary (n = 1) or adenoid cystic carcinomas (n = 1). According to the modified Bloom and Richardson score [10] most tumors were grade 2 (44 %) or grade 3 (32%). Mitotic activity was assessed as before [11] with a mean mitotic index of 12 per 2 mm2 (range 0–56). For all cases, hormone receptor and HER2 status were re-assessed as described previously [9]. Tissue microarray (TMA) slides were used for immunohistochemical staining of ER, PR and chromogenic *in situ* hybridization (CISH) for HER2 assessment, the latter showing HER2 amplification in only 5 cases (3%). Most tumors were ER positive (131/145; 90%) and PR positivity was also common (97/145; 67%). TMA slides were also stained and scored for E-cadherin, considering cases with no membranous staining as E-cadherin negative. Six cases were scored as E-cadherin negative, three lobular and three ductal carcinomas.

Intrinsic subtypes

Immunohistochemical stainings were used to classify the tumors into five different subtypes: luminal type A (ER+ and/or PR+, HER2- and Ki-67 low), Luminal type B (ER+ and/or PR+, and HER2+ and/or Ki67 high), HER2 driven (HER2+ and ER-/PR-), basal-like (ER-/PR-/HER2-, and CK5/6+ and/or CK14+ and/or EGFR+), and unclassifiable triple negative (negative for all six markers) as described before [9].

DNA extraction and multiplex ligation-dependent probe amplification analysis

Representative tumor areas were identified in HE stained slides and corresponding tumor areas (at least 1 cm2) were dissected with a scalpel from 8 µm paraffin slides [12]. DNA was extracted by overnight incubation in proteinase K (10 mg/ml; Roche, Almere, The Netherlands) at 56°C, boiling for 10 min and centrifugation. Five µl of this DNA solution was used for multiplex ligation-dependent probe amplification analysis. multiplex ligation-dependent probe amplification was performed according the manufacturers' instructions (MRC Holland, Amsterdam, The Netherlands), using a Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA, USA). The novel X043-1A kit (MRC Holland), containing 6 probes for 6 16p genes and 28 probes for 21 16q genes, was used. All tests

were performed in duplicate. Negative reference samples (normal breast and blood) were included in each multiplex ligation-dependent probe amplification run as before [1]. The PCR products were separated by electrophoresis on an ABI 3730 capillary sequencer (Applied Biosystems). Mean probe peaks were used for final gene copy number analysis with Genescan v4.1 (Applied Biosystems) and Coffalyser v9.4 (MRC-Holland) software. Cut-off values were set as before with 1.3–2.0 for gene copy number gain, >2.0 for amplification and <0.7 for loss. Values between 0.7 and 1.3 were regarded normal [13,14]. Whole arm loss was defined as copy number loss of more than 75% of all the probes as defined before using array-comparative genomic hybridization [15]. Partial loss on the long arm of the chromosome was defined as any probe showing copy number loss.
To define smallest regions of overlap (SRO) between areas of copy number loss we analyzed the cases according to the previous definition, with an additional threshold defining retention 0.8-1.2 [14,16], values between 0.7-0.8 and 1.2-1.3 were regarded as gray areas.

Statistics

Statistical calculations were performed using IBM SPSS for Windows version 20.0. Associations between gene copy number and clinicopathological characteristics were calculated with Pearson Chi-square (or Fisher's exact test when appropriate) for categorical variables. Grade, tumor size and mitotic count were dichotomized. Unsupervised hierarchical clustering using the statistical program R (www.r-project.org) was performed to identify relevant clusters. We used the maximum distance and Ward's clustering method and calculated the stability of the clusters with pvclust as before [1].
Information regarding prognosis and therapy was requested from the Integral Cancer registration The Netherlands (IKNL). Survival data were available for 100 cases with a mean follow up of 5.6 years. Therefore, survival analysis was based on 5 year survival rates. For univariate survival analysis, Kaplan–Meier curves were plotted and analyzed with the log rank test. Multivariate survival analysis was done with Cox regression including the variables that were significant in univariate analysis.

Results

Copy number analysis by multiplex ligation-dependent probe amplification. In 10 cases the amount of DNA was insufficient, leaving 135 cases of male breast cancer for further analysis. Gene copy number status of the analyzed genes is presented in Table 1 and Fig. 1. In 23/135 (17%) of cases there were no copy number alterations in any of the studied

genes on 16q; 2 of these had partial gain of 16p.

The most common 16q alteration was copy number loss (71/135; 53%), either partial arm loss (66/135; 49%), or whole arm loss (5/135; 4%). Twenty-seven cases with partial 16q arm loss (27/66, 41%) also had partial gain of 16q. A total of 49% of the cases (n=66) had partial gain of 16q and in 38 cases this combined with copy number gain of 16p.

None of the cases showed a whole arm loss of 16p, while six cases (4%) had partial 16p loss. In 56 cases (42%), there was gain of 16p, either partial (39/135; 29%), or whole arm gain (17/135; 13%). Two cases (2%) showed whole arm gain of both 16q and 16p. In two cases (2%) there was no other alteration than copy number gain on 16p.

Of all cases, 26% (35/135) had both loss of 16q (either partial or whole arm loss) and gain of 16p (either partial or whole arm gain), 11 also had partial gain of 16q.

Loss was present in varying frequencies in all genes on 16q but in only one gene on 16p (*CREBBP*). Gene loss was most common for *CDH11*, *MLYCD*, *FOXF1*, *SPG7* and *FANCA*. Copy number gain was most frequently seen on the short arm but was also present throughout chromosome 16 (Figure 1).

Of all the cases with any loss on 16q, 58/71 (82%) showed alternating regions of retention and loss with more than two areas of loss. Two cases showed loss starting at the most centromeric region on the 16q arm alternated by gray areas and no retention. Two showed terminal loss of 16q starting from the *CYLD* gene position with retention on the area centrometric of this probe and another case showed terminal loss starting from the *MMP2* gene position with gray areas to the *CYLD* gene position and retention on the area centrometric of this probe. Eight cases showed two regions of loss with one or two areas of retention. The latter cases were used to determine SRO.

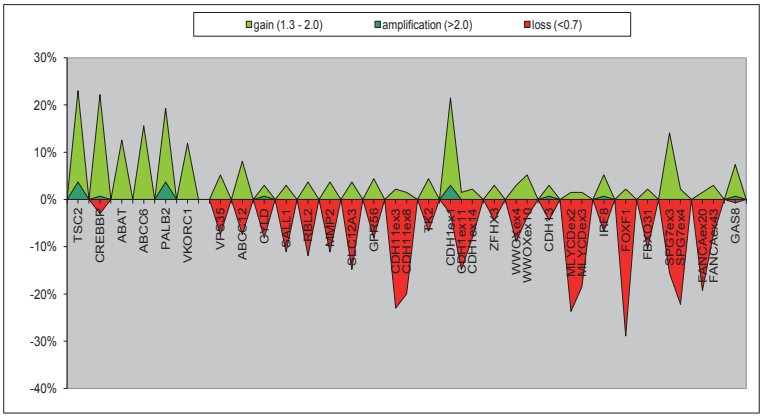

Figure 1 Copy number changes by multiplex ligation-dependent probe amplification in 27 genes on chromosome 16 in 135 male breast cancers

Table 1 Correlation between gene copy number losses by and clinicopathological features in 135 male breast cancers (only significant p-values shown)

Gene	Location	Grade G1	Size <2.0	Lymph node negative
TSC2	16p13.3 exon2			
CREBBP	16p13.3 exon12			
ABAT	16p13.2 exon4			
ABCC6	16p13.11exon13			
PALB2	16p12.1 exon6			
VKORC1	16p11.2 exon1			
VPS35	16q11.2 exon13			
ABCC12	16q12.1 exon28			
CYLD	16q12.1 exon19			0.009
SALL1	16q12.1 exon3			
RBL2	16q12.2 exon2	0.017		0.001
MMP2	16q12.2 exon14			0.017
SLC12A3	16q13 exon12			0.02
GPR56	16q13 exon10		0.04	0.009
CDH11	16q22.1 exon3			
CDH11	16q22.1 exon8			
TK2	16q22.1 exon6			
CDH1	16q22.1 exon1			0.047
CDH1	16q22.1 exon11			0.021
CDH1	16q22.1 exon14			
ZFHX3	16q22.3 exon3			
WWOX	16q23.1 exon4			
WWOX	16q23.1 exon10			
CDH13	16q23.3 exon1			
MLYCD	16q23.3 exon2			0.015
MLYCD	16q23.3 exon3			0.019
IRF8	16q24.1 exon9			0.017
FOXF1	16q24.1 exon2			
FBXO31	16q24.2 exon4			
SPG7	16q24.3 exon3			
SPG7	16q24.3 exon4			0.003
FANCA	16q24.3 exon20			0.006
FANCA	16q24.3 exon43			
GAS8	16q24.3 exon6			

Copy number alterations and clinicopathologic features

As shown in Table 1, copy number losses of the 16q genes *CYLD*, *RBL2*, *MMP2*, *SLC12A3*, *GPR56*, *CDH1* (exons 1 and 11), *MLYCD*, *IRF8*, *SPG7* (exon4) and *FANCA* (exon 20) were significantly correlated with negative node status. Loss of *GPR56* was associated with small tumor size (T1) and loss of *RBL2* with lower grade (G1). Age and mitotic count were not correlated with copy number changes in any of the studied genes (data not shown). As shown in Table 2, copy number gains of almost all genes on 16p were also significantly associated with negative lymph node status, as was true for some genes on 16q (*VPS35*, *ABCC12* and *GPR56*).

Copy number loss (partial loss and whole arm loss) on 16q was significantly correlated with lymph node negative status (n=112; p=0.032) irrespective of changes on 16p. When the cases had copy number loss on 16q combined with copy number gain on 16p the correlation was even stronger (n=112; p=0.012).

Loss on the long arm of chromosome 16 was not significantly different between grades and was present in 56% (18/32) of grade 1 tumors, 36% (21/59) of grade 2 tumors and 73% (32/44) of grade 3 tumors.

Of the six E-cadherin negative cases, two had no alterations on the *CDH1* gene, one lobular and one ductal tumor. Two cases, both lobular tumors, had loss of at least one probe of the *CDH1* gene, one case had copy number loss on the *CDH1* exon 11 and *CDH1* exon 14 probes together with gain on the *CDH1* exon 1 probe and the second one had loss on the *CDH1* exon 11 probe. The two remaining cases, both ductal tumors, had gain on the *CDH1* exon 1 probe. There was no correlation between alterations on the *CDH1* gene and expression of E-cadherin by immunohistochemical staining.

Cluster analysis

Unsupervised hierarchical clustering revealed two stable clusters ($p<0.001$) (Figure 2). Cluster A consisted of 78 cases and was characterized by partial and whole arm loss on 16q; 85% vs. 6% in cluster B. Combined loss on 16q and gain on 16p was also found in a higher percentage (40%) in cluster A than in cluster B. All of the genes analyzed on 16q showed loss in a significantly higher percentage in cluster A than in cluster B.

The male breast cancer cases in cluster A showed lymph node metastasis in a significantly lower percentage compared to cluster B (45% vs. 70%; p=0.008). Cluster B consisted of 57 cases and was characterized by a higher percentage of copy number gain; 72% vs. 35% in Cluster A. Cases showing no aberrations on chromosome 16 were also more frequently found in Cluster B. Distribution of other clinicopathological features was not significantly different between the clusters.

Table 2 Correlation between gene copy number gains by multiplex ligation-dependent probe amplification and clinicopathological features in 135 male breast cancers (only significant p-values shown)

Gene	Location	Grade G1	Size <2.0	Lymph node negative
TSC2	16p13.3 exon2			0.032
CREBBP	16p13.3 exon12			
ABAT	16p13.2 exon4			0.017
ABCC6	16p13.11exon13			0.004
PALB2	16p12.1 exon6			0.021
VKORC1	16p11.2 exon1			
VPS35	16q11.2 exon13			0.040
ABCC12	16q12.1 exon28	0.022		0.010
CYLD	16q12.1 exon19			
SALL1	16q12.1 exon3			
RBL2	16q12.2 exon2			
MMP2	16q12.2 exon14			
SLC12A3	16q13 exon12			
GPR56	16q13 exon10			0.014
CDH11	16q22.1 exon3			
CDH11	16q22.1 exon8			
TK2	16q22.1 exon6			
CDH1	16q22.1 exon1			
CDH1	16q22.1 exon11			
CDH1	16q22.1 exon14			
ZFHX3	16q22.3 exon3			
WWOX	16q23.1 exon4			
WWOX	16q23.1 exon10			
CDH13	16q23.3 exon1			
MLYCD	16q23.3 exon2			
MLYCD	16q23.3 exon3			
IRF8	16q24.1 exon9			
FOXF1	16q24.1 exon2			
FBXO31	16q24.2 exon4			
SPG7	16q24.3 exon3		0.039	
SPG7	16q24.3 exon4			
FANCA	16q24.3 exon20			
FANCA	16q24.3 exon43			
GAS8	16q24.3 exon6			

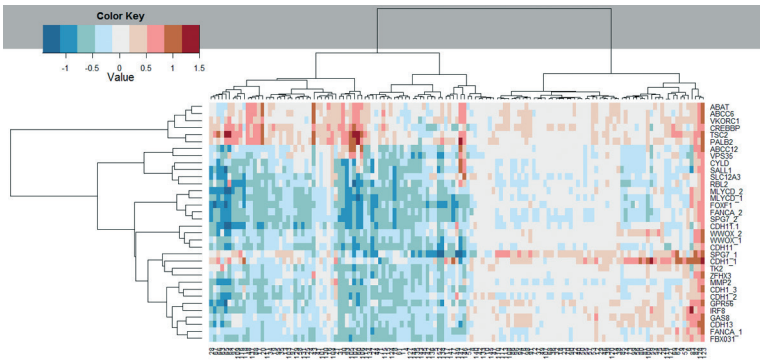

Figure 2 Unsupervised hierarchical clustering of copy number changes in genes on chromosome 16 in 135 male breast cancer patients. The identified clusters of patients (horizontal) and genes (vertical) are depicted in different color.

Intrinsic subtype analysis

The vast majority of cases were classified as Luminal type A (102/135; 76%), whereas 25 cases were of Luminal type B (18%). The remaining cases were basal-like (3/135; 2%) or unclassifiable triple negative (5/135; 4%). There were no HER2 driven cases. Cases with 16q loss (partial and whole arm loss) showed a similar intrinsic subtype distribution (73% Luminal A, 23% Luminal B and 4% unclassified triple negative). Combined loss on 16q (partial and whole arm loss) and gain on 16p occurred only in Luminal type, with an overrepresentation of Luminal A cases (89%). The Luminal type A cases were evenly dispersed over clusters A and B, as were the unclassifiable triple negative cases. All three basal-like cases clustered in cluster B (p=0.040).

Survival analysis

Grade 3 (p = 0.026), high mitotic count (>8; p = 0.028), large tumor size (>2.0 cm; p = 0.031) and Luminal type B (p = 0.042) were correlated with a decreased 5 year survival. In univariate survival analysis alterations on chromosome 16 did not predict survival. The cluster groups formed by unsupervised hierarchical clustering were not associated with survival. In multivariate Cox regression only tumor size and mitotic count emerged as independent prognostic factors.

Discussion

The aim of the present study was to characterize copy number changes on the long arm (and to a lesser extent the short arm) of chromosome 16 in a large group of male breast cancer using a novel multiplex ligation-dependent probe amplification kit. 53% of male breast cancer cases lost at least some part of 16q. This is a much lower percentage than previously described for female breast cancer (70% in luminal A tumors, 75% overall in invasive ductal cancer)[3,4,6], especially when considering the high ratio of Luminal A tumors in male breast cancer compared to female breast cancer. Most cases of male breast cancer showed at least partial loss of 16q. Of the 135 cases, 27 had regions of both loss and gain on 16q. These complex changes have been described to be more frequent in high grade ductal cancer [6], but in the present male breast cancer study these changes were randomly distributed over low and high grade tumors.

Cleton-Jansen et al, previously described loss of heterozygosity (LOH) mapping at 16q in a large cohort of female breast cancer cases [16], and found a similar percentage of 16q loss in male breast cancer compared with our results in male breast cancer ; 53% vs. 52%. However, ER positive tumors were more prevalent in their group with LOH on 16q. This suggests a higher percentage of loss when corrected for ER positivity. We found a clearly lower percentage of whole arm losses in male breast cancer; 4% vs. 28% compared with female breast cancer. These results must however be interpreted with caution as the multiplex ligation-dependent probe amplification technique and the LOH PCR techniques use different markers and probes in different exact locations on 16q for the analysis.

The Cleton-Jansen group defined SRO in order to determine the location of a putative tumor suppressor gene targeted by LOH. Two SRO were defined at region 16q24.3 and one at 16q22.1. When analyzing our data set with an additional threshold defining retention, we found a similar SRO at region 16q24.3 and two smaller SRO based on losses at regions 16q12.1 and 16q22.1. In male breast cancer there seem to be more complex losses with only three cases showing a single area of loss on 16q. Copy number loss of several genes on 16q was significantly correlated with negative lymph node status (*CYLD*, *RBL2*, *MMP2*, *SLC12A3*, *GPR56*, *CDH1*, *MLYCD*, *IRF8*, *SPG7*, *FANCA*), small tumor size (*GPR56*) and low tumor grade (*RBL2*). *CYLD* and *RBL2* are known tumor suppressor genes [17-20](16, 17, 18, 19). The latter is also known to be involved in several malignancies including female breast cancer [19,20]. *CDH1*, also known as E-cadherin, is a cell to cell adhesion glycoprotein. Loss of function is thought to contribute to progression of cancer by increasing proliferation, invasion, and/or metastasis [21]. The *SPG7* gene codes for a mitochondrial metalloprotease and *FANCA* encodes a protein involved in the preservation of genomic integrity through the *FA/BRCA*

pathway [22-24]. Both genes have been mapped to 16q23.3, previously described to be a region of frequent loss of heterozygosity in sporadic breast and prostate cancer [22]. In view of the present results, losses of these genes on 16q seem to play a role in tumorigenesis in the male breast as well.

Copy number gain of almost all genes on the short arm was also significantly correlated with negative lymph node status as was copy number loss of 16q (n=112; p=0.032), irrespective of changes on 16p. This is in line with findings in female breast cancer [25]. When loss of 16q and gain of 16p were both present, the correlation was even stronger (n=112; p=0.012). However, other than in female breast cancer, we found no or few correlations between 16q loss and other favorable clinicopathological features such as low grade, low mitotic count or small tumor size in male breast cancer.

Previous studies in male breast cancer used the comparative genomic hybridization method to analyze genetic alterations [7,8]. Largely in line with these two studies we found similarities but also differences in cytogenetic aberrations between female breast cancer and male breast cancer. In the study conducted by Rudlowski et al. gain on 16p and loss of 16q was reported in a lower percentage of cases compared with our results; 36% and 42% vs. 31% and 53%, respectively [8]. The difference in percentages could be due to the more sensitive technique used in our study. The group of Tommasi et al. used a more sensitive CGH technique (array CGH) and found in concordance with our study a lower frequency of loss on chromosome 16 in male breast cancer compared to female breast cancer [7]. The 16p11.2-p11.1 cytoband location was reported by Tommasi et al. to be frequently gained in male breast cancer. The single gene *VKOR1C*, located on 16p11.2, included in our assays was however found to be gained in a lower percentage (12% vs. 40%). The *PALB2* gene, also analyzed by Tommasi et al., was identified previously as a moderate penetrance breast cancer susceptibility gene, accounting for about 1% of BRCA1/2 negative familial early onset breast cancer [26]. In our male breast cancer group we found no copy number loss on the *PALB2* gene. These different results could be explained by the different techniques used, as we looked only at one specific exon on both genes (*VKORC1* exon 1 of 3 and *PALB2* exon 6 of 13).

In non-hierarchical cluster analysis the two major clusters reflected the correlation between copy number loss on 16q especially when combined with 16p gain and lower frequency of lymph node metastasis. Similar to all of the genes analyzed on 16q, loss of the *MMP2* gene was found in a higher percentage of tumors in Cluster A (28%) compared with tumors in Cluster B (0%). Matrix metalloproteinases (MMPs) promote tumor progression and metastasis in invasive cancers by degradation of the extracellular matrix, and loss of matrix metalloproteinases could in part account for the decreased propensity to develop lymph node metastasis.

Reasoning from the genes, we found a high percentage of copy number gain on *CDH1* exon 1 (coding for its signal peptide) and *SPG7* exon 3. In four patients there was even a combined gain on *CDH1* with consecutive losses on *CDH1* exon 13 and exon 14. These genes lie in regions previously reported as regions of copy number polymorphism and segmental duplications which define hotspots of chromosomal rearrangement. These earlier findings could explain our results [27]. Copy number gain of almost all genes on the short arm also showed a significant correlation with lymph node negative status as was true for several individual genes on 16q.

Loss of 16q was often associated with gain of 16p. According to conventional cytogenetics, in most cases this is due to isochromosome formation and typically requires a loss of the whole arm [28], which was only seen in three (3/35) of our cases. In eleven of these cases there was also partial gain on 16q. These results suggest that the combination of loss of 16q with gain of 16p may not entirely be explained by this mechanism and is a reflection of more complex rearrangements.

In conclusion, copy number loss on 16q and gain of 16p identify a group of male breast cancer with low propensity to develop lymph node metastasis. Although most male breast cancer are of Luminal intrinsic type, losses on 16q occur much less frequently in male breast cancer than in female breast cancer, providing further evidence that male breast cancer and female breast cancer are genetically different. 16q losses do not seem to have prognostic value in male breast cancer.

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Chapter 6

Analysis of copy number changes on chromosome 17 in male breast cancer by multiplex ligation-dependent probe amplification

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Submitted

Abstract

HER2 amplified female breast cancer (FBC) is associated with higher grade, more aggressive phenotype and worse prognosis. In male breast cancer (MBC), amplification of HER2, located on chromosome 17, occurs at much lower frequency than in FBC, where it is part of complex chromosome 17 (chr17) rearrangements. Only few studies have addressed genetic alterations on chromosome 17 in small cohorts of MBC. Multiplex ligation-dependent probe amplification (MLPA) and fluorescence hybridization (FISH) was used to characterize copy number changes on chromosome 17 in a group of 139 MBC. Results were compared to FBC and correlated with clinicopathological features and patients' outcome. In general, there was a lower frequency of copy number changes with a less complex pattern on chr17 in MBC compared to FBC. Chr17 changes in MBC concerned gain of 17q and loss of 17p, but polysomy of whole chr17 did not occur. Two recurrent amplicons were detected: 17q12 (including NEUROD2, HER2, GRB7 and IKZF3) and 17q23.1 (containing MIR21 and RPS6KB1). Whole arm copy number gain of 17q was associated with decreased 5 year survival (p=0.010). Amplification of HER2 was associated with high tumor grade but did not predict survival. Although copy number increases of HER2 and NEUROD2 were associated with high tumor grade, high mitotic count and decreased five year survival (p=0.015), only tumor size and copy number increase of NEUROD2 emerged as independent prognostic factors. In MBC chr17 shows a less complex pattern of rearrangements and fewer copy number changes compared to FBC. There is frequent increase on 17q with two distinct amplicons and loss of 17p, but no polysomy 17. Only NEUROD2 increase seems to have independent prognostic value. These results implicate a different role of chr17 aberrations in male and female breast carcinogenesis.

Introduction

Previous studies using multiplex ligation-dependent probe amplification (MLPA) and comparative genomic hybridization (CGH) in male breast cancer (MBC) showed clear differences in gene copy number changes when compared with female breast cancer (FBC), pointing towards differences in carcinogenesis between MBC and FBC [1,2]. Copy number changes on chromosome 17q have been extensively studied in different cancer types including FBC. This is firstly related to the presence of the oncogene (HER2) on chromosome 17q. Amplification of HER2 is present in about 10-20% of FBC, usually leads to overexpression of the protein and correlates with high grade, high mitotic index, worse prognosis and response to targeted therapy with trastuzumab [3-5]. Further, there are several other important oncogenes on chromosome 17 (chr17) such as TOP2A and PPM1D [6-8]. Lastly, to assess HER2 amplification status by hybridization, correction for polysomy of chr17 is still widely applied, although several studies have shown that polysomy of chr17 is very rare in FBC, and that copy number status of the centromere does not represent the number of chromosome 17. Rather, chr17 shows very complex rearrangements in FBC [9-12].
In MBC, HER2 amplification occurs at much lower frequency (2-8% versus 10-20% in FBC) [1,3,11,13-15]. Only a few (mainly CGH) studies have been published discussing genetic alterations on chr17 in small cohorts of MBC [2,16], but their association with outcome and polysomy 17 has not previously been studied in MBC.
In the present study we therefore aimed to characterize copy number changes on chr17 in a large group of MBC using a dedicated chr17 MLPA kit that was previously used to study polysomy 17 in FBC [11]. In addition HER2 chromogenic hybridization (CISH) was done and was correlated with clinicopathologic features and patients' outcome.

Materials and methods

Patient material

All consecutive cases of surgical breast specimens of invasive MBC from 1986 to 2011 were collected from four different pathology labs in The Netherlands (St. Antonius Hospital Nieuwegein, Diakonessenhuis Utrecht, University Medical Center Utrecht, and Laboratory for Pathology East Netherlands) as described in more detail previously [1,17,18] and from three pathology labs in Germany (Paderborn, Cologne, Kassel). Hematoxylin and eosin (HE) slides were reviewed by four experienced observers (PJvD, RK, AM, ML) to confirm the diagnosis and to type and grade according to current standards. Pathology reports were used to retrieve information on age, tumor size, and lymph node status.

Table 1 Baseline clinicopathological features of 139 male breast cancers

Characteristics	All cases (n=139)	Characteristics	All cases (n=139)
Age, years		Histological grade	
mean	67 (range 32-89)	I	33 (24%)
</= 50	13 (9%)	II	60 (43%)
>50	126 (91%)	III	46 (33%)
Histological type		Lymph node metastasis	*n= 114*
Ductal	124 (90%)	Absent	50 (44%)
Lobular	3 (2%)	Present	64 (56%)
Invasive cribriform	3 (2%)		
Mixed (ductal/lobular)	3 (2%)	Immunohistochemistry	
Mucinous	2 (1%)	ER	
Papillary	2 (1%)	(+)	128 (92%)
Invasive micropapillary	1 (1%)	(-)	11 (8%)
Adenoid cystic	1 (1%)	PR	
		(+)	93 (67%)
Tumor size (mean), cm	2.3 (n=135)	(-)	46 (33%)
T1	70 (50%)	AR	
T2	61 (45%)	(+)	112 (81%)
T3	4 (3%)	(-)	27 (19%)
		HER2 (CISH)	
Mitotic activity index/2 mm^2		(+)	5 (4%)
< 8 mitoses	*54 (39%)*	(-)	134 (96%)
8-14 mitoses	*34 (24%)*		
15 or > mitoses	*51 (37%)*		

A total of 139 cases from which the paraffin blocks contained enough tumor for DNA isolation were included. The clinicopathological features are summarized in Table 1. The average age of MBC patients was 67 years. Tumor size ranged from 0.2 to 7.2 cm. In 114 cases the lymph node status was known by axillary lymph node dissection or sentinel node procedure and 56% of these patients had lymph node metastases. The majority of cases were diagnosed (according to the WHO) as invasive ductal carcinoma. According to the modified Bloom and Richardson score [19] most tumors were grade 2 or grade 3. Mitotic activity was assessed as before [20] with a mean mitotic index of 12 per 2 mm2. For all cases, hormone receptor and HER2 status were re-assessed as described previously [17]. Tissue microarray (TMA) slides were used for immunohistochemical staining of ER and PR. Chromogenic in situ hybridization (SPoT-Light HER2 CISH kit, Invitrogen) was used for HER2 assessment. HER2 gene was considered to be amplified when more than 50% of tumor cells had 5-10 signal dots or small clusters per nucleus (low level amplification) or more than 10 signal dots or large clusters per nucleus were present in more than 50% of the tumor cells (high level amplification). There was HER2 amplification assessed by CISH in only 5 cases. Most tumors were ER positive and PR positivity was also common.

Intrinsic subtypes

Immunohistochemical stainings were used to classify the tumors into the five intrinsic subtypes: luminal type A (ER+ and/or PR+, HER2- and Ki-67 low), Luminal type B (ER+ and/or PR+, and HER2+ and/or Ki67 high), HER2 driven (HER2+ and ER-/PR-), basal-like (ER-/PR-/HER2-, and CK5/6+ and/or CK14+ and/or EGFR+), and unclassifiable triple negative (negative for all six markers) as described before [17].

DNA extraction and MLPA analysis

Representative tumor areas were identified on HE stained slides and dissected with a scalpel from 8 µm paraffin slides [21]. DNA was extracted by overnight incubation in proteinase K (10 mg/ml; Roche, Almere, The Netherlands) at 56°C, boiling for 10 min and centrifugation. Five µl of this DNA solution was used for MLPA analysis. MLPA was performed according the manufacturers' instructions (MRC Holland, Amsterdam, The Netherlands), using a Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA, USA). The novel (P004-C1) kit (MRC Holland), containing five probes for five 17p genes and twenty-six probes for seventeen 17q genes, was used. All tests were performed in duplicate. Negative reference samples (normal breast and blood) were included in each MLPA run as before [1]. The PCR products were separated by electrophoresis on an ABI 3730 capillary sequencer (Applied Biosystems) and final gene copy number ratios were calculated with Genescan v4.1 (Applied Biosystems) and Coffalyser v9.4 (MRC-Holland)

software. For genes with more than one probe present in the kit, the mean of all gene copy number ratios in duplicate was calculated. Cut-off values were set as before with >1.3 until 2.0 for gene copy number gain, >2.0 for amplification and <0.7 for loss. Copy number increase of a gene was defined by values above 1.3 including both gain and amplification. Values between 0.7 and 1.3 were regarded normal [3,22]. Whole arm loss or gain was defined as copy number loss of more than 75% of all the probes as defined before using array-CGH [23]. Partial gain on the long arm of the chromosome was defined as any probe showing copy number increase.

MBC chr17 copy number data was also compared to chr17 copy number data of 111 FBC cases derived from our previous chr17 FBC paper [11] for 11 of the 22 studied genes due to a different MLPA kit design in our previous FBC paper.

Statistics

Statistical calculations were performed using IBM SPSS for Windows version 20.0. Associations between gene copy numbers and clinicopathological characteristics were calculated with Pearson Chi-square (or Fisher's exact test when appropriate) for categorical variables. Grade, tumor size and mitotic count were dichotomized as usual [1,18]. Unsupervised hierarchical clustering using the statistical program R (www.r-project.org) was performed to identify relevant clusters. We used the maximum distance and Ward's clustering method and calculated the stability of the clusters with pvclust as before [1]. Information regarding prognosis and therapy was requested from the Integral Cancer registration The Netherlands (IKNL). Survival data were available for 100 cases with a mean follow up of 5.6 years. Therefore, survival analysis was based on 5 year survival rates. For univariate survival analysis, Kaplan–Meier plots were analyzed with the log rank test. Multivariate survival analysis was done with Cox regression including the variables that were significant in univariate analysis. Correction for multiple comparisons was applied according to Holm-Bonferroni.

Table 2 Copy number changes on chromosome 17 in 139 male breast cancer cases

Gene	Chromo-some	# probes	% increase >1.3		% amplification >2.0		% loss <0.7	
MNT	17p13.3	1	1.4		.		13.7	
TP53	17p13.1	1	7.9		.		13.7	
PMP22	17p12	1	.	*(3.6)*	.	*(.)*	15.1	*(13.5)*
MFAP4	17p11.2	1	5.0		1.4		7.2	
USP22	17p11.2	1	5.8		0.7		6.5	
WSB1	17q11.1	3	7.2	*(24.3)*	0.7	*(5.4)*	.	*(0.9)*
NOS2	17q11.1	1	5.8	*(19.8)*	.	*(5.4)*	0.7	*(1.8)*
TRAF4	17q11.2	1	26.6	*(33.3)*	0.7	*(8.1)*	.	*(2.7)*
CPD	17q11.2	1	2.9	*(28.8)*	.	*(6.3)*	3.6	*(0.9)*
NEUROD2	17q12	1	26.6	*(40.5)*	7.2	*(22.5)*	.	*(7.2)*
ERBB2	17q12	4	20.9	*(23.4)*	5.8	*(17.1)*	.	*(3.6)*
GRB7	17q12	2	23.0	.	4.3	.	.	
IKZF3	17q12	1	27.3	.	7.2	.	.	
RARA	17q21.2	1	6.5	*(7.2)*	1.4	*(0.9)*	0.7	*(18.9)*
TOP2A	17q21.2	3	15.1	*(27.0)*	2.2	*(4.5)*	0.7	*(1.8)*
BRCA1	17q21.31	2	7.2	*(8.1)*	.	*(.)*	2.9	*(3.6)*
SGCA	17q21.33	1	19.4	*(16.2)*	2.9	*(4.5)*	.	*(3.6)*
MIR21	17q23.1	1	32.4		5.8		.	
RPS6KB1	17q23.1	1	30.9		2.9		.	
PPM1D	17q23.2	1	16.5		2.9		2.9	
AXIN2	17q24.1	1	15.1		0.7		.	
UNC13D	17q25.1	1	23.0		0.7		.	

(*) percentages loss, gain and amplification in female breast cancer derived from our previous Chr 17 FBC paper [11]

Results

Copy number analysis of 17p and 17q by MLPA

Gene copy number status of the analyzed genes in MBC in comparison to FBC is presented in Table 2 and Figure 1. In general, there was a lower frequency of copy number changes with a less complex pattern on chr17 in MBC compared to FBC. In 51/139 (36.7%) cases there were no copy number alterations in any of the studied genes in MBC. Copy number increase was the most common alteration on 17q present in 56% (78/139) of cases, and copy number loss was most common on 17p (36/139; 26%). Six of the 139 cases (4%) showed whole arm gain of 17q. None of the cases showed gain/polysomy of whole chr17. NEUROD2, IKZF3, HER2, MIR21 were most commonly amplified. Copy number gain was most common for MIR21 and RPS6KB1. Losses were most frequent in the MNT, TP53 and PMP genes, all three located on 17p (Figure 2).

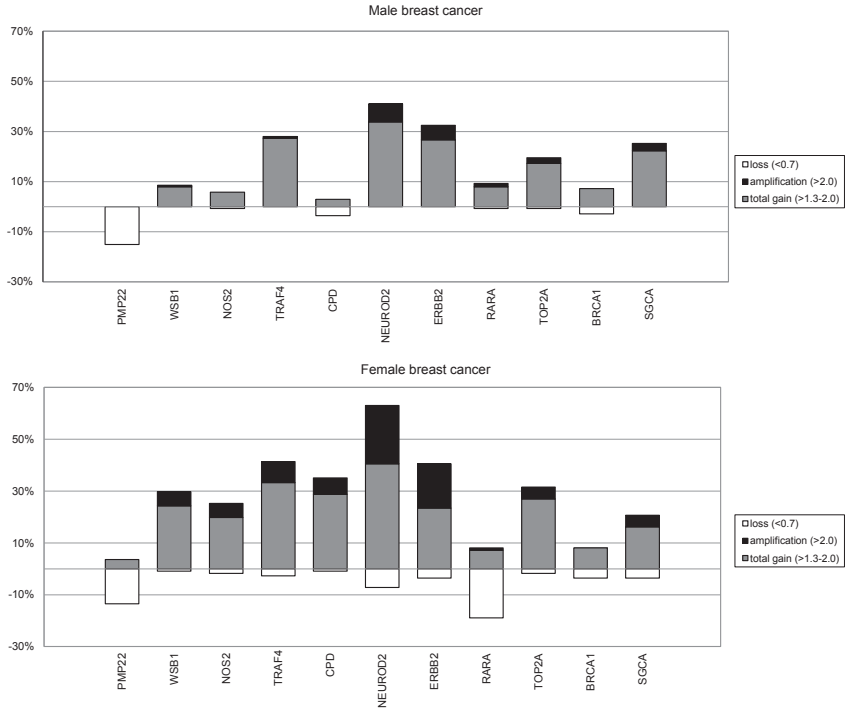

Figure 1 Copy number changes by MLPA of 11 genes on chromosome 17 in 139 male breast cancers compared to 111 female breast cancers (female data derived from our previous Chr 17 FBC paper 11)

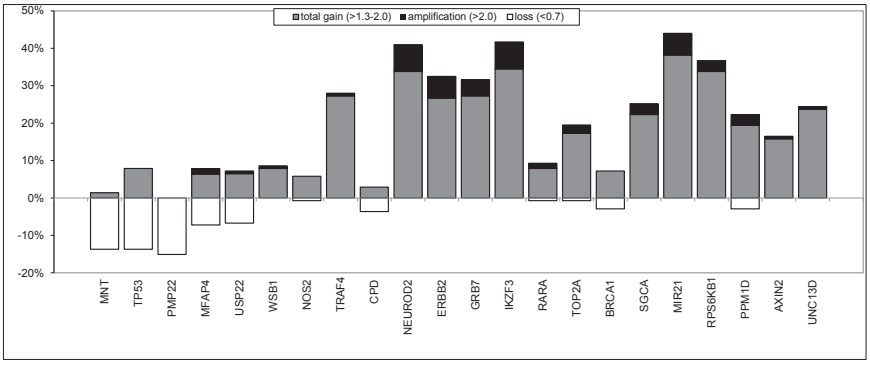

Figure 2 Copy number changes by MLPA in 22 genes on chromosome 17 in 139 male breast cancers

Copy number alterations and clinicopathological features

In 5.8% (8/139) of MBC HER2 was amplified by MLPA. Four of these eight cases had MLPA amplification ratios between 2.0 and 2.5 and four had amplification ratios above 2.5. The cases showing amplification between 2.0 and 2.5 showed no amplification by CISH. Four of the five cases with HER2 amplification by CISH showed amplification by MLPA with ratios above 2.5. One case interpreted as having low level amplification by CISH showed gain but no amplification by MLPA (Table 3).

Three of the eight cases (37.5%) with amplification of HER2 by MLPA also had whole arm gain of 17q including gain of the WSB1 gene located near the centromere. Two of these cases also had partial gain on the short arm combined with copy number loss on the short arm. Amplification of the whole 17q12 region including the NEUROD2, GRB7 and IKZF3 genes was present in 75% (6/8) of cases with HER2 amplification, and two of these cases had additional amplification of the RARA/TOP2A gene region on 17q21.2. The NEUROD2, HER2, GRB7 and IKZF3 genes were also frequently gained, whereas copy number loss of this region was never present. Another region of frequent copy number increase was found on 17q23.1 were the MIR21 and RPS6KB1 genes are located (Figure 2). In summary, two recurrent amplicons were detected: 17q12 (including NEUROD2, HER2, GRB7 and IKZF3), and 17q23.1 (MIR21 and RPS6KB1).

As shown in Table 4, copy number increase of several genes on 17q was correlated with unfavorable clinicopathological features such as high mitotic count and high grade (NEUROD2, HER2, GRB7, IKZF3, RPS6KB1, PPM1D, AXIN2 and UNC13D), high grade and greater size (SGCA) or high grade alone (BRCA1, MIR21).

Table 3 Status of Her2Neu based on immunohistochemistry and CISH in correlation with Her2Neu amplified status by MLPA

Her2Neu immuno-histochemistry	Chromogenic in situ hybridization (C-ISH) Her2Neu	Her2Neu status	Multiplex ligation probe amplification (MLPA)
positive (3+)	high amplification	positive	3.795
positive (3+)	low amplification	positive	4.859
positive (3+)	high amplification	positive	4.292
positive (3+)	high amplification	positive	2.536
negative (2+)	low amplification	positive	*1.448**
negative (0)	no amplification	negative	2.100
negative (0)	no amplification	negative	2.003
negative (1+)	no amplification	negative	2.023
negative (2+)	no amplification	negative	2.269

*only gain by MLPA, no amplification

Table 4 Correlation between gene copy number increase (>1.3 (including amplified cases)) and clinicopathological features in 139 male breast cancers. Blank cells indicate non-significant results

Gene	Location	Mitotic index	Size	Grade	lymph node status
MNT	17p13.3				
TP53	17p13.1				
PMP22	17p12				
MFAP4	17p11.2				
USP22	17p11.2				0.006
WSB1	17q11.1				0.042
NOS2	17q11.1				
TRAF4	17q11.2				
CPD	17q11.2				0.035
NEUROD2	17q12	**<0.0001**		**<0.0001**	
ERBB2	17q12	0.046		0.017	
GRB7	17q12	0.016		0.006	
IKZF3	17q12	**0.001**		0.028	
RARA	17q21.2				0.01
TOP2A	17q21.2				
BRCA1	17q21.31			0.01	0.021
SGCA	17q21.33		0.025	0.021	
MIR21	17q23.1			0.019	
RPS6KB1	17q23.1	**0.002**		**0.001**	
PPM1D	17q23.2	0.047		0.033	
AXIN2	17q24.1	0.008		**<0.0001**	
UNC13D	17q25.1	**<0.0001**		**<0.0001**	

Bold = after correction for multiple comparisons

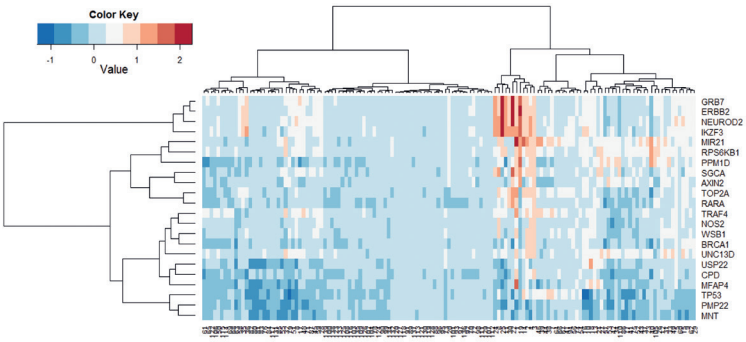

Figure 3 Unsupervised hierarchical clustering of copy number changes in genes on chromosome 17 in 139 male breast cancer patients. The identified clusters of patients (horizontal) and genes (vertical) are depicted in different colors

Amplification of NEUROD2 was correlated with high mitotic count and high grade. Amplification of the HER2 and GRB7 gene was correlated with high grade and amplification of the IKZF3 gene with high mitotic count (Table 5). After correction for multiple comparisons the correlation between copy number increase of NEUROD2 amongst others and high mitotic count and high grade remained significant (Table 4).

Cluster analysis

In unsupervised hierarchical clustering the NEUROD2, HER2, GRB7 and IKZF3 genes clustered together ($p<0.001$) (Figure 2). Reasoning from the cases, an interesting cluster emerged consisting of 12 cases characterized by chr17 whole arm copy number gain and amplification of NEUROD2, HER2, GRB7 and IKZF3 with significantly more Luminal type B cases than Luminal type A cases ($p=0.010$). Distribution of other clinicopathological features (age, grade, mitotic index, size and lymph node status) was not significantly different in this cluster compared with the remaining cases.

Survival analysis

Survival data were available for 100 cases with a mean follow up of 5.6 years. Grade 3 ($p = 0.026$), high mitotic count (>8 mitoses/2 mm2; $p = 0.028$), large tumor size (>2.0 cm; $p = 0.031$), Luminal type B ($p = 0.042$), positive HER2 status by CISH (low and high level amplification) ($p=0.039$), copy number increase of NEUROD2 ($p=0.015$), copy number increase of HER2 ($p=0.015$) and whole arm gain of chr17q ($p=0.010$) were associated with a decreased 5 year survival. The earlier described clusters showed no correlation to survival. In multivariate Cox regression only tumor size and increase of NEUROD2 emerged as independent prognostic factors. (Figure 4)

Table 5 Correlation between gene amplification (>2.0) and clinicopathological features. Blank cells indicate non-significant results

Gene	Location	Mitotic index	Grade
MNT	17p13.3		
TP53	17p13.1		
PMP22	17p12		
MFAP4	17p11.2		
USP22	17p11.2		
WSB1	17q11.1		
NOS2	17q11.1		
TRAF4	17q11.2		
CPD	17q11.2		
NEUROD2	17q12	0.049	0.015
ERBB2	17q12		0.016
GRB7	17q12		**0.001**
IKZF3	17q12	0.007	
RARA	17q21.2		
TOP2A	17q21.2		
BRCA1	17q21.31		
SGCA	17q21.33		
MIR21	17q23.1		
RPS6KB1	17q23.1		
PPM1D	17q23.2		
AXIN2	17q24.1		
UNC13D	17q25.1		

Bold = after correction for multiple comparisons

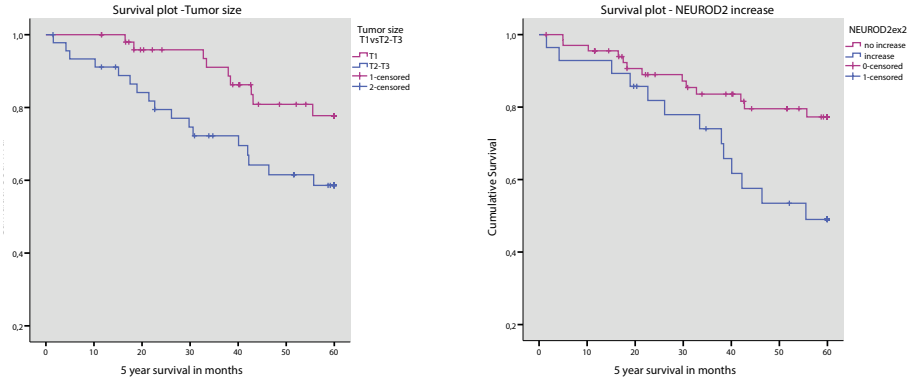

Figure 4 Survival plots of 100 male breast cancers stratified for NEUROD2 copy number status (right) and tumor size (left)

Discussion

The aim of the present study was to characterize copy number changes on chr17 in a large group of MBC using MLPA. The majority of cases showed aberrations on chr17, mainly copy number increase on 17q (78/139; 56%) and copy number loss on 17p (36/139; 26%). Only six of the 139 cases (4%) showed whole arm gain of 17q. None of the cases showed gain/polysomy of whole chr17, which is in line with previous FBC studies [10-12]. Compared to FBC [9] there was however a lower frequency of chr17 copy number changes with a less complex pattern of genomic rearrangements in MBC.

Previous studies in MBC used chromosome CGH to analyze gains and losses [2,16]. Rudlowski et al. reported gains on 17q in 36% of MBC. In their study 17q gain was not associated with any of the clinicopathological features studied [16]. In the present study 58% of MBC showed partial gain on chromosome 17q, and 14% (19/139) showed partial amplification on 17q. In line with our study, Tommasi et al. found both losses and gains on chromosome 17 to be less prominent in MBC than in FBC [2].

Whole arm gain of 17q and gain of the WSB1 gene located near the centromere were frequently seen in association with amplification of the HER2 gene (3/8; 37.5%). However the short arm only showed partial copy number gain and partial loss or no alteration at all in these cases, arguing against true polysomy 17 in MBC, as in FBC [9].

Although in itself rare, the co-amplification and co-clustering of the genes on the neighboring locus containing the NEUROD2, GRB7, IKZF3 genes in 75% (6/8) of cases with HER2 amplification points towards a bigger amplicon on 17q, other than in FBC [9,24,25]. This

amplicon includes both the HER2 and NEUROD2 loci, this is in line with the prognostic value of NEUROD2 and HER2 copy number increase in the present study.

The HER2 gene showed copy number gain in 21% (29/139) but true amplification was only seen in 5.8%, in line with previous HER2 expression studies in MBC [13,15], but lower than Tommasi et al. who found HER2 to be amplified in 30% of 25 MBC cases by CGH [2], probably caused by the different techniques and their smaller sample size. HER2 was amplified in a lower percentage compared to previous MLPA studies in FBC (5.8% vs. 20%) [3]. In concordance with our previous studies in FBC and MBC, HER2 amplification by MLPA correlated strongly with HER2 status by CISH [1,11].

A positive HER2 status defined by CISH (p=0.039) and copy number increase of the HER2 gene by MLPA (p=0.015) were correlated with a decreased 5 year survival. Amplification of HER2 was rare (5.8%) and not by itself a predictor of survival in univariate survival analysis. This could be due to the small number of cases showing amplification of HER2. Copy number increase of NEUROD2 (p=0.015) was also correlated with decreased 5 year survival as was whole arm gain of chromosome 17q (p=0.010). In multivariate Cox regression analysis, copy number increase of NEUROD2 had independent prognostic value next to tumor size. As NEUROD2 itself, a protein coding gene which plays a role in neuronal differentiation and neuronal cell fate, is unlikely to be involved in breast cancer prognosis, other neighbouring genes might rather be drivers of this amplicon.

Some important genes such as MED1, MED24 and DARPP-32 are located in the NEUROD2 locus. MED1 is a subunit of the master transcriptional co-regulator Mediator/TRAP coactivator complex [26] and is a key ERα coactivator [27,28]. The MED1/MED24 unit was previously shown to be often and simultaneously overexpressed in FBC and to play an important role in the growth of breast cancer cells via the ras-mitogen-activated protein (MAP) kinase pathway [29]. Several studies suggest that the MED1 gene is located within the HER2 amplicon [24,25] and that it seems to play a key role in HER2-mediated tamoxifen resistance [30].

The DARPP-32 gene encodes for a Dopamine and cAMP-regulated phosphoprotein, and its overexpression has been implicated in the resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor targeted therapy in cancer [31].

The second amplicon contains RPS6KB1, a protein coding gene reported to be amplified and over expressed in 10-30% of FBC. We found an increase of RPS6KB1 in 30% of MBC cases. The coded protein, a ribosomal protein S6 kinase, is positioned downstream of the PI3K and mTOR pathways and is involved in protein synthesis, cell growth and cell proliferation which makes it a interesting target for therapy considering its position.

The TOP2A oncogene encodes for topoisomerase 2 alpha, a nuclear protein which is important for DNA replication and mitosis. It is the main target of adjuvant anthracycline-

based chemotherapy. TOP2A has been previously reported to be commonly amplified in FBC. The prognostic value of TOP2A gene amplification in FBC is controversial [6]. In our study there was a lower percentage of TOP2A increase in MBC compared to FBC (15% vs. 27%) [11]. Gain or amplification of TOP2A does not seem to be of prognostic value in MBC.
The PPM1D oncogene, also reported previously to be frequently amplified in FBC (25%), was amplified in a much lower percentage (2.9%) and gained in 16.5% of MBC. In our MBC study, an increase of PPM1D showed a trend towards correlation with high grade and high mitotic count, but it did not seem to be a predictor of survival as in FBC [8].
In conclusion, in MBC, chr17 is characterized by copy number increase on 17q with two distinct amplicons and loss of 17p. Like in FBC, there is no polysomy of chr17. MBC shows a similar but less complex pattern of rearrangements and fewer copy number changes than in FBC. Whole arm copy number gain of 17q was associated with HER2 copy number increase. Copy number increase of HER2 and NEUROD2 were associated with high tumor grade, high mitotic count and decreased five year survival. NEUROD2 increase seems to have independent prognostic value but is unlikely to be a driver aberration. These results implicate a different role of chr17 in male and female breast carcinogenesis.

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Chapter 7

Copy number profiling by array comparative genomic hybridization identifies frequent BRCA2-like male breast cancer patients

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Abstract

Genomic aberrations can be used to subtype breast cancer. In this study, we investigated DNA copy number profiles of 69 cases of male breast cancer (MBC) by array comparative genomic hybridization (aCGH) to detect recurrent gains and losses in comparison with female breast cancers (FBC). Further, we classified these profiles as BRCA1-like, BRCA2-2 like or non-BRCA-like profiles using previous classifiers derived from FBC, and correlated these profiles with pathological characteristics.

We observed large copy number gains on chromosome 1q, 5p, 8q, 10p, 16p, 17q, 20 and X. Large losses were on chromosome 1p, 6p, 8p, 9, 11q, 13, 14q, 16q, 17p and 22. The pattern of gains and losses in ER+ MBC was largely similar to ER+ FBC, except for gains on chromosome X in MBC, which are not common in FBC.

Out of 69 MBC patients, 15 patients (22%) had a BRCA2-like profile, of which 2 (3%) were also BRCA1-like. One patient (1%) was only BRCA1-like; the remaining 53 (77%) patients were classified as non-BRCA-like. BRCA2-like cases were more often p53 accumulated than non-BRCA-like cases (p=0.014).

In conclusion, the pattern of gains and losses in ER+ MBC was largely similar to that of its ER+ FBC counterpart, except for gains on chromosome X in MBC, which are not common in FBC. A significant proportion of MBC has a BRCA2-like aCGH profile, pointing to a potentially hereditary nature, and indicating that they could benefit from a drug regimen targeting BRCA defects as in FBC.

Introduction

Male breast cancer (MBC) is a rare disease. Of all breast cancer patients, less than 1% is male.[1–4]

However, the overall incidence of MBC is globally increasing.[4] Compared with female breast cancer (FBC), male patients have a later onset of disease and a more advanced stage of disease at first diagnosis.[2,3] Prognosis of MBC depends on the stage at first diagnosis, and when corrected for stage is similar to FBC .[4]

Often, MBC is assumed to be comparable with FBC in postmenopausal women with a high prevalence of positive estrogen receptor (ER) and progesterone receptor (PR) status, infrequent HER2 overexpression and and low histological grade. Treatment for MBC is thereby still largely extrapolated from its female counterpart, with adjuvant or neo-adjuvant chemotherapy or hormonal (e.g. tamoxifen) therapy being administered based on TNM stage, grade, and ER/PR/HER2 status.[5,21]

Nevertheless, previous studies have shown several differences between MBC and FBC on the phenotypic and genotypic level. The most common histological type of MBC is invasive ductal cancer, which is seen in 90% of the cases. Lobular and medullary cancers are very rare and account for less than 1% of MBC cases, in contrast to female breast cancer.[5] Invasive micropapillary cancers seem to be more prevalent and associated with BRCA2 mutations.[6] Most MBC are of luminal intrinsic type and HER2 driven and basal-like cancers are rare.[7] On the genetic level, MBC studies have mainly investigated amplifications and other copy number variations (CNVs).[8–14] Overall, large genomic aberrations found include gains on chromosome 1q, 8q, 16p, 17q, and 20q. Frequent losses are found on 8p and 13q.

Thera are also differences on the hereditary level of the disease. About 10% if MBC is estimated to have a genetic predisposition[1,15–17] in contrast to about 5% in females. Further, *BRCA2* is the most prevalent germline mutation, in contrast to females where BRCA1 germline mutations are much more prevelant. Male *BRCA2* mutation carriers develop breast cancer in 5-10% of the cases, which is 80-100 times higher than for men without this genetic predisposition.[16–20], but still much lower than in females (about 40%). Male *BRCA1* mutation carriers have only a 1% susceptibility to develop breast cancer in contrast to 60-80% in female carriers.[17] Other low prevalent genes associated with MBC are *PTEN*, *p53*, and *CHEK2*.[1,5] Men with Klinefelter's syndrome have a 20-50 times higher risk for breast cancer than men with a normal 46XY karyotype.[5]

Therefore, when comparing MBC with FBC, there seem to be differences hidden behind similarities. Optimal treatment is best based on the biology of the cancer, so it is important to further molecularly characterize MBC to better define biologically

and clinically relevant subgroups[23–26] to catch up with FBC where many new treatment options and biomarkers are becoming available.

We therefore analyzed a group of MBC by array Comparative Genomic Hybridization (aCGH) in search of gains and losses specific for MBC, while paying special attention to the prevalence of BRCA1-like and BRCA2-like copy number (CN) profiles.[27,28]it is unclear whether current breast cancer diagnostics misses many BRCA1-associated cases. A reliable test that is able to indicate the involvement of BRCA1 deficiency in cancer genesis could support decision making in genetic counselling and clinical management. To find BRCA1-specific markers and explore the effectiveness of the current diagnostic strategy, we designed a classification method, validated it and examined whether we could find BRCA1-like breast tumours in a group of patients initially diagnosed as non-BRCA1/2 mutation carriers. A classifier was built based on array-CGH profiles of 18 BRCA1-related and 32 control breast tumours, and validated on independent sets of 16 BRCA1-related and 16 control breast carcinomas. Subsequently, we applied the classifier to 48 breast tumours of patients from Hereditary Breast and Ovarian Cancer (HBOC These profiles have been associated with sporadic or hereditary defects in respectively *BRCA1* and *BRCA2* and predict good response to high dose platinum-based chemotherapy[29] and probably to poly(ADP)ribose polymerase (PARP) inhibitors, agents that induce DNA double-strand breaks. In view of the assumed high prevalence of *BRCA2* mutations in MBC, a fairly high proportion of MBC could qualify for such treatment.

Material and methods

Patient material

We collected tissue samples from 75 MBC cases (n=24 from the Netherlands Cancer Institute, and n=51 from the University Medical Center Utrecht). The clinical characteristics of the 69 patients left after analysis (see below) are described in table 1. They were diagnosed at a median age of 65 years (range: 36-86). Sixty seven patients underwent surgery, one only biopsy, and one patient did not undergo surgery due to insufficient lung function. Radiotherapy was administered in 30 cases, chemotherapy in 14 cases, and 36 patients received hormonal therapy. In addition, we analyzed a cohort of FBC cases matched for biologically relevant characteristics (ER+/HER2-).

This study was conducted according to Dutch law and guidelines that allow for the analysis of residual tissue specimens obtained for diagnostic purposes and anonymized publication of the results (http://www.federa.org/code-goed-gebruik-van-lichaamsmateriaal-2011).

Pathology

Tumors were marked on a hematoxylin and eosin (HE) stained slides from paraffin blocks and the percentage of invasive tumor was estimated by experienced pathologists (JS, PJvD). DNA was isolated from tumors that contained at least 60% tumor percentage in the paraffin slides [30]. Briefly, slides were deparaffinized and stained with hematoxylin. Decrosslinking with NaSCN was done and the tumor region marked by the pathologists was scraped off. Proteinase K digestion was done for 2 days. DNA was further isolated by the QIAamp DNA mini kit (Qiagen). DNA concentrations were measured by Nanodrop (Thermo Scientific).

Immunohistochemistry (IHC) was performed on a BenchMark Ultra autostainer (Ventana Medical Systems) Briefly, paraffin sections were cut at 3 µm, heated at 75°C for 28 minutes and deparaffinized in the instrument with EZ prep solution (Ventana Medical Systems). Heat-induced antigen retrieval was carried out using Cell Conditioning 1 (CC1, Ventana Medical Systems) for 36 minutes. ER (clone SP1), PR (clone 1E12) and HER2 (clone 4B5) were detected using antibodies from Roche (ready-to-use dispensers, 32 minutes for ER and PR, and 12 minutes for HER2). Ki67 was detected by incubating sections with antibody clone MIB1 (DAKO) diluted 1:125 for 32 minutes followed by amplification (Ventana Medical Systems). p53 (clone DO-7) antibody (DAKO) was used at 1:3000 dilution for 32 minutes. Specific reactions were detected using UltraView Universal DAB Detection Kit (Ventana Medical Systems), and slides were counterstained with hematoxylin.

ER and PR were scored positive when ≥10% of the cells stained positive. HER2 status was scored negative (0 or 1+), borderline (2+), or positive (3+). We only distinguished between HER2 positive and negative for statistical analyses; borderline HER2 samples were checked for amplification using the copy number data on the two *HER2* probes of our 12x135K Nimblegen aCGH (see below), considering the 2+ cases with gene amplification as HER2 positive. For p53, a cutoff of 5% was used and for Ki67 we used a cutoff of 14% [31]. We designated intrinsic subtypes based on IHC as luminal A (ER and/or PR positive, HER2 negative, and low (<14%) Ki67), luminal B (ER and/or PR positive, HER2 positive and/or high (≥14%) Ki67), HER2 driven (ER and PR negative, HER2 positive) or triple negative (ER-, PR-, HER2-) as before [5]. Histological grade was assigned according to the modified Bloom and Richardson score [32,33].

Array CGH

Array CGH was performed as described before [34]. Briefly, we labeled 500 ng of tumor and reference DNA (Promega G1471)with the ENZO labeling kit (ENZO Life Sciences, ENZ-42670). Excess nucleotides were removed using Qiagen MinElute Purification kit (Qiagen, 28004). We pooled tumor and reference DNA and hybridized it on Nimblegen 12x135K

Table 1 Clinical characteristics of male breast cancer patients studied for copy number changes by a CGH

	Total (percentage) (n=69)	BRCA1-like (n=3)	BRCA2-like (n=15)	non-BRCA-like (n=53)
Radiotherapy				
Yes	30 (43%)	1	7	23
No	37 (54%)	2	7	29
Unknown	2 (3%)	0	1	1
Chemotherapy				
Yes	14 (20%)	0	3	11
No	53 (77%)	3	11	41
Unknown	2 (3%)	0	1	1
Endocrine/ Hormonal therapy				
Yes	36 (52%)	3	9	26
No	31 (45%)	0	5	26
Unknown	2 (3%)	0	1	1

Table 2 Pathological characteristics of male breast cancers studied for copy number changes by a CGH. All tests are performed on pathological characteristics with non-BRCA2-like and BRCA2-like status by 2x2 tables and Chi square tests or FE tests if appropriate. P-values with * are derived from 3x2 tables. (For luminal subtyping, we only distinguished between luminal A and luminal B by using 2x2 tables.)

	total (percentage) (n=69)	BRCA1-like (n=3)	BRCA2-like (n=15)	non-BRCA-like (n=53)	P-values
Tumor size (cm)					0.796*
≤2.0	29 (42%)	1	6	22	
>2.0 and ≤5.0	30 (43%)	2	7	23	
>5.0	4 (6%)	0	0	4	
unknown	6 (9%)	0	2	4	
ER					1
pos.	66 (96%)	3	15	50	
neg.	3 (4%)	0	0	3	
unknown	0 (0%)	0	0	0	
PR					0.321
pos.	50 (72%)	1	13	37	
neg.	19 (28%)	2	2	16	
unknown	0 (0%)	0	0	0	
HER2					1
pos.	3 (4%)	0	0	3	
neg.	66 (96%)	3	15	50	
unknown	0 (0%)	0	0	0	
Ki67					1
pos.	16 (23%)	1	3	12	
neg.	51 (74%)	2	12	39	
unknown	2 (3%)	0	0	2	
p53					0.014
pos.	25 (36%)	1	10	15	
neg.	43 (62%)	2	5	37	
unknown	1 (2%)	0	0	1	
Histological grade					0,932*
I	24 (35%)	1	6	18	
II	29 (42%)	0	6	23	
III	16 (23%)	2	3	12	
Luminal subtyping					0,74
Luminal A	47 (68%)	2	12	35	
Luminal B	17 (25%)	1	3	13	
Other/unknown	5 (7%)	0	0	5	

arrays (Roche Nimblegen) according to manufacturers' protocol. Slides were scanned at 2.0 micron double pass in an Agilent High Resolution Microarray Scanner (G2505C, Agilent).The image files were further processed using NimbleScan (v2.5) software (Roche Nimblegen). Grids were automatically aligned on the picture, manually checked, and per channel pair files were generated. The NimbleScan DNACopy algorithm was applied at default settings and the unaveraged DNAcopytext files were used for further analyses.

Bioinformatics

SVM (support vector machine) quality control and BRCA1 and BRCA2 shrunken centroid classification was performed as described before. [27,28,34,35]

We defined bad profile quality as having a larger number of segments than the median + 2 times the median absolute deviation value of the cohort. The BRCA1 and BRCA2 classifiers assign a probability score (between 0 corresponding for non-BRCA1-like and 1, corresponding for BRCA1-like) to the CN profiles to belong to the BRCA1- or BRCA2-like group, respectively, versus belonging to the non-BRCA-like group [27,28]. The cut-off for BRCA1-like was 0.63 and for BRCA2-like 0.5 [27,29]. Because we found a strong separation on principal component analysis between MBC and FBC based on the X chromosome, and the classifier contains probes for this chromosome, we excluded it for classification. This did not substantially influence FBC classification.

To identify recurrent CN gains and losses in ER+/HER2-MBC (ER negative and HER2 positive groups were too small for subgroup analysis) we used the ADMIRE tool [36]. We dewaved the aCGH profiles smoothing with a bandwidth of 1 using the NoWaves R package, segmented these profiles using the R package cghseg and used these as input for ADMIRE [37,38]. The ADMIRE settings were default, except that we removed systematic bias and prevented optimistic results.

Statistics

Associations between BRCA1- or BRCA2-like status and ER, PR, HER2, p53, and Ki67 status were tested using Chi square testing for contingency tables and Fisher Exact (FE) tests where appropriate. For the stainings and subtyping (i.e. luminal A or B), the samples were divided into positive and negative groups. These statistical analyses were performed using IBM SPSS statistics (version 20).

Results

Genomic aberrations

We obtained proper copy number profiles of 69 samples after excluding 3 samples because of poor DNA labeling and 3 after failed segmentation of the profiles (see supplementary figure 1). An overview of genomic aberrations of all our MBC profiles combined is shown in figure 1. We observed large copy number gains on chromosome 1q, 5p, 8q, 10p,16p,17q, 20 and X. Large losses were seen on chromosome 1p, 6p, 8p, 9, 11q, 13, 14q, 16q, 17p and 22. Supplementary table 1 shows all the called aberrations. When we ran the same analysis on the control FBC we did not observe any striking differences in the recurrent copy number aberrations except for gain of chromosome X which is preferentially lost in FBC (see figure 1 for MBC and supplementary figure 2 for FBC).

BRCA1/2-like profiles

There were 15 (22%) cases that were classified as *BRCA2*-like, of which 2 (3%) were also *BRCA1*-like, and 1 (1%) was *BRCA1*-like only. The remaining 53 (77%) cases were classified as non-*BRCA*-like.

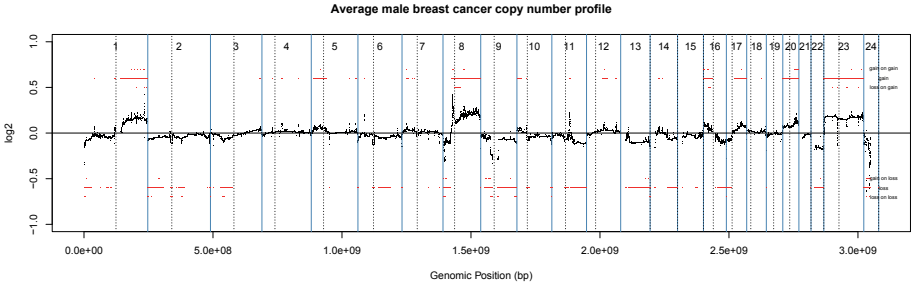

Figure 1 Recurrent copy number by array CGH in male breast cancer.
Significant aberrations (i.e. gains, losses) are presented by the middle line of red dots ('gain' and 'loss', respectively). The four remaining lines represent the significant gains or losses within these 1st level gains or losses.

Correlations between aCGH and pathology

The pathological characteristics of the tumors, in relation to the BRCA-like status, are shown in table 2. Out of 69 samples, 66 (96%) were ER+ and 50 (72%) were PR+. Two samples (3%) were scored as borderline HER2. Both samples did not show amplifications on the two *HER2* probes of the aCGH and were considered HER2 negative. Three samples

(4%) were HER2+. Furthermore, 47(68%) samples were classified as luminal A and 17 (25%) as luminal B. Two samples (3%) could not be divided into luminal A or B subtypes, because of unknown Ki67 status. The three remaining samples (4%) were scored as triple-negative (ER-, PR-, and HER2-). There were no samples classified as HER2-driven breast cancer.

BRCA2-like MBC were significantly more often p53 positive (p=0.014), but no other significant correlation were found with *BRCA1-2* profiles.

Discussion

In this retrospective study we analyzed copy number profiles of MBC by array CGH, assessed BRCA1/2-like profiles as previously established for FBC, and correlated these with clinicopathologic characteristics and copy number profiles of ER/HER2 matched FBC. Our cohort, the largest analyzed to date by the highest resolution aCGH so far, seems to be overall comparable to other MBC cohorts regarding ER status (96% positive, literature range 54-100%), PR status (72% positive vs. 50-96%), HER2 status (4% positive vs. 2-56%), Ki67 status (23% high vs. 20-47%, although different cutoffs for high/low Ki67 have been used), p53 positivity (36% vs. 9-58%), intrinsic subtype (luminal A 68% vs. 44-83%, luminal B 25% vs. 10-51%), and histological grade (23% high vs. 3-85%) [9,39-53].

We observed large copy number gains on chromosome 1q, 5p, 8q, 10p, 16p, 17q, 20 and X, and large losses on chromosome 1p, 6p, 8p, 9, 11q, 13, 14q, 16q, 17p and 22. Four earlier reports describe copy number profiles of MBC [13-16]. Tommasi *et al.* used a 44K (100Mb resolution) Agilent aCGH on 25 MBC cases, Johansson *et al.* BAC aCGH (32,000 probes) on 56 cases, and Rudlowski *et al.* and Tirkkonen *et al.* applied metaphase CGH on 39 and 24 MBC cases, respectively [13-15]. We summarized the most frequent aberrations they found in figures 2 and 3. Some differences may be expected due to the lower resolution (e.g. BAC or metaphase) platforms used before [16,34]. Nowadays, oligonucleotide-based arrays are used that have much more resolution. Generally, large aberrations, e.g. +1q, -8p, +8q, -13q, +16p, +17q, and +20q, were observed in all datasets, in line with literature describing similarities between different methods to obtain CN profiles [34,54-57].

We did not find significant differences in aCGH copy number changes between our luminal MBC and control luminal FBC. Gains on 1q, 8q, 16p, 17q, and 20q, and losses on 6q, 8p, 9p, 9q, 11q, 13q, and 16q as seen in the present MBC study were also common in FBC. This is in line with Rudlowski *et al.*, Tirkkonen *et al.*, and Johansson *et al.* who all concluded that copy number aberrations are similar in MBC and FBC [14-16].

However, in the present study, we found frequent gains on the X chromosome in

MBC, which are not frequent in FBC. Rudlowski *et al.* also conclude that chromosomal differences exist between MBC and FBC cases and that +Xp and +Xq are often seen in MBC, while these aberrations are not found in FBC [14]. X chromosomal abnormalities have already been implicated in both MBC and FBC, although the mechanism is unclear [58,59]. Given that MBC and FBC respectively gain and lose the X chromosome, a role for the X chromosome in MBC carcinogenesis can be proposed. Men with Klinefelter's syndrome already have a higher risk of MBC [7].

When applying *BRCA1/2*-like profiles as previously established for FBC on or MBC cases, we found BRCA2-like profiles in 22% of MBC. Largely, this may be due to the fact that these cases are in fact hidden hereditary cases, but some may be due to sporadic BRCA2 mutations or BRCA2 promoter methylation [60]. *BRCA1*-like aCGH profiles were seen in 3% of cases with BRCA2-like profiles, and in 1% of cases exclusively. This is in line with the fact that *BRCA1* germline mutations are quite rare in MBC.

We also compared *BRCA1/2*-like status with pathological characteristics. *BRCA1*-associated FBC show a quite distinct ER-/PR-/HER2- pattern, while they are mostly high grade and p53 mutated. *BRCA2*-associated are usually ER+/PR+/HER2- and more often p53 mutated and high grade than FBC that are not BRCA-like [61]. In MBC, significant associations between *BRCA2* germline mutations and high tumor grade, PR-, and HER2 positivity have been found [39]. Other studies on correlations between *BRCA*-mutation status and the pathological characteristics did not find any (significant) correlations, which might be due to small sample sizes [9,44]. In the present study, *BRCA2*-like patients were more often p53 positive than non-*BRCA2*-like cases. p53 loss has been proposed to be required for tumorigenesis in the context of *BRCA2* loss [62-65]. These MBC cases with *BRCA1/2*-like profiles may well respond to high dose platinum-based chemotherapy because FBC with such profiles do, urging a prospective clinical trial stratifying MBC patients for platinum-based or conventional chemotherapy according to aCGH profile [26].

In conclusion, the pattern of gains and losses in ER+ MBC was largely similar to that of its ER+ FBC counterpart, except for gains on chromosome X in MBC, which are not common in FBC. A significant proportion of MBC has a *BRCA2*-like aCGH profile, which may point to a hidden *BRCA2* associated hereditary nature or sporadic changes in *BRCA2*. *BRCA1*-like aCGH profiles were rare as expected. These *BRCA2*-like (and *BRCA1*-like) MBC cases deserve to be further tested in relation to survival and predictive value for treatment targeting BRCA deficiency, such as platinum compounds and PARP inhibitors. This urges a prospective clinical trial stratifying MBC patients for PARP inhibitors/platinum-based or conventional chemotherapy according to aCGH profile.

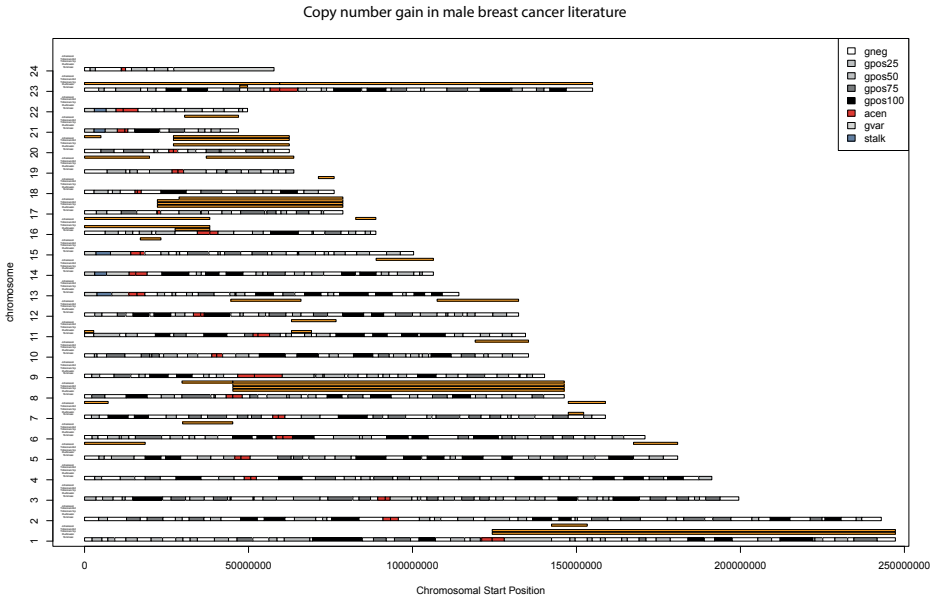

Figure 2 Overview of male breast cancer copy number gain by CGH in literature. Gains (in orange) based on four articles on CN profiles of male breast cancer [13-16]. The most common aberrations are presented.

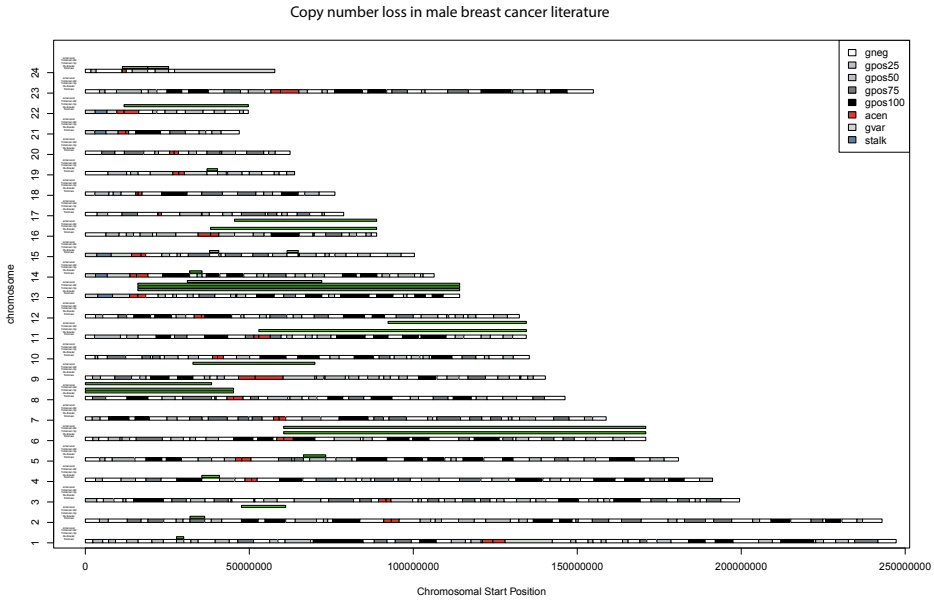

Figure 3 Overview of male breast cancer copy number loss by CGH in the literature. Losses (in green) based on four articles on CN profiles of male breast cancer [13-16]. The most common aberrations are presented.

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Chapter 8

Summary and future perspectives **Samenvatting in het Nederlands**

Summary

Bcl2 and mitotic index

In **chapter 2** we describe our study investigating the prognostic value of Bcl2 in combination with mitotic index in MBC. The vast majority of MBC (94%) showed Bcl2 expression, more frequently than previously described for FBC. Bcl2 expression had no significant associations with clinicopathologic features such as tumor size, mitotic count and grade. In univariate survival analysis, Bcl2 had no prognostic value, and showed no additional prognostic value to tumor size and histological grade in Cox regression. In addition, the Bcl2/mitotic index combination did not predict survival in MBC, as opposed to FBC.

CTGF

In **chapter 3** we set out to investigate the prognostic value of CTGF in MBC, as well as to evaluate clinicopathological correlations, since CTGF was previously described to be an important player in carcinogenesis and a biomarker of aggressive FBC. stromal CTGF expression was seen in a high percentage of MBC and was correlated with high grade and high proliferation index. In view of the important role of the microenvironment in cancer progression, this might suggest that stromal CTGF could be an interesting target for novel therapies and molecular imaging. However, neither stromal nor tumor epithelial cell CTGF expression had prognostic value in MBC and FBC. The lack of association with prognosis warrants caution. In FBC, stromal CTGF expression positively correlated with mitotic count. But other than in MBC there was no correlation with tumor grade. Tumor CTGF expression was correlated with triple negative status of the tumor (p=0.002) in FBC. In MBC this association was not found, possibly due to the low percentage of triple negative tumors in this group. The potential role of CTGF as a therapeutic target for triple negative FBC deserves to be further studied.

Prognostic index

Accurate prognostication is essential for advising on adjuvant systemic treatment and informing patients. Several predictive models are available for FBC including, chronologically, the Morphometric Prognostic Index (MPI), Nottingham Prognostic Index (NPI), Adjuvant! and Predict. In **Chapter 4** we evaluated the prognostic performance of these models in a group of 166 MBC patients. All models were moderately able to discriminate between good and poor survivors and although originally developed and validated for FBC, also perform quite well for MBC. These models may therefore help in MBC prognostication and decisions on adjuvant systemic therapy.

MLPA chromosome 16q

Gene copy number changes have an important role in carcinogenesis and could serve as potential biomarkers for prognosis and targets for therapy. In **chapter 5**, we characterized copy number changes on chromosome 16 in male breast cancers using a novel multiplex ligation-dependent probe amplification (MLPA) kit. The majority (83%) of MBC showed copy number changes on chromosome 16, with frequent loss of 16q. Losses on 16q were however less often seen in MBC than previously described in FBC providing further evidence that MBC and FBC are genetically different. Loss on 16q was significantly correlated with favorable clinicopathologic features such as negative lymph node status, small tumor size, and low grade. We found that a combination of 16q loss and 16p gain correlated even stronger with negative lymph node status, which was also underlined by unsupervised clustering. Our findings suggest that assessing copy number change on chromosome 16 in male breast cancer could help in predicting lymph node status at time of presentation.

MLPA chromosome 17

In **Chapter 6** we characterized copy number changes on chromosome 17 in MBC using a novel MLPA kit. In general, there was a lower frequency of copy number changes with a less complex pattern on chr17 in MBC compared to FBC. Chr17 changes in MBC concerned increase on 17q and loss of 17p, but polysomy of whole chr17 did not occur. Two recurrent amplicons were detected: 17q12 (including NEUROD2, HER2, GRB7 and IKZF3) and 17q23.1 (containing MIR21 and RPS6KB1). Whole arm copy number gain of 17q was associated with decreased 5 year survival (p=0.010). Amplification of HER2 was associated with high tumor grade but did not predict survival. Although copy number increase of HER2 and NEUROD2 were associated with high tumor grade, high mitotic count and decreased five year survival (p=0.015), only copy number increase of NEUROD2 emerged as an independent prognostic factor. These results implicate a different role of chr17 aberrations in male and female breast carcinogenesis.

aCGH

In **Chapter 7** we analyzed a group of MBC by array Comparative Genomic Hybridization (aCGH) in search of gains and losses specific for MBC, paying special attention to the prevalence of BRCA1-like and BRCA2-like copy number profiles. Copy number changes in ER+ MBC and ER+ FBC are generally similar. Gains on 1q, 8q, 16p, 17q, and 20q, and losses on 6q, 8p, 9p, 9q, 11q, 13q, and 16q as seen in the present MBC study were also common in FBC in line with previous studies. However we found frequent gains on the X chromosome in MBC, which are not frequent in FBC. X chromosomal abnormalities

have already been implicated in both MBC and FBC, although the mechanism is unclear. Men with Klinefelter syndrome who have extra copies of the X chromosome have a predisposition for MBC. A role for the X chromosome in MBC carcinogenesis can be proposed. A significant proportion of MBC has a *BRCA2*-like aCGH profile, which may point to a hidden *BRCA2* associated hereditary nature or sporadic changes in *BRCA2*. *BRCA1*-like aCGH profiles were rare as expected. These *BRCA2*-like (and *BRCA1*-like) MBC cases deserve to be further tested in relation to survival and predictive value for treatment targeting BRCA deficiency, such as platinum compounds and PARP inhibitors.

Conclusion and future perspectives

Based on our findings we conclude that although MBC and FBC are similar in many ways, behind these similarities clear differences are hidden. We therefore support the notion that MBC should be considered separately when in search for novel prognosticators and targets for therapy.

In view of the important role of the microenvironment in cancer progression, stromal CTGF is an interesting target for novel therapies and molecular imaging. In the light of our findings in chapter 3 it would be interesting to further explore and unravel the interactions of CTGF with other members of the CCN family and with other pathway effectors. The potential role of CTGF as a therapeutic target for triple negative FBC deserves to be further studied.

In chapter 5 we describe that copy number loss on the long arm of chromosome 16 and gain of the short arm identify a group of male breast cancer with low propensity to develop lymph node metastasis and in chapter 6 copy number gain of NEUROD2 emerged as an independent prognostic factor.

It would be interesting to verify these findings on a larger group of MBC and using more extensive techniques to shed a light on the possible mechanisms behind these observations. Having said that, work continues on the massive parallel sequencing of our MBC cases using a next generation sequencing platform. This will enable us to thoroughly study many known and interesting oncogenes moving forward in unraveling the carcinogenesis in MBC and finding novel possible targets for therapy.

In Chapter 7 we propose a role for chromosome X in male carcinogenesis. Further studies are needed to clarify this role and its possible clinical value in terms of therapy. The predictive and prognostic value of BRCA2-like profile in MBC also deserves further study in light of uncovering better regimens to treat men with breast cancer. This urges a prospective clinical trial stratifying MBC patients for PARP inhibitors/platinum-based or conventional chemotherapy according to aCGH profile.

Nederlandse samenvatting (ook voor niet medici)

Introductie

Borstkanker bij mannen is een zeldzame ziekte. In Nederland komen gemiddeld 91 gevallen per jaar voor met een vijf jaar overlevingskans van 82%. De morbiditeit (mate van ziek zijn) en mortaliteit (sterftecijfer) van de ziekte is significant en het aantal nieuwe gevallen is stijgende. Omdat het een weinig voorkomende ziekte is, is er ook weinig onderzoek naar verricht, vooral in vergelijking met borstkanker bij vrouwen.
De kennis omtrent borstkanker bij mannen is gebaseerd op onderzoek op kleine series. Hierdoor is de optimale klinische behandeling van borstkanker bij mannen mogelijk onbekend gezien het feit dat behandelingsstrategieën overgenomen zijn van onderzoek gedaan op borstkanker bij vrouwen. Een panel van experts heeft er onlangs op aangedrongen dat borstkanker bij mannen beschouwd moet worden als een unieke ziekte en verschillend van borstkanker bij vrouwen.

Bij mannen is de borst een niet functioneel orgaan opgebouwd uit vet, bindweefsel en enkele verspreid gelegen spaarzame klierbuizen. Anders dan bij vrouwen worden er in het borstweefsel bij mannen geen lobjes (klierweefsel) gevormd tenzij er blootstelling is aan endogene of exogene oestrogeenhormonen. In vergelijking met vrouwen zijn mannen bij openbaring van de ziekte vaak ouder, hebben vaker uitzaaiingen naar de klieren en een verder gevorderd stadium van de ziekte. Bij mannen is borstkanker voor het grootste deel, en vaker dan bij vrouwen, hormoongevoelig. Er zijn verder verschillen beschreven tussen borstkanker bij mannen en vrouwen op het niveau van eiwitten en genen.

In deze studie hebben we in samenwerking met verschillende instituten in Nederland en Duitsland een grote groep van borstkankergevallen bij mannen verzameld. Ons doel was om de verschillen tussen borstkanker bij mannen en bij vrouwen beter te identificeren. Daarnaast was het doel om betere voorspellers van prognose en aangrijppunten voor therapie te vinden en op die manier de mechanismen die leiden tot het ontstaan van borstkanker bij mannen beter te begrijpen.

Bevindingen in dit proefschrift
In het eerste gedeelte van dit proefschrift focussen we met name op onderzoek op eiwitniveau. Hiervoor hebben we gebruik gemaakt van tissue micro-array blokjes (TMA's). In deze blokjes worden meerdere tumorsamples ingebed, van elke patiënt 3 kleine tumor

biopten van 0.6mm. Met deze methode kan op een efficiënte manier het expressiepatroon van eiwitten met immunohistochemische kleuringen worden onderzocht.
In hoofdstuk twee onderzoeken we de expressie van het eiwit Bcl2 in combinatie met delingsactiviteit van de tumoren. Deze combinatie bleek in eerder onderzoek zinvol als prognostische marker in borstkanker bij vrouwen. In onze groep toont het overgrote deel van borstkankergevallen expressie van het eiwit Bcl2. Dit is meer frequent dan in borstkanker bij vrouwen. Er worden echter geen significante verbanden gevonden met klinische of pathologische kenmerken. In tegenstelling tot borstkanker bij vrouwen blijkt Bcl2 dan ook niet van waarde te zijn bij het voorspellen van de prognose of overlevingskans bij mannen, ook niet in combinatie met de delingsactiviteit van de tumor.

In hoofdstuk 3 onderzoeken we de prognostische betekenis van de expressie van het eiwit CTGF in onze groep. Tevens evalueren we klinische en pathologische correlaties omdat CTGF eerder is omschreven als een belangrijke speler in het ontstaan van borstkanker bij vrouwen en als marker voor agressiviteit van de ziekte. CTGF expressie in het bindweefsel rond de tumor wordt in een hoog percentage van de borstkankergevallen in onze groep gezien en staat in verband met hogere graad van de tumor en een hogere delingsactiviteit. Het kan zijn dat CTGF een interessante target is voor therapie en moleculaire beeldvorming. CTGF expressie heeft echter geen prognostische waarde in borstkanker bij mannen of vrouwen, wat enige terughoudendheid vraagt. In de vrouwengroep is er een verband gevonden tussen de expressie van CTGF in het omgevend bindweefsel en de delingsactiviteit van de tumor, maar anders dan in de mannen groep niet met de tumorgraad.
In borstkankergevallen bij vrouwen is er ook een verband tussen CTGF expressie in de tumorcellen en de hormoon negatieve status van de tumor. Dit verband wordt niet gevonden in de mannengroep, mogelijk door het lage percentage van hormoonnegatieve tumoren in deze groep. De potentiële rol van CTGF als een therapeutische target voor hormoonnegatieve borstkanker bij vrouwen verdient verder onderzoek.

Het accuraat stellen van een prognose is essentieel voor het geven van advies omtrent de behandeling van en voorlichting aan de patiënt. In hoofdstuk 4 evalueren we het functioneren van meerdere prognostische modellen die voor borstkanker bij vrouwen zijn gemaakt in onze groep. Deze zijn de Morphometric Prognostic Index (MPI), Nottingham Prognostic Index (NPI), Adjuvant! en Predict. Alle modellen waren geschikt om onderscheid te kunnen maken tussen een lange en korte overleving. Deze prognostische modellen, ontwikkeld en gevalideerd voor borstkanker bij vrouwen, werken ook goed in de mannengroep.

In het tweede gedeelte van dit proefschrift ligt de nadruk op het niveau van de genen. Met moleculaire technieken hebben we in verschillende regio's van het DNA gekeken naar genetische veranderingen en hun prognostische waarde in borstkanker bij mannen. Hiervoor is DNA materiaal uit al onze tumor samples geïsoleerd.

In hoofdstuk 5 en 6 kijken we naar veranderingen in het aantal kopieën van de genen. Hiervoor gebruiken we de techniek multiplex ligation-dependant probe amplification (MLPA) waarmee in één keer meerdere samples en meerdere genen gelijktijdig kunnen worden onderzocht.
In hoofdstuk 5 doen we dit in chromosoom 16. In onze groep hebben 83% van de gevallen genkopie veranderingen op chromosoom 16, met frequent verlies van de lange arm van dit chromosoom. Dit is een lager percentage dan beschreven in de vrouwengroep. Er worden verbanden gevonden tussen verlies van genkopieën op de lange arm van chromosoom 16 en positieve klinische en pathologische kenmerken. We hebben gevonden dat een combinatie van verlies van de lange arm en een toename van de korte arm in verband staat met een gunstige klierstatus (geen uitzaaiingen in de klieren). Onze bevindingen suggereren dat het bepalen van genkopie veranderingen op chromosoom 16 in borstkanker bij mannen zou kunnen helpen in het voorspellen van de klierstatus.

In hoofdstuk 6 onderzoeken we chromosoom 17 en vergelijken we onze resultaten met een eerder vergelijkbaar onderzoek in een vrouwengroep. In het algemeen is er een lagere aanwezigheid van genkopie veranderingen met een minder complex patroon op chromosoom 17 bij de mannengroep. Chromosoom 17 veranderingen in de mannengroep bevatten een toename op de lange arm van chromosoom 17 en afname op de korte arm, maar vermenigvuldiging van het complete chromosoom komt niet voor. Twee terugkerende gebieden met een toename van genkopieën zijn gevonden. Complete arm kopie toename van 17q is geassocieerd met een afname van de 5 jaar overlevingskans. Sterke vermenigvuldiging van de HER2 gen op chromosoom 17 wijst op een hoge tumor graad, maar voorspelt niet de overlevingskans. Hoewel een aantal factoren verband houden met hoge graad van de tumor, hoge delingsactiviteit en afgenomen 5 jaar overlevingskans, is slechts een genkopie toename van NEUROD2 gebleken als onafhankelijke prognostische factor. Deze resultaten impliceren een andere rol van chromosoom 17 afwijkingen in borstkanker bij mannen dan bij vrouwen.

In hoofdstuk 7 onderzoeken we een mannen groep met borstkanker met een andere techniek namelijk array Comparative Genomic Hybridization (aCGH) op zoek naar veranderingen in genkopieën die specifiek zijn voor borstkanker bij mannen met

speciale aandacht voor de prevalentie van BRCA1- en BRCA2-gelijkende genkopie profielen. Genkopie veranderingen in oestrogeen receptor positieve tumoren zijn in het algemeen hetzelfde in borstkanker bij mannen als bij vrouwen. Veranderingen op diverse chromosomen komen ook overeen. We vinden echter vaak toename op het X chromosoom bij mannen, wat niet vaak voorkomt bij vrouwen. Veranderingen op het X chromosoom zijn al beschreven in borstkanker bij mannen en vrouwen, maar het mechanisme is onduidelijk. Mannen met het Klinefelter syndroom welke extra kopieën van het X chromosoom hebben lopen een groter risico op het ontwikkelen van borstkanker. Een rol voor het X chromosoom bij het ontstaan van borstkanker bij mannen kan worden gesuggereerd. Een groot deel van de mannengroep heeft een BRCA2 gelijkend genkopie profiel, wat kan wijzen op een verborgen BRCA2 geassocieerde erfelijkheid of sporadische veranderingen in BRCA2. BRCA1 gelijkende genkopie profielen zijn zoals verwacht zeldzaam. Deze BRCA2 (en BRCA1 gelijkende) gevallen dienen verder te worden onderzocht met betrekking tot overleving en voorspellende waarde voor behandeling gericht op BRCA afwijkingen.

Conclusie en toekomstperspectief

Gebaseerd op de bevindingen kunnen we concluderen dat, hoewel er veel overeenkomsten zijn tussen borstkanker bij mannen en vrouwen er ook duidelijke verschillen zijn. We ondersteunen de theorie dat borstkanker bij mannen apart beschouwd moet worden bij het zoeken naar voorspellers van prognose en aangrijppunten voor therapie.

Met betrekking tot de belangrijke rol van de micro-omgeving in de progressie van kanker is CTGF eiwit expressie in het omgevend tumorweefsel een interessante target voor nieuwe behandelingen en moleculaire beeldvorming. In het licht van de bevindingen van hoofdstuk 3 zou het interessant kunnen zijn om interacties tussen CTGF en andere leden van de CCN familie verder te onderzoeken. De potentiële rol van CTGF als aangrijppunt van therapie voor drievoudig negatieve borstkanker bij vrouwen verdient verder te worden onderzocht.

In hoofdstuk 5 omschrijven we dat een combinatie van genkopie verlies van de lange arm en toename van de korte arm van chromosoom 16 een groep van mannen met borstkanker onderscheidt met een lage neiging om uitzaaiingen naar de klieren te ontwikkelen. In hoofdstuk 6 omschrijven we dat toename van NEUROD2 naar voren komt als een onafhankelijke prognostische factor. Dit heeft mogelijk verband met genkopie toename van de hiernaast gelegen genen.
In hoofdstuk 7 vinden we een rol van het X chromosoom in het ontstaan van borstkanker

bij mannen. Er is verdere studie nodig om de rol en de klinische waarde hiervan te verduidelijken. De voorspellende en prognostische waarde van BRCA2 gelijkende profielen in borstkanker bij mannen verdient verder onderzoek in het licht van het opsporen van beter behandelingsstrategieën voor mannen met borstkanker.

Het zou interessant zijn om onze resultaten te controleren op een grotere groep mannen met borstkanker en om meerdere technieken hierop los te laten om een licht te kunnen werpen op de mogelijke mechanismen achter onze observaties. Dit gezegd hebbende, het werk gaat door met het onderzoek naar de genen in onze groep borstkanker bij mannen met de allernieuwste techniek 'next generation sequencing (NGS)' . Dit maakt het mogelijk om diepgravend onderzoek te doen naar vele interessante genen om uiteindelijk een beter begrip van het ontstaan van borstkanker bij mannen te krijgen en nieuwe aangrijppunten voor therapie te ontdekken.

Dankwoord

Curriculum vitae

List of publications

Dankwoord

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Curriculum Vitae

Miangela Marie Laclé is geboren op 4 mei 1980 te Oranjestad Aruba als dochter van Diana Cecilia Catharina Amatha Croes en Francisco Mariano Laclé. Van 1992 tot 1998 volgde ze het voortgezet wetenschappelijk onderwijs op de Colegio Arubano op Aruba met als vakkenpakket; Nederlands, Engels, Spaans, Wiskunde A, Natuurkunde, Scheikunde en Biologie. Na een jaar op het eiland en in Nederland actief te hebben geparticipeerd op de arbeidsmarkt, vertrok ze definitief naar Nederland waar ze in het jaar 1999 begon aan de studie Medisch Beeldvormende en Radiotherapeutische Technieken op de Hanzehogeschool te Groningen. Hier behaalde ze in het jaar 2000 haar propedeuse.

In hetzelfde jaar besloot ze een carrière switch te maken en begon ze met de opleiding tot operatieassistent aan het Opleidingscentrum VU Ziekenhuis en het Medisch Centrum Alkmaar met succesvolle afronding in het jaar 2003. Met deze titel werd ze toegelaten in het zijinstroom traject van de opleiding Geneeskunde aan de Rijksuniversiteit Groningen. Een jaar later in 2004 startte ze in het vierde jaar van de reguliere opleiding Geneeskunde. Haar masterscriptie behandelde het terugkeer vaan patiënten op de arbeidsmarkt na een cardiale interventie. Hierin werd ze begeleidt door Dr. M.J.L. de Jongste, cardioloog. In de maand mei van het jaar 2007 behaalde ze haar doctoraalaantekening en diploma tot Arts aan de Rijksuniversiteit Groningen.

Aansluitend was ze werkzaam als arts-assistent dermatologie in het Medisch Centrum Leeuwarden waar ze eerder haar oudste coassistentschap had gelopen. In deze periode publiceert ze samen met Dr. Blanken en Dr. Vodegel een case report betreffende Juveniele Dermatomyositis in het Nederlands Tijdschrift voor Dermatologie en Venereologie. Vanaf januari 2008 werkte ze als arts-assistent interne geneeskunde in het Albert Schweitzer Ziekenhuis te Zwijndrecht. Na klinische ervaring te hebben opgedaan koos ze voor het vak pathologie waarmee ze eerder tijdens haar keuze coschap dermatopathologie op de afdeling pathologie van de Isala Klinieken te Zwolle nauwer in aanraking was gekomen. In het jaar 2008 begint ze aan de opleiding pathologie in het Universitair Medisch Centrum Utrecht. Voor het perifere deel van haar opleiding was ze werkzaam in het St. Antonius Ziekenhuis te Nieuwegein. Onder leiding van Dr. Matthijs F. van Oosterhout publiceert ze een tweede case report in The American Journal of Thoracic Oncology getiteld An unusual presentation of malignant pleural mesothelioma.

Terug in het Universitair Medisch Centrum Utrecht werd ze enthousiast gemaakt door Prof. Dr. P.J. van Diest voor het onderzoek naar borstkanker bij mannen.

Haar onderzoeksperiode resulteert in dit proefschrift onder begeleiding van Prof. Dr. P.J. van Diest. Ondertussen heeft ze in het kader van haar opleiding tot patholoog het Britse pathologie examen behaald en is ze Fellow of the Royal College of Pathologists. Op 1 december 2013 begint ze haar carriere als Patholoog in het UMC Utrecht met een fellowship op het gebied van de gastro-enterologie bij Prof. Dr. G.J.A. Offerhaus.

List of Publications

1. Prognostic value of mitotic index and Bcl2 expression in male breast cancer.
Miangela M. Lacle, Carmen van der Pol, Arjen Witkamp , Elsken van der Wall, Paul J. van Diest.
PLoS One. 2013;8(4):e60138. doi: 10.1371/journal.pone.0060138. Epub 2013 Apr 1

2. Expression of Connective Tissue Growth Factor in male breast cancer: clinicopathologic correlations and prognostic value.
Miangela M. Lacle, Paul J. van Diest, Roel Goldschmeding, Elsken van der Wall, and Tri Nguyen.
Submitted

3. Prognostic models in male breast cancer.
Carmen van der Pol, **Miangela M. Lacle**, Arjen J. Witkamp, Robert Kornegoor, Miao Hui, Christine Bouchardy, Elsken van der Wall, Helena Verkooijen, Paul J. van Diest.
Manuscript in preparation

4. Analysis of copy number changes on chromosome 16q in male breast cancer by multiplex ligation-dependent probe amplification.
Miangela M. Lacle, Robert Kornegoor, Cathy B. Moelans, Anouk H. Maes-Verschuur, Carmen van der Pol, Arjen J. Witkamp, Elsken van der Wall, Josef Rueschoff, Horst Buerger, Paul J. van Diest.
Mod Pathol. 2013 Nov;26(11):1461-7. doi: 10.1038/modpathol.2013.94. Epub 2013 Jun 7

5. Analysis of copy number changes on chromosome 17 in male breast cancer by multiplex ligation-dependent probe amplification.
Miangela M. Lacle, Cathy B. Moelans, Robert Kornegoor, Carmen van der Pol, Arjen J. Witkamp, Elsken van der Wall, Josef Rueschoff, Horst Buerger, Paul J. van Diest.
Submitted

6. Copy number profiling by array comparative genomic hybridization identifies frequent *BRCA2*-like male breast cancer.
*Hedde D. Biesma, *Philip C. Schouten, **Miangela M. Lacle**, Joyce Sanders, Wim Brugman, Ron Kerkhoven, Ingrid Mandjes, Petra van der Groep, Paul J. van Diest, Sabine C. Linn.
Manuscript in preparation