GENOTYPING AND PHENOTYPING OF MALE BREAST CANCER

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GENOTYPERING EN FENOTYPERING
VAN BORSTKANKER BIJ DE MAN
(met een samenvatting in het Nederlands)

PROEFSCHRIFT

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door

Robert Kornegoor
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Promotor Prof.dr. P.J. van Diest

CONTENTS

1

General introduction

Male breast tissue lies posterior to the nipple on the chest wall and is composed of fat, stroma and sparse rudimentary ducts. There is no lobule formation, unless exposed to high levels of endogenous or exogenous estrogens[1]. The male breast is considered non functional, however it is claimed that males, due to specific hormonal changes, can develop to mature female-like breasts[1] and can even lactate[2,3]. Gynecomastia is the most common disease of the male breast. Male breast cancer, although rare, does occur and accounts for less than 1% of all breast cancers[4]. In general, breast cancer is considered to be a female disease and a diagnosis of male breast cancer is often met with sense of disbelief[5]. These men are not surprised by the diagnosis cancer per se, but many are shocked to have breast cancer.

"Now when I first knew I had got it, I thought to myself …well how the Dickens did I get breast cancer. I'm not a woman. I'm a man."

In The Netherlands there are approximately 100 men diagnosed with male breast cancer each year[6]. Morbidity and mortality of this disease is significant and the incidence of male breast cancer is rising[7,8]. Understanding of male breast cancer tumor biology is essential for guiding therapy, but because large series are lacking, knowledge on male breast cancer is limited and based on small single institutional studies. Therefore, the optimal treatment for male breast cancer is not known and most treatment algorithms are derived from studies done in females. Recently, an expert panel emphasized that male breast cancer should be considered a unique disease, rather than being considered analogous to (postmenopausal) female breast cancer[9].

RISK FACTORS

Due to the rarity of male breast cancer, establishing precise risk factors is challenging. Still, several risk factors have been identified of which genetic and endocrine factors seem to be the most important. Approximately 15-20% of the male breast cancer patients report a family history of breast / ovarian cancer and 10% of the men probably have a genetic predisposition[9]. *BRCA2* mutation carriers have the highest chance of developing male breast cancer with a 6.8% cumulative breast cancer risk at age 70 years[10,11]. In females, *BRCA1* germline mutations are more important, while in men with *BRCA1* germline mutations the cumulative breast cancer risk is relative low (<2%)[10]. Although the life time risk is relative low, men with breast cancer should be considered for genetic counseling and a family history should always be obtained[12]. There is some evidence indicating that men with *CHEK2* and *CYP17* mutations are susceptible for developing male breast cancer, although this has been questioned by

others[13-16]. Men with hormonal imbalances (increased estrogen and decreased testosterone) also seem to have an increased risk for developing breast cancer. Klinefelter's syndrome is characterized by testicular dysgenesis and these patients have a 20-50 times increased risk of developing breast cancer[17-19]. Also other causes of testicular abnormalities, liver cirrhoses, exogenous estrogen and obesity, have been correlated with male breast cancer[20-23]. The role of gynecomastia in male breast cancer is still debated. Gynecomastia occurs physiologically during (transient) periods of increased estrogen levels and/or decreased testosterone levels[24]. Gynecomastia is often seen alongside invasive breast cancer, but in healthy men gynecomastia is also frequently encountered[24-26]. Still, several studies found a significant correlation between gynecomastia and male breast cancer and atypical ductal hyperplasia and ductal carcinoma *in situ*, two known precursor lesions for breast cancer in females, have been reported in male breasts with gynecomastia[19,27-30]. It remains unclear whether gynecomastia and male breast cancer share similar etiological changes or whether (some cases of) gynecomastia should be regarded a precursor lesion for male breast cancer.

CLINICAL FEATURES

Male breast cancer patients differ from females breast cancer patients. Male breast cancer occurs in older patients with a mean age of 67 years[31,32]. The most common symptom is a painless lump. Nipple involvement (retraction, discharge or ulceration) is often seen and is an early event[25,33,34]. Mammography and high resolution ultrasound are accurate in discriminating benign and malignant lesions in the male breast[33,35]. Most male breast cancer cases are discovered in an advanced stage, which is particularly due to early lymph node metastases[31,32,34]. Glandular tissue in the male breast is sparse, which places even small tumors close to the overlying skin and nipple, which is drained by lymphatic channels. It has been speculated that this anatomic situation could explain the presence of early lymph node metastases[34]. However, some genetic events probably also play a role here. Lymph node metastases and tumor size remain, until now, the most important prognostic factors[32,36,37]. The overall survival seems to be worse compared with female breast cancer, but when corrected for stage and age, patients' outcome is similar[32,34,38,39]. Most common therapy strategies in men are extrapolated from the female counterpart. Surgery is the primary approach and (modified) radical mastectomy is most commonly used[37]. Lumpectomy is less often performed, probably due to relatively large tumors and male breast anatomy. Like in female breast cancer, sentinel node procedure is an accurate method in male breast cancer[40,41]. Endocrine therapy is the mainstay of adjuvant treatment of male breast cancer, as the vast majority of these tumors are ER and/or PR positive. Indeed male breast cancer patients do

benefit from adjuvant hormonal therapy[42]. However compliance to tamoxifen is poor and many patients discontinue endocrine treatment due to side effects. Common problems include hair loss, skin rash, impotence, decrease in libido, weight gain, hot flashes, mood changes, depression and insomnia, which occurs in more than half of the patients[43-45]. Aromatase inhibition seems to result in a reduction of the estrogen levels[46]. Although more studies are needed, there seems to be a potential role for aromatase inhibitors in the treatment of male breast cancer[7,47].

PATHOLOGY

The male breast differs histologically from the female breast by the lack of lobules. The male breast is composed of rudimentary ducts ending in terminal buds. The vast majority of male breast cancers are ductal type (>90%)[25]. Protein expressions levels are different between male and female breast cancer. Estrogen receptor (ER) and progesterone receptor (PR) expression is more common in male breast cancer[31,32,48]. This is probably due to differences in hormone levels between men and women, particularly between men and premenopausal women. Like in postmenopausal women, there are low levels of circulating estrogens in men. Most of these estrogens are synthesized in the peripheral tissue, by aromatisation of precursors, and have local effects[49,50]. These locally produced estrogens seem to be very potent in stimulating growth of ER positive cells and are probably important in the development of ER positive breast cancer in men and postmenopausal women[51].

The expression of important oncogenes and tumor suppressor genes, like p53, p21, CyclinD1 and Bcl-2 also seem to be different between male and female breast cancer[48,52-54.] However, the majority of these studies are small (<50 patients) and different methods have been used. Therefore, these data should be interpreted with caution.

Only a few studies have been performed studying the genetic makeup of male breast cancer. Some of these studies have reported that there are important differences between male and female breast cancer. Amplification was more common in female breast cancer and whole chromosome arm gains or polysomy was more often seen in male breast cancer[55]. Also different regions of the genome with different biological processes seem to be involved in male breast cancer[56,57]. On the other hand some studies concluded that similar genetic changes are selected during tumor progression in male and female breast cancer[58,59]. Along with genetic alterations, also epigenetic changes are involved in the development and progression of (breast) cancer. These changes can also be used as a marker for patients' outcome and therapy response[60-64]. The role of epigenetics in the development and progression of male breast cancer is unknown.

In conclusion, knowledge of male breast cancer is sparse. No clinical and/or prognostic relevant biomarkers have been identified and tested in large series. Genetic alterations are also poorly characterized and no studies have been performed on epigenetic changes in male breast cancer. Until now, treatment principles based on studies in females are used in male breast cancer as well, despite the fact that there seem to be important differences.

STUDY DESIGN AND OUTLINE OF THIS THESIS

To gain more insight in male breast cancer we collected our material from six different institutions (University Medical Center Utrecht, St. Antonius hospital Nieuwegein, Diakonessenhuis Utrecht, Pathology Laboratory East Netherlands, Institute of Pathology Paderborn and Centre for Pathology and Cytology Cologne). A total of 134 male breast cancer cases were collected, which represents one of the largest groups published until now. From these patients, clinicopathological data like age, tumor size and lymph node involvement was extracted from corresponding pathology reports. All original hematoxylin and eosin (H&E) slides were reviewed to confirm the diagnosis and to characterize the tumor (mitotic activity, histological grade, presence of a fibrotic focus). From all male breast cancer cases a tissue block was available. Tissue microarray (TMA) blocks were constructed for high throughput. TMA is nowadays accepted as a fast and accurate approach for evaluation of immunohistochemical stainings in large groups[65,66].

Molecular subtypes of breast cancer have been described by gene expression studies, but can also be defined according their immunohistochemical profile. These molecular subtypes have distinctive prognostic and therapeutic implications. **Chapter 2** describes the distribution of molecular subtypes in male breast cancer. These molecular subtypes are correlated with clinicopathological features and the clinical relevance is discussed.

The presence of a fibrotic focus (FF) and overexpression of hypoxia related markers have been correlated with aggressive tumor phenotype and adverse patients' outcome in female breast cancer. **Chapter 3** describes the clinicopathological and prognostic implications of these markers in male breast cancer.

In **Chapter 4** the immunophenotype of male breast cancer is described using 14 widely used immunohistochemical markers. These data give insight in the carcinogenesis of male breast cancer and several clinical and prognostic relevant biomarkers were identified.

Cancer occurs due to accumulation of genetic and epigenetic events. Oncogene amplification was studied by MLPA in **Chapter 5**, and **Chapter 6** describes promoter hypermethylation in male breast cancer using MS-MLPA. With these high throughput techniques a variety of genes could be evaluated in one reaction. These two chapters give more in insight in the (epi)genetic makeup of male breast cancer and these findings were correlated with patients' outcome.

The role of gynecomastia in male breast cancer is still debated. **Chapter 7** describes a morphological and immunohistochemical study of gynecomastia, to further characterize gynecomastia and to study whether or not gynecomastia should be regarded as a precursor lesion for male breast cancer.

Finally, **Chapter 8** provides a summary of the thesis with future perspectives, followed by a Dutch summary in **Chapter 9** and additional information, such as acknowledgements, authors curriculum vitae and a list of publications.

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2

Molecular subtyping of male breast cancer by immunohistochemistry

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ABSTRACT

Introduction

Molecular subtyping of breast cancer by gene expression has proven its significance in females. Immunohistochemical surrogates have been used for this classification, because gene expression profiling is not yet routinely feasible. Male breast cancer is rare and large series are lacking. In this study, we used immunohistochemistry for molecular subtyping of male breast cancer.

Methods

A total of 134 cases of male breast cancer were immunohistochemically stained on tissue microarrays for estrogen receptor (ER), progesterone receptor (PR), *HER2* and epidermal growth factor receptor (EGFR), as well as for CK5/6, CK14 and Ki67. *HER2* was also assessed by chromogen *in situ* hybridization. Cases were classified as luminal A (ER+ and/or PR+ and *HER2*- and Ki67 low), luminal B (ER+ and/or PR+, and *HER2+* or Ki67 high), *HER2* driven (ER-, PR-, *HER2*+), basal-like (ER-, PR-, *HER2*-, CK5/6+ and/or CK14+ and/or EGFR+) or unclassifiable triple-negative (negative for all six markers).

Results

Luminal type A was by far the most encountered type of male breast cancer, representing 75% of the cases. Luminal type B was seen in 21% and the remaining 4% of cases were classified as basal-like (n=4) and unclassifiable triple-negative (n=1). No *HER2* driven cases were identified. Patients with basal-like cancer were significantly younger (p=0.034). Luminal B type cancers showed significantly higher histological grade (p<0.001), mitotic index (p<0.001) and PR negativity (p=0.005) compared with luminal type A cancers.

Conclusion

Most male breast cancers are luminal A and luminal B types, whereas basal-like, unclassifiable triple-negative and *HER2* driven male breast cancers are rare. Luminal type B seem to represent a subtype with an aggressive phenotype. This distribution of molecular subtypes in male breast cancer is clearly different compared with female breast cancers, pointing to possible important differences in carcinogenesis.

INTRODUCTION

Male breast cancer is a relatively uncommon disease accounting for less than 1% of breast cancer incidence[1]. Despite the rarity of this disease, mortality and morbidity are nevertheless significant. Men generally present with higher stage compared with their female counterparts, which is thought to be mainly due to early lymph node metastases formation[2-5]. Overall prognosis has been reported to be poor in male breast cancer, but prognosis of male and female breast cancer seems to be similar when adjusted for stage and age[5,6]. Classification and therapy of male breast cancer has largely been extrapolated from female breast cancer, because large clinical series of male breast cancer are lacking. Several small studies showed, however, differences between female and male breast cancer in hormonal expression[4,7], expression of oncogenes and tumor suppressor genes[7,8] and molecular profile[9,10].

In female breast cancer, gene expression profile studies have identified several distinctive breast cancer "molecular" subtypes[11-13]. As gene expression analysis by microarray is not (yet) routinely feasible, immunohistochemical surrogates have been used for breast cancer classification[14,15]. Using a panel consisting of estrogen receptor (ER), progesterone receptor (PR), *HER2*neu, CK5/6, CK14 and epidermal growth factor receptor (EGFR), female breast cancers could be classified as luminal (A or B), *HER2* driven or basal-like, with prognostic significance[15]. It has been proposed to optimize this algorithm by adding Ki67 for more accurate classification of luminal type B breast cancers[16]. These distinctive breast cancers subtypes could reflect specific genetic alterations in the progression from progenitor cells to tumor cells, which give rise to, eg, a basal expression program (*EGFR* amplification, loss of *BRCA1*) or a luminal program (16q-losses)[17].

Only a few published reports on small series[18,19] have tried to classify male breast cancer using immunohistochemistry with conflicting results. In this study we study the molecular subtypes in a large group of male breast cancer patients by immunohistochemistry in correlation with clinicopathological features.

MATERIALS AND METHODS

All consecutive cases of surgical breast specimens of invasive male breast cancer from 1986-2010 were collected from four different pathology laboratories in The Netherlands (St. Antonius Hospital Nieuwegein, Diakonessenhuis Utrecht, University Medical Center Utrecht, Laboratory for Pathology East Netherlands) and two hospitals in Germany (Paderborn and Cologne). Pathology reports were used to extract age, tumor size and lymph node status, regarding cases with isolated tumor cells as lymph node positive. In total 134 cases were included.

Hematoxylin and eosin (HE) slides were reviewed by three experienced observers (PJvD, RK, AM) to confirm the diagnosis and to characterize the tumor. Histological type (WHO), tubule formation, nuclear grade, mitotic activity index according to the protocol described before[20] and histological grade according to the modified Bloom and Richardson score[21] were recorded.

Immunohistochemical stainings were performed using tissue microarray blocks. HE stained slides were used to identify representative tumor areas. From these areas three 0.6 mm punch biopsies from formalin fixed and paraffin embedded tissue blocks were obtained and embedded in a recipient paraffin block, using a precision tissue array instrument (Beecher Instruments, Sun Prairie, WI, USA). Sections of 4 µm were cut and immunohistochemistry for ER, PR, *HER2*, CK5/6, CK14 and Ki67 was performed using a Bond-Max autostainer (Leica Microsystems, Wetzlar, Germany) with the Bond polymer refine detection kit (Leica Microsystems, DS9800). EGFR staining was done manually (Table 1). Appropriate positive and negative controls were used throughout.

Scoring of the immunohistochemical stainings was done by consensus of two experienced observers (RK, PJvD) who were unaware of other tumor characteristics or staining results. Mean staining percentages for available punches were used. ER and PR stainings were considered positive if 10% or more cells showed nuclear staining. In addition, we also evaluated the 1% threshold as recommended in the latest American Society of Clinical Oncology/College of American Pathologists guidelines[22]. *HER2* staining was interpreted according to the DAKO scoring system. Any cytoplasmic staining for CK5/6 or CK14[17] and any membrane staining for EGFR[23] was scored positive. Ki67 staining was interpreted as low or high using a 14% threshold[16].

For triple-negative (ER-, PR- and *HER2*-) tumors not showing reactivity for any of the basal markers (CK5/6, CK14, EGFR) whole tumor tissue sections were cut and stained for CK5/6, CK14 and EGFR in order not to miss focal staining due to the limited sampling for a tissue microarray.

Table 1 Antibodies used for immunohistochemical characterization of male breast cancer

Antibody	Source	Clone	Dilution	Antigen retrieval
ER	DAKO	1D5	1:200	EDTA
PR	DAKO	PgR636	1:100	Citrate buffer
HER2	Neomarkers	SP3	1:100	EDTA
CK5/6	DAKO	D51/16B4	1:50	Borat buffer pH:8.9
CK14	Neomarkers	LL002	1:400	EDTA
EGFR	Zymed	31G7	1:30	Prot K
Ki67	DAKO	MIB-1	1:100	Citrate buffer

HER2 chromogenic in situ hybridization (CISH) was performed and interpreted using the Spot-light *HER2* CISH kit (Invitrogen) according to the manufacturer's instructions as before [24]. Cases were also considered to be *HER2* positive when they were CISH amplified.

The immunohistochemical stainings were used to classify the breast cancer cases into five different subtypes: luminal type A (ER+ and/or PR+, *HER2*- and Ki67 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).

Statistical calculations were performed using SPSS for Windows version 15.0. Differences between breast cancer subtypes regarding clinicopathological characteristics were calculated with ANOVA for continuous variables and with Pearson χ^2 test (or Fisher's exact test when appropriate) for categorical variables. Significance level was set at $p<0.05$.

RESULTS

Patients' age ranged from 32 to 89 years (average: 66 years). Tumor size ranged from 0.4 to 5.5 centimeters (average: 2.13 centimeters). Lymph node status was known in 83% of cases by axillary lymph node dissection or sentinel node procedure, 54% of these showing lymph node metastases. During the tissue microarray procedure four cases were lost, leaving 130 cases. Table 2 shows the biomarker profile for the 130 cases of male breast cancer.

Molecular sybtyping using the 10% ER/PR threshold

Using the 10% ER/PR threshold, most cases were ER positive (123/130, 95%) and PR positive (88/130, 68%). Only four cases (4/130, 3%) showed *HER2* overexpression/amplification (3 *HER2* 3+ and CISH amplified, 1 *HER2* 2+ and CISH amplified). Expression of the basal markers CK5/6 (12/130, 9%), CK14 (1/130, 1%) and EGFR (15/130, 12%) was also encountered infrequently.

Characteristics according to the immunohistochemically defined molecular subtypes are presented in Table 3, together with the classical pathological features. The vast majority of cases were classified as luminal type A (98/130, 75%), whereas 27/130 (21%) were luminal type B. No *HER2* driven cases were identified. The remaining 4% of cases were basal-like (4/130, 3%) or unclassifiable triple-negative (1/130, 1%).

All 27 luminal type B cases were ER positive and only four cases showed *HER2* amplification, the rest was considered luminal type B because of high Ki67. PR positivity was seen in only 48% luminal B cases, which was significantly less frequent compared with luminal type A tumors ($p=0.005$). Luminal type B breast cancers were furthermore characterized by little

tubule formation (p=0.008), high nuclear grade (p=0.036), high mitotic activity (p<0.001) and consequently high histological grade (p<0.001) compared to luminal type A cancers. There were no differences in age, tumor size and the presence of lymph node metastasis between luminal type A and B cancers. EGFR positivity was seen in 5/27 (19%) of luminal type B cancers, which was higher than in luminal type A tumors (8/98, 8%), but not significantly (p=0.120). In three of the four cases with *HER2* overexpression/amplification, EGFR overexpression was seen as well (p=0.04).

Three basal-like breast cancers showed CK5/6 positivity and one basal-like breast cancer was identified after staining whole tumor tissue sections for EGFR. In one case, an adenoid cystic carcinoma (considered to be low grade basal), simultaneous expression of all basal markers was seen (Figure 1). The remaining three cases were grade 2-3 carcinomas. In one case, lymph node metastases were present. Patients with the basal-like cancer subtype had an average age of 54 years, which was significantly younger than patients with luminal type A breast cancers (p=0.034) who had a mean age of 67 years.

There was only one unclassifiable triple-negative case, which did not show any expression of basal markers. This tumor was a moderately differentiated ductal carcinoma. There were no such cases which fulfilled the criteria of the *HER2* driven subtype. The cases which showed *HER2* overexpression/amplification all showed ER and/or PR positivity.

Table 2 Biomarkers of 130 cases of male breast cancer

Biomarker	Grouping	N	%
Estrogen receptor	-	7	5
	+	123	95
Progesterone receptor	-	42	32
	+	88	68
HER2	Non-amplified	126	97
	Overexpressed/amplified	4	3
CK5/6	-	118	91
	+	12	9
CK14	-	129	99
	+	1	1
EGFR	-	115	88
	+	15	12
Ki67	Low	106	82
	High	24	18

Table 3 Classical pathological features of 130 cases of male breast cancer and their distribution over molecular subtypes

Characteristics	All cases (n=130)	Luminal A (n=98)	Luminal B (n=27)	Basal-like (n=4)	Unclassifiable (n=1)
Age (mean)	66	67	65	54*	59
<50 years	12 (9%)	9 (9%)	2 (7%)	1 (25%)	0
>50 years	118 (91%)	89 (91%)	25 (93%)	3 (75%)	1 (100%)
Histological type					
Ductal	117 (90%)	89 (91%)	24 (89%)	3 (75%)	1 (100%)
Lobular	3 (2%)	2 (2%)	1 (4%)	0	0
Invasive cribriform	2 (2%)	1 (1%)	1 (4%)	0	0
Mixed (ductal/lobular)	2 (2%)	2 (2%)	0	0	0
Mucinous	2 (2%)	2 (2%)	0	0	0
Papillary	2 (2%)	2 (2%)	0	0	0
Invasive micropapillary	1 (1%)	0	1 (4%)	0	0
Adenoid cystic	1 (1%)	0	0	1 (25%)	0
Tumor size (mean)	2.13 cm	2.09 cm	2.24 cm	2.43 cm	2.00 cm
< 2.0 cm	63 (50%)	47 (50%)	14 (52%)	2 (50%)	0
> 2.0 cm	63 (50%)	47 (50%)	13 (48%)	2 (50%)	1 (100%)
Tubule formation					
> 75%	12 (9%)	10 (10%)	2 (7%)	0	0
10 - 75%	54 (42%)	48 (49%)	5 (19%)	1 (25%)	0
< 10%	64 (49%)	40 (41%)	20 (74%)*	3 (75%)	1 (100%)
Nuclear atypia					
Mild	12 (9%)	11 (11%)	1 (4%)	0	0
Moderate	77 (59%)	61 (62%)	12 (44%)	3 (75%)	1 (100%)
Severe	41 (32%)	26 (27%)	14 (52%)*	1 (25%)	0
Mitotic activity index /2 mm^2	11.0	9.1	18.3 *	9.1	1.0
0-12 mitoses	73 (56%)	66 (67%)	4 (15%)	2 (50%)	1 (100%)
>12 mitoses	57 (44%)	32 (33%)	23 (85%)*	2 (50%)	0
Histological grade					
I	31 (24%)	29 (30%)	2 (7%)	0	0
II	52 (40%)	42 (43%)	6 (22%)	3 (75%)	1 (100%)
III	47 (36%)	27 (28%)	19 (70%)*	1 (25%)	0
Lymph node metastasis					
Absent	50 (46%)	39 (46%)	8 (42%)	2 (67%)	1 (100%)
Present	58 (54%)	46 (54%)	11 (58%)	1 (33%)	0

* Significantly different compared with luminal type A breast cancer

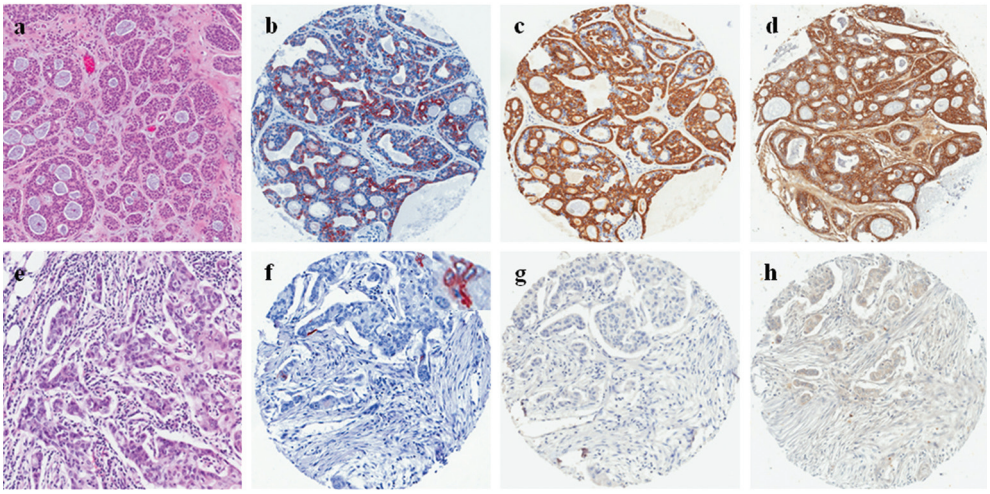

Figure 1 Two cases of basal-like breast cancer. One case, an adenoid cystic carcinoma (a; HE), showed CK5/6 (b), CK14 (c) and EGFR (d) reactivity. The other case, a high grade basal like breast cancer (e; HE) showed single positive tumor cells in the CK5/6 staining (f), but no reactivity in the CK14 (g) or EGFR (h) stainings.

Molecular sybtyping using the 1% ER/PR threshold

Using the 1% ER/PR threshold, a minor shift of male breast cancer cases towards other molecular groups was seen: two basal-like breast cancers and the unclassifiable triple-negative case were between 1-10% ER/PR positive and (being Ki67 low) moved to the luminal type A group, which now comprised 78% of cases (101/130), whereas 27/130 (21%) were luminal type B. No *HER2* driven cases were identified. The remaining 1% of cases were basal-like (2/130) and there were no more unclassifiable triple-negative cases. Statistical analyses revealed similar differences between the groups as was found with a 10% cutoff value for ER and PR. Patients with basal-like breast cancer were significant younger ($p=0.007$) and luminal type B showed a high malignant phenotype with high nuclear ($p=0.038$) and histological grade ($p<0.001$), few tubule formation ($p=0.012$) and high mitotic count ($p<0.001$) compared with luminal type A tumors. However, luminal type B tumors were not more often PR negative in case 1% staining was regarded positive.

DISCUSSION

In female breast cancers, molecular subtyping have extensively been studied and proven its significance[11,12,25]. In male breast cancer, only a few studies have been conducted in this field, which showed conflicting results, because of small groups and different immunohistochemical

definitions[18,19]. The present study, one of the largest series of male breast cancer published until now, demonstrates that luminal A and to a lesser extent luminal B types represent the vast majority of breast cancers in men. *HER2* driven, basal-like and unclassifiable triple-negative breast cancers seem to be very rare in men.

Luminal type A was the dominating subtype of male breast cancer representing 75% of the cases using the 10% ER/PR threshold and even 78% using the 1% ER/PR threshold, and is apparently more often encountered in men compared with female breast cancer[12,16]. In female breast cancer, these tumors are associated with older age and postmenopausal status[15]. Like in postmenopausal women, there are only low levels of circulating estrogen in males. Most of the estrogen is synthesized in the peripheral tissue and has local effects in a paracrine or autocrine fashion, which is important for the development of hormone dependent breast cancers[26,27] and probably explains the high incidence in males. Other reports also demonstrated high rates of ER positive male breast carcinomas[4,5,7,18].

None of the 130 cases were classified as *HER2* driven, as all *HER2* positive cases showed ER positivity and were therefore classified as luminal type B. High rate of EGFR positivity in these tumors has been seen before[18] and is in line with previous gene expression studies in women [12,13]. This profile may contribute to the higher malignant phenotype of these tumors also reflected by their poor differentiation, high mitotic activity and more often PR negativity compared with luminal type A tumors. In females, luminal type B breast cancers are associated with local and regional relapse and bad survival compared with luminal type A tumors[12,16,25,28]; in male breast cancer this has yet to be studied. In the present study, we added Ki67 to the standard biomarker panel for a more accurate classification of luminal type B tumors, as this was shown in previous studies[16] to improve the immunohistochemical surrogate molecular classification (only 30% of luminal B cancers are *HER2* positive). Nevertheless, there is discussion in the literature on the optimal threshold for Ki67, and the 14% threshold that we chose according to Cheang et al.[16] did not have optimal sensitivity and specificity in their study. The Ki67 threshold will therefore likely need to be refined in the future.

The frequency of basal-like breast cancer in female breast cancer is around 16%[15], is associated with high grade tumors[29,30], younger age[15,30], *BRCA1* mutations[31,32] and an overall worse prognosis[14,15]. Our study shows that basal-like breast cancer in men is very rare at 3.0%, in line with previous smaller studies, based on immunohistochemistry[18] and high resolution genomic profiling[9]. One of our cases was a low grade basal-like cancer (an adenoid cystic carcinoma) and three were high grade basal-like with moderate - high nuclear and histological grade[29,30]. The patients with basal-like breast cancers were significantly younger, which is also a characteristic of basal-like breast cancer in females[15,30]. The low incidence of basal-like breast cancer in men could be associated with their relatively high age at time of diagnosis (66 years)

compared to women with breast cancer[4,5] and the low frequency of *BRCA1* mutations in men[33-36]. As stated, young age and *BRCA1* mutations are associated with basal-like breast cancer in females[15,30-32].

Seemingly in contrast with our findings, Ciocca *et al.*, identified four basal-like breast cancers in a small group of male breast cancer (n=28), representing 14% of their studied cases[19]. However, in their study a now outdated definition of basal-like breast cancer was used classifying also ER positive cases with expression of basal markers as basal-like. Only one of their cases with expression of basal markers had no expression of hormone receptors and in fact, according to our definition, basal-like breast cancer was in their study also rare. In our study we defined basal-like breast cancer as triple-negative tumors (ER-, PR- and *HER2*-) with expression of any basal marker (CK5/6, CK14 and/or EGFR), which is currently probably the most pragmatic approach[14,15,37].

Similar to previous studies[14,38-40], we used tissue microarrays for defining immunohistochemical profiles, in which focal or heterogeneous staining can be missed. To minimize this sampling error for the basal markers, we stained additional whole tumor tissue sections for CK5/6, CK14 and EGFR in case a tumor was triple-negative (ER-, PR- and *HER2*-) and did not show any expression of basal markers in the tissue microarray. In these whole tumor tissue sections, one additional case of basal-like breast cancer was identified.

In conclusion, our study, one of the largest series of male breast cancers published until now, demonstrates that luminal type A is by far the most common breast cancer subtype in males. Luminal type B breast cancer is less common and represents a subgroup of ER positive tumors with highly malignant phenotype. *HER2* driven, basal-like and unclassifiable triple-negative breast cancers in men seem to be very rare. The distribution of breast cancer subtypes in men is different compared with females, pointing to possible important differences in carcinogenesis.

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3

Fibrotic focus and hypoxia in male breast cancer

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ABSTRACT

Introduction

Fibrotic focus is a scar-like lesion near the centre of a carcinoma and has been associated with high grade, lymph node metastases and poor survival in female breast cancers. Hypoxia is suggested to be the crucial link between fibrotic focus and aggressive tumor phenotype and is also itself a poor prognostic marker. We here set out to study fibrotic focus and hypoxia in male breast cancer for the first time.

Methods

In a group of 134 male breast cancer patients the presence and size of a fibrotic focus and the expression of three hypoxia related immunohistochemical stainings, hypoxia inducible factor-1α, carbonic anhydrase IX and Glut-1, were studied in correlation with clinicopathological features and prognosis.

Results

Fibrotic focus was seen in 25% of the male breast cancer cases and was correlated with hypoxia inducible factor-1α overexpression (p=0.023), high grade (p=0.005), high mitotic activity (p=0.005) and lymph node metastases (p=0.037). Hypoxia inducible factor-1α positive tumors were more often high grade (p=0.003) and HER2 amplified (p=0.005). Glut-1 expression was also more common in grade 3 tumors (p=0.038), but no association between carbonic anhydrase IX and any clinicopathological feature was found. Fibrotic focus >8mm and hypoxia inducible factor-1α overexpression were correlated with decreased patients' outcome (p=0.035 and p=0.008 respectively). Hypoxia inducible factor-1α overexpression was an independent and the most powerful predictor of survival in multivariate analysis (p=0.029; hazard ratio 2.5).

Conclusion

The presence of a fibrotic focus is associated with hypoxia inducible factor-1α overexpression, and both are associated with aggressive tumor phenotype and poor survival in male breast cancer. These markers seem to have similar clinical importance as previously reported in female breast cancer.

INTRODUCTION

Male breast cancer is uncommon and represents less than 1% of all breast cancers[1]. Large series in male breast cancer are lacking and much of the knowledge is generalized from breast cancer in females. The limited data published on male breast cancer indicates that there are genetic and phenotypic differences between male and female breast cancer[2-6]. Initially, prognosis in men was claimed to be poor with higher incidence of lymph node metastases at time of discovery, but more recent studies showed that prognosis of male and female breast cancers corrected for stage and age is similar[7-9]. Although tumor size and lymph node status are independent prognosticators in male breast cancer[9,10], there still is a need for more accurate outcome predictors of male breast cancer.

A fibrotic focus is a scar like lesion consisting of an area of mainly collagen and fibroblasts, often located near the centre of a carcinoma. Fibrotic focus is associated with poor survival in female breast cancer and with high grade, high mitotic activity, lymph node metastases, and HER2 overexpression[11-14]. In lymph node negative female breast cancer patients fibrotic focus was along with mitotic activity index the most important prognostic factor[15]. It has been claimed that it is an easily assessable histological determinant that should be incorporated in histopathological reports[15,16]. Fibrotic focus has extensively been studied in female breast cancer, but in other malignancies, like carcinomas of the lung, pancreas and colon, fibrotic focus also appeared to be a useful marker with prognostic implications[17-19]. Hypoxia is thought to be the crucial link between fibrotic focus and tumor phenotype and progression. Fibroblasts in fibrotic focus and tumor cells in carcinomas with fibrotic focus express hypoxia inducible factor-1α (HIF-1α) and carbonic anhydrase IX (CAIX), two hypoxia related factors[20,21]. Expression of these markers in female breast cancer tumor cells has also been correlated with high grade and decreased survival[22-26].

The incidence, clinicopathological correlations and prognostic implications of fibrotic focus and hypoxia had not yet been studied in male breast cancer, which was the aim of this study.

MATERIALS AND METHODS

Patient material

In the present study we used a previously described group of male breast cancer[27]. This group is composed of 134 consecutive cases from 1986 - 2010, collected from 4 different pathology labs in The Netherlands (St. Antonius hospital Nieuwegein; n=28, Diakonessenhuis Utrecht; n=22, University Medical Center Utrecht; n=23, and Laboratory for Pathology East Netherlands;

n=40) and 2 hospitals in Germany (Paderborn; n=8 and Cologne; n=13). The age ranged from 32 to 89 years (average: 66 years). Tumor size ranged from 0.4 to 5.5 centimeters (average: 2.1 centimeters). In 4 cases tumor size was not recorded. In 84% lymph node status was known and 54% of these patients had lymph node metastases (including isolated tumor cells; n=4). The majority of cases were (according to the WHO) invasive ductal carcinomas (90%), with some lobular (n=3), mixed type (ductal/lobular; n=2), invasive cribriform (n=2), papillary (n=2), mucinous (n=2), invasive micropapillary (n=1) and adenoid cystic carcinomas (n=1). Most tumors were grade II (40%) and grade III (36%)[28]. Mitotic activity index per 2mm^2 was assessed as before[29]. Estrogen receptor (ER) was positive in 94% (125/133) of the cases, and progesterone receptor (PR) was also common (90/133; 68%). In 1 case hormone receptor status was missing. HER2 overexpression/amplification was rare (4/134; 3%).

Fibrotic focus

Fibrotic focus was defined following criteria first described by Hasebe et al[11]. In short, fibrotic focus is a scar like area with a radially expending fibrosclerotic core, usually in the centre of a carcinoma (Figure 1). It consists of variably dense and sometimes hyalinized collagen bundles and fibroblasts. A diameter of at least 1 mm was required and fibrotic areas smaller than 3 mm did not contain tumor cells. With increasing size, solid nest and strands of tumor cells were more often seen within a fibrotic focus. Sometimes remnants of necrosis were seen,

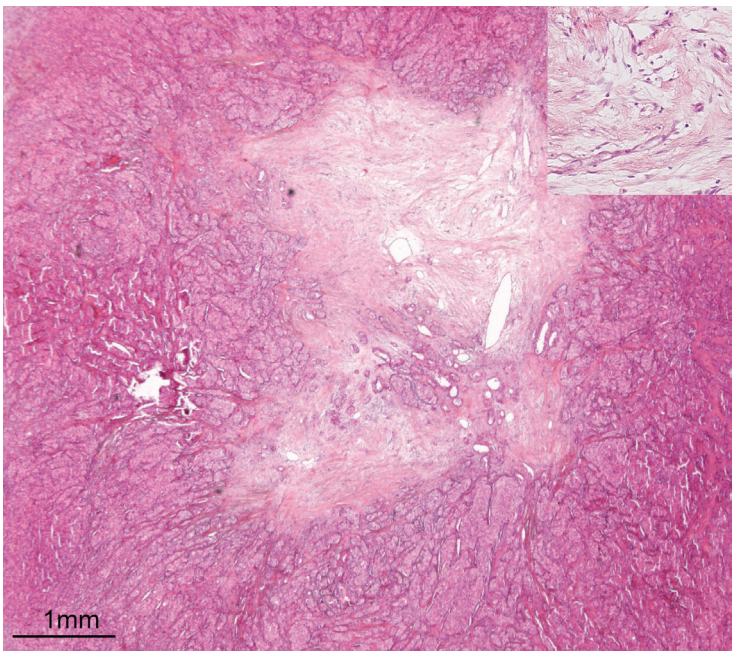

Figure 1 Invasive ductal carcinoma with a fibrotic focus. This example demonstrates the histological appearance of a fibrotic focus with a fibrosclerotic core and disorganized vessels.

but foci of necrosis without a fibrotic core were not classified as fibrotic focus. Fibrotic focus was surrounded by more cellular parts of infiltrating carcinoma. In case secondary changes were seen indicating core needle biopsy artefacts, like the presence of a needle track or fat necrosis, the fibrotic areas were not scored as a fibrotic focus. The presence and size of a fibrotic focus was recorded. The size of a fibrotic focus was dichotomized using a threshold of >8 mm and the fibrotic focus / tumor diameter ratio was also calculated (threshold >1/3), for correlation with patients' outcome[12,15].

Immunohistochemistry

Immunohistochemical stainings were performed using tissue microarray blocks, which were constructed as described before[27]. In short, Hematoxylin and eosin (HE) stained slides were used to identify representative tumor areas. From the areas richest in tumor cells, three 0.6 mm punch biopsies from formalin fixed and paraffin embedded tissue blocks were obtained and embedded in a recipient paraffin block, using a precision tissue array instrument (Beecher Instruments, Sun Prairie, WI, USA). Four µm thick sections were cut and stained for Glut-1, HIF-1α and CAIX at the University Medical Center Utrecht. Glut-1 stainings were performed using a Bond-max automated immunostainer (Leica Microsystems, Wetzlar, Germany) and the stainings for HIF-1α and CAIX were done manually as before (Table 1)[30]. Antigen retrieval for Glut-1 was done with epitope retrieval buffer 1, 20 min at 99°C (AR9961, Leica Microsystems). The slides were incubated with the primary antibody Glut-1 for 15 minutes at room temperature and afterwards with the Bond refine polymer kit (DS9800; Leica Microsystems). For CAIX antigen retrieval was performed in citrate buffer (pH = 6.0, for 20 min at 100°C). After incubation for 60 minutes at room temperature with the primary antibody we used Powervision ready to use (Poly-HRP-anti Ms/Rb/RtIgG biotin free; ImmunoLogic, ImmunoVision technologies, Brisbane CA, USA) for the recognition of the primary antibody. EDTA buffer (pH= 9.0, for 20 min at 100°C) was used for antigen retrieval for HIF-1α. Slides were incubated with the primary antibody HIF-1α overnight at 4°C.

Table 1 Overview of the hypoxia related antibodies and tissue processing details used to characterize male breast cancer.

Antigen	Type	Source	Dilution	AR	Incubation time antibody	Positive control
Glut-1	Polyclonal	DAKO	1:200	Citrate	15 minutes	Placenta
CAIX	Polyclonal	Abcam	1:1000	Citrate	1 hour	Renal cell carcinoma
HIF-1α	Monoclonal	BD Bioscience	1:50	EDTA	Overnight (4°C)	Breast carcinoma

AR: antigen retrieval. DAKO, Glostrup, Denmark ; Abcam, Cambridge Science Park, Cambridge, UK; BD Bioscience, Franklin Lakes, NJ, USA.

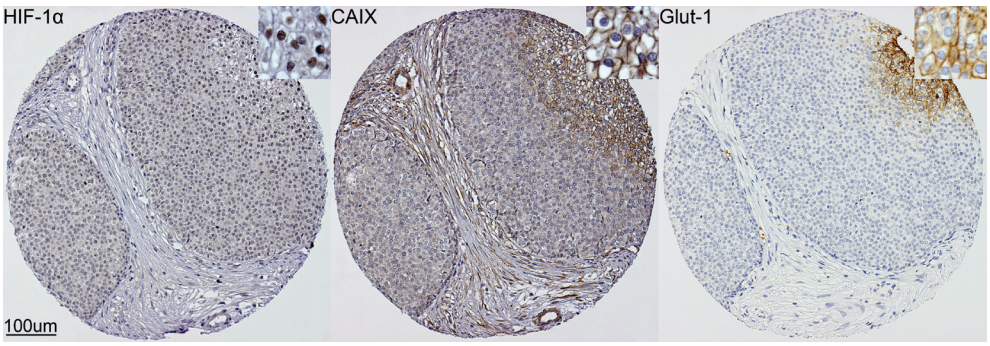

Figure 2 Tissue micro array slide stained for HIF-1α, CAIX and Glut-1, all scored positive in the same case of male breast cancer.

Table 2 Clinicopathological features in 134 cases of male breast cancers with and without fibrotic focus.

Feature	Grouping	All cases (%)	Fibrotic Focus present (%)	Fibrotic Focus absent (%)	P-value
Tumor size	< 2.0 cm	65 (50%)	14 (41%)	51 (53%)	0.231
	> 2.0 cm	65 (50%)	20 (59%)	45 (47%)	
Tubule formation	> 75%	13 (10%)	0 (0%)	13 (13%)	0.030
	10 - 75%	55 (41%)	12 (35%)	43 (43%)	
	< 10%	66 (49%)	22 (65%)	44 (44%)	
Nuclear atypia	Mild	12 (9%)	0 (0%)	12 (12%)	0.038
	Moderate	80 (60%)	19 (56%)	61 (61%)	
	Severe	42 (31%)	15 (44%)	27 (27%)	
Mitoses/2mm²	mean	11	15	10	0.005
	0-8	61 (46%)	12 (35%)	49 (49%)	0.166
	>8	73 (54%)	22 (65%)	51 (51%)	
Grade	I/II	86 (64%)	15 (44%)	71 (71%)	0.005
	III	48 (36%)	19 (56%)	29 (29%)	
Lymph node metastases	absent	51 (46%)	8 (29%)	43 (51%)	0.037
	present	61 (54%)	20 (71%)	41 (49%)	
ER	-	8 (6%)	1 (3%)	7 (7%)	0.679
	+	125 (94%)	33 (97%)	92 (93%)	
PR	-	43 (32%)	13 (38%)	30 (30%)	0.394
	+	90 (68%)	21 (62%)	69 (70%)	
HER2	-	130 (97%)	32 (94%)	98 (98%)	0.266
	+	4 (3%)	2 (6%)	2 (2%)	

For detection of the primary antibody Novolink polymer was used (Novocastra Laboratories Ltd, Newcastle Upon Tyne, United Kindom). All slides were developed with diaminobenzidine. Formalin fixed and paraffin embedded clear cell renal cell carcinoma, placenta and breast carcinoma tissue were used as positive control for CAIX, Glut-1 and HIF-1α, respectively. Appropriate negative control steps were used throughout the procedure. All stainings were scored by two experienced observers (PJvD/RK).

For HIF-1α, mean nuclear staining percentages of the available punches were used, regarding 5% or more tumor cells with nuclear staining as positive[22]. Any clear membrane staining in the Glut-1 and CAIX was scored positive (Figure 2). For all markers two patterns of staining were scored as before[25]: a diffuse pattern with staining throughout tumor cells and the perinecrotic pattern with staining restricted to perinecrotic tumor cells.

Statistics

Prognostic information was requested from the Integral Cancer registration The Netherlands (IKNL). Outcome data were available for 101 cases (101/134; 75%) with a median follow-up of 5.7 years (range 0.1 - 20.3 years). Therefore, survival analysis was based on 5 years survival rates.

Statistical calculations were performed using SPSS for Windows version 15.0. χ^2 Pearson test (or Fisher's exact test when appropriate) was used to evaluate correlation between fibrotic focus and the clinicopathological features age (>50 years), size (>2cm), lymph node status, tubule formation, nuclear grade, mitotic activity (>8 mitoses per 2 mm^2), histological grade (grade 1/2 versus 3) and ER, PR and HER2 status. To compare mean age, size and number of mitoses, ANOVA was performed. Spearman's Rho was used to calculate correlations between the hypoxia related immunohistochemical markers. Expression of these markers were also correlated with clinicopathological features using Pearson χ^2 test and ANOVA. Two sided P values <0.05 were regarded significant. For univariate survival analysis Kaplan-Meier curves were plotted and analysed with the logrank test. Multivariate survival analysis was done with Cox regression including the variables that were significant in univariate survival analysis.

RESULTS

Histopathological features of the 134 cases of male breast cancers with and without fibrotic focus are summarized in Table 2. Fibrotic focus was seen in 25% (34/134) of the cases and was significantly associated with high nuclear (p=0.038) and histological grade (p=0.005), few or no tubule formation (p=0.030) and presence of lymph node metastases (p=0.037). Tumors with fibrotic focus showed significantly higher mean mitotic activity with on average 15

mitoses versus 10 mitoses per 2mm2 in tumors with and without fibrotic focus (p=0.005). However, in case a cut-off value of 8 mitoses was used no significant difference was found (p=0.166) No correlation was found between fibrotic focus and tumor size. Mean age was similar and no significant differences were found in ER, PR and HER2 status between tumors with and without fibrotic focus.

HIF-1α, CAIX and Glut-1 showed overexpression in respectively 27% (34/125), 7% (9/132) and 31% (41/131) of cases (some cases were lost on the TMA slides). A diffuse staining pattern was most commonly seen for these markers. HIF-1α showed diffuse expression in 22% (27/125) of the cases, which represents 79% of all HIF-1α positive tumors. There was a significant correlation between overexpression of HIF-1α and the other two hypoxia related proteins Glut-1 (p<0.001; correlation coefficient: 0.32) and CAIX (p=0.034; correlation coefficient: 0.21). Correlations between expression of the hypoxia related markers and clinicopathological features are presented in Table 3.

Table 3 Clinicopathological features of male breast cancer cases with and without staining of HIF-1α, CAIX and Glut-1.

Feature	Grouping	HIF-1α		CAIX		Glut-1	
		+ (%)	- (%)	+ (%)	- (%)	+ (%)	- (%)
Tumor size	< 2.0 cm	14 (44%)	48 (54%)	6 (75%)	57 (47%)	19 (47%)	44 (51%)
	> 2.0 cm	18 (56%)	41 (46%)	2 (25%)	63 (53%)	21 (53%)	43 (49%)
Mitoses/ 2mm²	mean	13	10	12	11	13	10
	0-8	12 (35%)	46 (51%)	2 (22%)	58 (47%)	12 (29%)	47 (52%)
	>8	22 (65%)	45 (49%)	7 (78%)	65 (53%)	29 (71%)	43 (48%)*
Grade	I/II	15 (44%)	66 (73%)	6 (67%)	79 (64%)	21 (51%)	63 (70%)
	III	19 (56%)	25 (27%)*	3 (33%)	44 (36%)	20 (49%)	27 (30%)*
LN meta	absent	11 (41%)	38 (50%)	3 (43%)	47 (46%)	16 (46%)	34 (46%)
	present	16 (59%)	38 (50%)	4 (57%)	56 (54%)	19 (54%)	40 (54%)
ER	-	0	7 (8%)	0	7 (6%)	0	7 (8%)
	+	34 (100%)	84 (92%)	9 (100%)	116 (94%)	41 (100%)	83 (92%)
PR	-	11 (32%)	29 (32%)	2 (22%)	41 (33%)	12 (29%)	31 (34%)
	+	23 (68%)	62 (68%)	7 (78%)	82 (67%)	29 (71%)	59 (66%)
HER2	-	30 (88%)	91 (100%)	9 (100%)	119 (97%)	39 (95%)	88 (98%)
	+	4 (12%)	0*	0	4 (3%)	2 (5%)	2 (2%)

LN meta: lymph node metastases. * significantly different (p<0.05)

HIF-1α positive tumors showed higher mean mitotic count (13 versus 10 mitoses per 2 mm^2), but this did not reach significance (p=0.086). HIF-1α overexpression was significantly more common in grade 3 tumors (p=0.003). All HER2 amplified tumors (n=4) were also HIF-1α positive (p=0.005). In case only diffuse staining of more than 5% of the tumor cells was regarded positive, there was a significantly increased number of mitoses in HIF-1α positive tumors (p=0.038) and an even stronger correlation between HIF-1α and high histological grade (p<0.001). A diffuse staining pattern of HIF-1α was also associated with the presence of fibrotic focus (p=0.023). When subgroup analysis was performed for tumors with and without lymph node metastases we found that the aggressive phenotype of HIF-1α positive tumors was particularly seen in the group of male breast cancer patients with lymph node metastases, since in the lymph node negative group no correlation with mitotic count and histological grade was seen. Glut-1 overexpression was correlated with high mitotic count (>8; p=0.014) and with high histological grade (p=0.038). CAIX overexpression was rare (n=9) compared to the other two hypoxia related markers and no correlation with any clinicopathological features was seen.

In Table 4 and Figure 3 univariate survival rates are presented according to clinicopathological features, presence of a fibrotic focus and expression of hypoxia related markers. Grade 3 (p=0.027), high mitotic count (>8; p=0.015) and large tumor size (>2.0 cm; p=0.036) were predictors of poor prognosis. The presence of a fibrotic focus or the relative size of a fibrotic focus (fibrotic focus / tumor size) did not influence patients' outcome. However, fibrotic focus >8 mm was correlated with decreased survival (p=0.035). HIF-1α overexpression was also a marker for adverse patients' outcome (p=0.008). When HIF-1α expression was separated into perinecrotic and diffuse staining, perinecrotic staining showed the most unfavorable patients' outcome (survival rate: 40%), compared to a survival rate of 54% and 75% for diffuse HIF-1α staining and HIF-1α negative tumors (p=0.014). However, these results need to be interpreted with caution because there were only five cases with perinecrotic HIF-1α staining with available follow up data and therefore no significant difference was found in case perinecrotic and diffuse HIF-1α staining were compared. The other hypoxia related markers (CAIX and Glut-1) did not correlate with patients' outcome. In the subgroups of lymph node positive and negative patients, no prognostic correlations were found.
In Cox regression, HIF-1α overexpression appeared to be an independent and the most powerful predictor of patients' survival (p=0.029; hazard ratio 2.5). Tumor size was the only other independent prognosticator in this model (p=0.047; hazard ratio 2.4) and fibrotic focus >8 mm was not retained as an independent prognostic factor (Table 5).

Table 4 Univariate (log rank) survival rates of 101 male breast cancer patients according to classic clinicopathological features, fibrotic focus and hypoxia related markers.

Feature	Grouping	5 years survival	
		Survival rate	P-value
Tumor size	< 2.0 cm	79%	0.036
	> 2.0 cm	59%	
Mitoses/2mm²	0-8	81%	0.015
	>8	57%	
Grade	I/II	77%	0.027
	III	53%	
Lymph node metastases	absent	82%	0.132
	present	65%	
Fibrotic Focus	absent	65%	0.581
	present	75%	
Fibrotic Focus / tumor size ratio	≤ 1/3	69%	0.447
	> 1/3	61%	
Size of Fibrotic Focus	≤ 8 mm	70%	0.035
	> 8mm	43%	
HIF-1α	-	75%	0.008
	+	51%	
CAIX	-	67%	0.448
	+	83%	
Glut-1	-	68%	0.797
	+	67%	

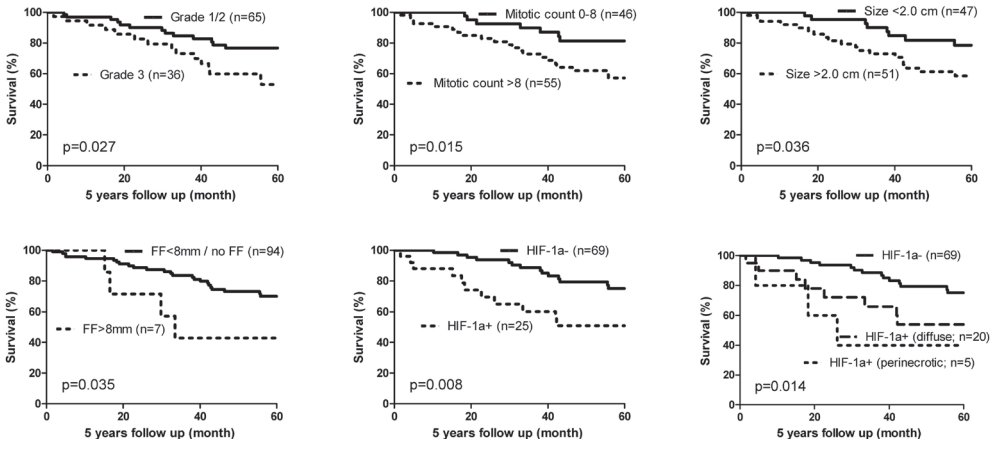

Figure 3 Kaplan Meier survival curves according to histological grade, mitotic count, tumor size, the presence of a fibrotic focus >8mm, HIF-1α overexpression and pattern of HIF-1α overexpression.

Table 5 Multivariate survival analysis (Cox regression).

Feature	Hazard ratio	95% CI	p-value
Tumor size	2.4	1.0-5.9	0.047
Mitoses/2mm²	1.8	0.75-4.6	0.182
Grade	1.2	0.36-4.0	0.780
Size of Fibrotic Focus	1.9	0.59-5.9	0.291
HIF-1α	2.5	1.1-5.6	0.029

95% CI: 95% confidence interval

DISCUSSION

Fibrotic focus is considered an easily assessable histological determinant with prognostic implications in several malignancies. Hypoxia is deemed crucial in the formation of fibrotic focus and is also on its own a marker for aggressive course in cancer. The present study is the first to investigate the clinicopathological relevance of fibrotic focus and hypoxia in male breast cancer. We demonstrate that fibrotic focus is associated with overexpression of HIF-1α and that both are associated with aggressive tumor phenotype. Fibrotic focus >8mm and HIF-1α overexpression were correlated with decreased survival. HIF-1α was even an independent prognosticator.

Our findings are consistent with previous studies in female breast cancer, which demonstrated that in female breast cancer fibrotic focus is also correlated with high histological grade, high mitotic activity, presence of lymph node metastases and adverse patients' outcome[11,12,14]. However, in the present study fibrotic focus >8 mm was not an independent prognostic factor when tumor size and/or histological grade and/or mitotic count were taken into account. Although in female breast cancer fibrotic focus is also more often seen in large and HER2 positive tumors[11,12,14], we did not find such significant correlations in male breast cancer. In HER2 positive cancers, fibrotic focus was seen in 50%, but because HER2 positivity was rare (n=4) no significance was reached. Like in female breast cancer, no correlation was found between fibrotic focus on the one hand and ER status and age on the other[11].
The role of fibrotic focus in tumor phenotype and progression may be explained by the presence of hypoxic fibroblast and tumor cells with expression of HIF-1α and CAIX[20,21]. Low oxygen induces hypoxia modulated gene expression, with transcription of HIF-1α, a key player in the adaptive process of cells that allow them to escape from dying during hypoxia. HIF-1α induces transcription of genes that are involved in angiogenesis, cell survival, proliferation and promotes an aggressive tumor phenotype[31,32]. Several studies demonstrated high

microvessel count and high microvessel density in tumors with fibrotic focus, especially in case a fibrotic focus is relative large and harbors necrosis[13,14]. The fibroblasts occupying a fibrotic focus are reported to be highly proliferative and have the ability to express proteinase, which is crucial for developing metastases[33-36].
There was indeed a significant correlation between the presence of fibrotic focus and overexpression of HIF-1α in tumor cells, suggesting that hypoxia is important in fibrotic focus formation in male breast cancer as well, and confirming previous results in female breast cancer[21]. Unlike previous studies, no association was found between CAIX expression and the presence of a fibrotic focus[20,21]. We used TMA slides for high throughput and focal staining may therefore have been missed. However, usual correlations were found for HIF-1α overexpression on TMAs, so differences in antibody and staining procedures may also play a role here.

Expression of HIF-1α in tumor cells have been reported in a variety of malignancies[37]. Our results are in line with studies in female breast cancer, which demonstrated that HIF-1α overexpression was correlated with highly malignant phenotype and bad outcome[22-25]. For the first time, we demonstrated that HIF-1α overexpression is an independent predictor of survival in male breast cancer patients and could be used as an important prognosticator, more powerful in predicting adverse male breast cancer patients' outcome than classical clinicopathological features. The correlation between HER2 overexpression/amplification and HIF-1α overexpression has been described in female breast cancer[22]. HER2 is able to induce HIF-1α synthesis, which depends on activation of PI-3-Kinase/protein kinase B (PI3K-AKT) pathway[38]. There are conflicting results regarding the prognostic relevance of HIF-1α in female breast cancer groups with or without lymph node metastases[22,24]. In our group of male breast cancer aggressive phenotype associated with HIF-1α overexpression was particularly seen in patients with lymph node metastases. However, survival analysis did not reveal prognostic differences for HIF-1α in tumors with lymph node metastases alone. Furthermore in the present study a diffuse staining pattern of HIF-1α was the strongest predictor of high malignant phenotype and the presence of a fibrotic focus. This could point to different pathways of HIF-1α up regulation which might be in part hypoxia independent and reflect oncogenic adaptations of tumor cells. However, perinecrotic staining of HIF-1α seems to be the strongest predictor of adverse patients' outcome, which is in line with findings in female breast cancer[25]. Conclusions concerning HIF-1α staining patterns should be made with caution based on our results, because there were only a few cases (n=7) with perinecrotic staining, which may have been underscored in TMA slides[39].
The frequency of HIF-1α overexpressing tumors in male breast cancer in the present study is hard to compare with previously reported data in female breast cancer, because different

methods and different cut off values were used. In the present study 27% (34/125) of the tumors were HIF-1α positive, which is slightly lower compared to a previous study, reporting 34% (51/150) HIF-1α positive female breast cancers, using a similar cut off value of 5%[22]. Further research is needed to investigate possible differences in expression of hypoxia related immunohistochemical markers between male and female breast cancers.

In conclusion, we demonstrate that fibrotic focus and HIF-1α overexpression in male breast cancer are associated with high grade tumors and poor prognosis and should be regarded as markers for aggressive behavior. These markers seem to have similar clinical importance as previously reported in female breast cancer.

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4

Immunophenotyping of male breast cancer

Histopathology. 2012 in press

ABSTRACT

Introduction

Male breast cancer is a rare disease and knowledge on carcinogenesis is limited. Conflicting results, based on small series, have been reported on clinically relevant biomarkers.

Methods

134 cases of male breast cancer were immunohistochemically stained on tissue microarrays for estrogen receptor, progesterone receptor (PR), androgen receptor, HER2, BRST2, CyclinD1, Bcl-2, p53, p16, p21, Ki67, CK5/6, CK14 and EGFR. Data were correlated with clinicopathological features and patients' outcome.

Results

High mitotic count and high grade were correlated with high Ki67, HER2 amplification/overexpression, p53 accumulation, high p21, low PR and Bcl-2 expression. PR negativity (p=0.009) and p53 accumulation (p=0.042) were correlated with decreased 5-year survival and were independent markers for patients' outcome, in Cox regression. In unsupervised hierarchical clustering four groups were identified correlated with distinctive clinicopathological features. The hormone negative/ER+/high grade cluster was significantly associated with decreased survival (p=0.011) and was an independent prognostic factor, in Cox regression.

Conclusion

Several tissue biomarkers are associated with aggressive phenotype in male breast cancer. PR and p53 are the most promising individual prognostic markers. Based on immunophenotype, four distinctive and prognostic relevant male breast cancer groups were identified, indicating that protein expression profiling may be clinically useful in male breast cancer.

INTRODUCTION

Male breast cancer is a rare disease and is often discovered in advanced stages with lymph node metastases [1-4]. The overall survival is poor compared to females, but adjusted for age and stage prognosis seems to be fairly similar[4,5]. Tumor size and lymph node status are the most important prognostic factors in male breast cancer[4,6].

In contrast to female breast cancers, little is known about carcinogenesis of male breast cancers, because large series are lacking. Using immunohistochemistry several proteins have been identified, which are involved in the development and progression of female breast cancer. These proteins have prognostic and therapeutic implications as well, and some of them are used in daily practice[7-10].

The immunophenotype of male breast cancer has been only partly elucidated. Compared to female breast cancer, previous studies reported a higher rate of estrogen receptor (ER), and progesterone receptor (PR) positive tumors, wheras HER2 amplification/overexpression was rare[3,4,11]. In a previous study we demonstrated that the distribution of molecular subtypes in male breast cancer is different (predominantly luminal and only rarely HER2 driven, basal-like and unclassifiable triple-negative tumors) from that in female breast cancer, pointing to differences in carcinogenesis[12]. The expression of other important oncogenes and tumor suppressor genes, like p53, p21, CyclinD1 and Bcl-2 also seems to be different compared to women, but these results need to be interpreted with caution due to small series and conflicting results, and the clinical relevance of these biomarkers in male breast cancer still needs to be determined[11,13,14]. These biomarkers can also be used for unsupervised hierarchical clustering, which seem to be a potent tool for subdividing breast cancer patients in novel clinically relevant groups[15,16]. This approach has not previously been used in male breast cancer patients.

In this study we therefore investigated the immunophenotype of male breast cancer and applied unsupervised hierarchical cluster analysis. Data were correlated with clinicopathological features and prognosis.

MATERIALS AND METHODS

The study population comprised 134 cases of invasive male breast cancer collected from 4 different pathology labs in The Netherlands (St. Antonius Hospital Nieuwegein, Diakonessenhuis Utrecht, University Medical Center Utrecht and Laboratory for Pathology East Netherlands) and 2 hospitals in Germany (Paderborn and Cologne), as described previously[12]. Age ranged from 32 to 89 years (average: 66 years). Tumor size ranged from

0.4 to 5.5 centimeters (average: 2.1 centimeters). In 84% lymph node status was known and of these cases 54% of the patients had lymph node metastases. The vast majority of cases were invasive ductal carcinomas (90%), and there were some lobular (n=3), mixed (ductal/lobular) (n=2), invasive cribriform (n=2), papillary (n=2), mucinous (n=2), invasive micropapillary (n=1) and adenoid cystic carcinomas (n=1). According to the modified Bloom and Richardson score[17] most tumors were grade II (40%) or grade III (36%). Mitotic activity was assessed as before[18].

Immunohistochemical stainings were performed on four µm sections from tissue microarray blocks, which were constructed as described before[12]. For ER, PR, HER2, AR, BRST2, CyclinD1, Bcl-2, p53, p16, p21, Ki67, EGFR, CK5/6 and CK14 (Table 1) a Bond-max immunostainer (Leica Microsystems, Wetzlar, Germany) was used with the Bond polymer refine detection kit (Leica Microsystems, DS9800). EGFR staining was done manually as before[19]. Appropriate positive and negative controls were used throughout.

Chromogenic *in situ* hybridization (CISH) for HER2 was performed and interpreted as previously described using the Spot-light HER2 CISH kit (Invitrogen) according to the manufacturer's instructions[20].

Scoring of the immunohistochemical stainings was done by two experienced observers (PJvD/RK) blinded to other features. The percentage of positive cells was estimated for each core separately and mean staining percentages for available punches were used for final analysis. ER and PR were considered positive if 10% or more cells showed nuclear staining. Ki67 staining was interpreted as low or high using a 14% threshold[21], and p53 staining was considered to be positive if 5% or more cells showed accumulation[22]. The remaining nuclear proteins AR, CyclinD1, p16 and p21 were considered positive when at least 10% of nuclei stained, as previously described[13,23,24]. Cases were considered to be HER2 positive when they were CISH amplified or immunohistochemically 3+ according to the DAKO score. Any cytoplasmic staining for CK5/6 or CK14[25] and any membrane staining for EGFR[26] was scored positive. Cytoplasmic staining for Bcl-2 and p16 were scored as 0 (no staining), 1 (mild staining), 2 (moderate staining) and 3 (intense staining) and regarded positive in case moderate (2) or intense (3) staining was seen. Any cytoplasmic staining for BRST2 was considered to be positive.

Molecular subtyping using immunohistochemistry was done as described before[12]. In short, Luminal A cancers were ER+ and/or PR+, HER2-, Ki67 low, Luminal B cancers were ER+ and/or PR+, HER2+ and/or Ki67 high, HER2 driven cancers were ER-/PR-/HER2+, Basal-like cancers were ER-/PR-/HER2- and CK5+ and/or CK14+ and/or EGFR+, and unclassifiable triple negative cancers were negative for all six markers (ER-/PR-/HER2-/CK5-/CK14-/EGFR-). Prognostic information was obtained from the National Cancer Registry of The Netherlands (IKNL).

Table 1 Antibodies used for immunohistochemical characterization of male breast cancer.

Antibody	Source	Clone	Dilution	Antigen retrieval
ER	DAKO	1D5	1:200	EDTA
PR	DAKO	PgR636	1:100	Citrate buffer
HER2	Neomarkers	SP3	1:100	EDTA
Ki67	DAKO	MIB-1	1:100	Citrate buffer
AR	Novocastra	AR27	1:20	Citrate buffer
Bcl-2	DAKO	124	1:200	Citrate buffer
CyclinD1	Neomarkers	SP4	1:40	EDTA
BRST2	Signet	D6	1:400	none
p16	Neomarkers	16P07	1:640	EDTA
p21	DAKO	SX118	1:40	EDTA
p53	Biogenex	BP53-12	1:100	Citrate buffer
CK5/6	DAKO	D51/16B4	1:50	Borat buffer pH:8.9
CK14	Neomarkers	LL002	1:400	EDTA
EGFR	Zymed	31G7	1:30	Prot K

DAKO, Glostrup, Denmark; Neomarkers, Fremont, CA, USA; Novocastra, Newcastle, United Kingdom; Signet laboratories, Dedham, MA, USA; Biogenex, San Ramon, CA, USA; Zymed, Carlsbad, CA, USA.

Overall survival data were available for 101 cases (101/134; 75%) with a median follow-up of 5.7 years (range 0.1 – 20.3 years). Therefore, survival analysis was based on 5 years survival rates.

Statistics

The dichotomized results (positive or negative) of all immunohistochemical markers were analyzed by unsupervised hierarchical clustering with the statistical program R (www.r-project.org). Clinicopathological data were correlated with the identified clusters of male breast cancer and with individual immunohistochemical markers, using ANOVA for continuous variables and the Pearson χ^2 test (or Fisher's exact test when appropriate) for categorical variables. The following clinicopathological features were dichotomized: age (>50 years), tumor size (>2.0cm), mitotic activity (>8 mitoses/2mm^2) and histological grade (grade 1/2 versus 3). The distribution of biomarkers between previously described molecular subtypes was also tested using Pearson χ^2 test. *P*-values of 0.05 were regarded significant. Survival analyses were calculated using Kaplan Meier survival curves and the log-rank test. Multivariate survival analysis was done with Cox regression, taking the clinicopathological features that showed significance in univariate analysis into account. SPSS for Windows version 15.0 was used for statistical calculations.

RESULTS

Table 2 shows the frequency of expression of the various biomarkers in male breast cancer. Most tumors were hormone positive (ER, PR and AR). Expression of Bcl-2 (98/131; 75%) and CyclinD1 (101/131; 77%) was also very common. Approximately half of the tumors were positive for p21 (63/131; 48%) and BRST2 (73/131; 56%). In contrast, HER2 overexpression/amplification (4/134; 3%) and p53 accumulation (20/132; 15%) were rare. Most tumors were also negative for the basal markers CK5/6, CK14 and EGFR. In fact only one case, an adenoid cystic carcinoma, showed CK14 expression.

Immunohistochemistry was also used for molecular subtyping. As described before[12], the vast majority of cases were classified as luminal type A (98/130, 75%), and 27/130 (21%) were luminal type B. No HER2 driven cases were identified. The remaining 4% of cases were basal-like (4/130, 3%) and unclassifiable triple negative (1/130, 1%).

Correlations between biomarkers and clinicopathologic features

Patients with ER negative tumors were younger compared to ER positive tumors (56 versus 67 years; p=0.016). Smaller tumor size was seen in Bcl-2 (2.0 versus 2.5 cm; p= 0.017) and CyclinD1 (2.0 versus 2.6 cm; p=0.008) expressing tumors. High mean mitotic activity was associated with HER2 overexpression/amplification (p<0.001), high Ki67 (p<0.001), and p21 positivity (p<0.001). Significantly lower mean mitotic activity was found in BRST2 positive tumors (p=0.038). When mitotic count was dichotomized (≤8 or >8 mitoses per 2mm2) Bcl-2 (p=0.006) and PR positive (p=0.033) tumors were also associated with low mitotic count. Histological grade 3 tumors showed a significant association with HER2 overexpression/amplification (p=0.015), high Ki67 (p=0.001), p53 accumulation (p=0.049) and p21 positivity (p=0.002). An inverse correlation was seen for PR (p=0.008) and Bcl-2 (p=0.010) positive tumors. p53 (p=0.010) and BRST2 (p=0.001) were associated with axillary lymph

Table 2 Frequency of expression of various biomarkers in male breast cancer.

Biomarker	N	Positive	% positive
ER	133	125	94
PR	133	90	68
HER2	134	4	3
Ki67	131	24	18
AR	132	107	81
Bcl-2	131	98	75
CyclinD1	131	101	77
BRST2	131	73	56
p16 cytoplasm	131	59	45
p16 nuclear	131	31	24
p21	131	63	48
p53	132	20	15
CK5/6	132	12	9
CK14	133	1	1
EGFR	133	15	11

node metastases. AR, p16, CK14 and CK5/6 did not show any significant correlation with clinicopathologic features.

Immunohistochemical profile was also significantly different between the analyzed molecular subtypes of male breast cancer. Luminal type B tumors were more often p21 positive (p=0.026) and Bcl-2 negative (p=0.028). All four basal-like breast cancers were negative for AR and CyclinD1, whereas the luminal type breast cancers were positive in respectively >81% and >78% of the cases.

Cluster analysis

Data for one or more markers were missing in three cases, leaving 131 cases for cluster analysis. Two major clusters (cluster A and cluster B) were identified, which could be subdivided into four distinctive groups (A1, A2, B1, B2), graphically presented in the clustergram and corresponding dendrogram (Figure 1). The two cases which did not cluster were two basal-like breast cancers.

Clinicopathological features and immunohistochemical profiles of the four distinctive groups are presented in Table 3. The groups were designated as hormone receptor negative (A1), ER+ high grade (A2), ER+ intermediate grade (B1) and ER+ low grade (B2). In the hormone negative group (A1) the ER negative cases clustered together with PR and AR negative cases. Protein expression of the remaining markers was also low. These tumors were relative large with a mean size of 2.6 cm (p=0.023) and showed intermediate histological grade.

Like the A1 (hormone negative) group, the A2 (ER+ high grade) group also showed low PR expression. In addition, the A2 group was characterized by HER2 amplification, high Ki67, and p21, p16 and p53 accumulation. Expression of basal markers (CK5/6 and EGFR) was also more common, but did not reach significance. These tumors were high grade (p=0.001) with high mitotic activity (p<0.001) and often had lymph node metastases (p=0.033).

The ER+ intermediate and low grade tumors (B1 and B2) had a remarkably similar immunohistochemical phenotype with hormone receptor, Bcl-2 and CyclinD1 positivity and low Ki67. The most striking difference between the B1 and B2 groups was the fact that all ER+ intermediate grade tumors were BRST2 negative and all ER+ low grade tumors were BRST2 positive. p21 was more often negative in the ER+ low grade cluster. The tumors in the ER+ low grade group were well differentiated, with low mitotic count and small size. Interestingly, lymph node metastases were common (64%) in this cluster of low grade tumors and were as frequent as in the ER+ high grade group. Lymph node metastases were seen in only 31% of the ER+ intermediate grade tumors.

The molecular subtypes of male breast cancers also showed a significant distinctive distribution between the four groups (p<0.001). Luminal type B tumors clustered in the A2 ER+ high grade group. A few luminal type B cases were seen in the A1 hormone negative (2/18; 11%) and

the B1 ER+ intermediate grade groups (4/34; 12%) and none in the B2 ER+ low grade group. Two basal-like breast cancer clustered in the hormone negative group and the other two did not fit into any of the groups. The unclassifiable triple negative case clustered in the hormone negative group.

Reasoning from the other direction, the hormone receptors clustered with Bcl-2, CyclinD1, BRST2 and p21. Another cluster was formed by markers which were less frequently positive (CK5/6, CK14, p53, p16, EGFR, HER2, and Ki67).

Survival analysis

Univariate survival analysis results are presented in Table 4 en Figure 2. High histological grade (grade 3), high mitotic count (>8) and large tumor size (>2.0 cm) were predictors of adverse patients' outcome (p=0.027, p=0.015 and p=0.036, respectively). *HER2* positive tumors were also associated with decreased survival (p=0.046), but this needs to be interpreted with caution as only four tumors were *HER2* positive. The other two biomarkers correlated with decreased survival were p53 accumulation (p=0.042) and PR negativity (p=0.009). There was no significant association between the other biomarkers and patients' outcome. Patients with luminal type A tumors seemed to have a better survival than those with luminal type B tumors, but this did not reach significance (p=0.084).

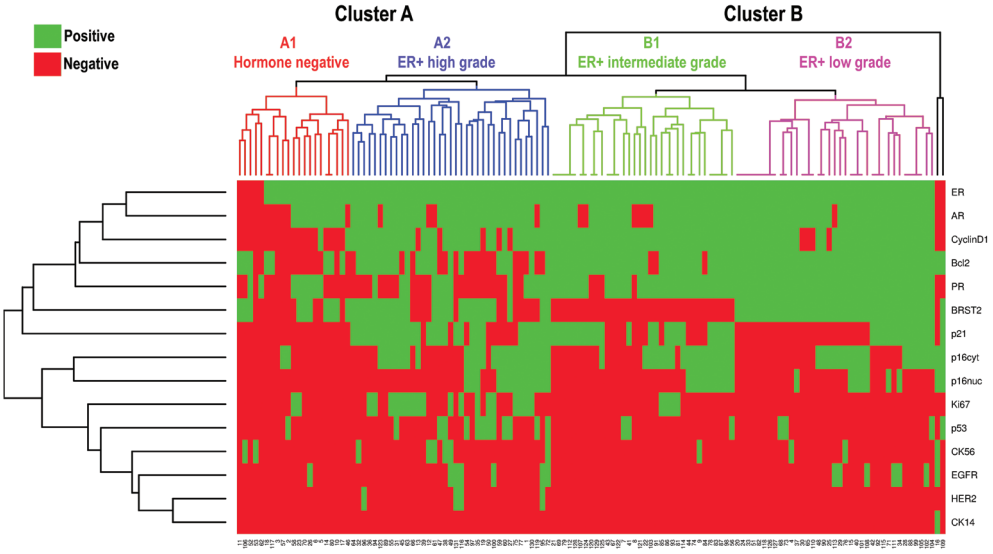

Figure 1 Unsupervised hierarchical clustering of 14 immunohistochemical markers in 131 male breast cancer cases. The clustergram and corresponding dendrogram indicate relations between male breast cancer cases and immunohistochemical markers. P16 cytoplasmic staining (p16 cyt) and nuclear staining (p16 nuc) were scored separately.

Table 3 Clinicopathological and immunohistochemical phenotype according to identified clusters of male breast cancer patients. A1: hormone negative, A2: ER + high grade, B1: ER + intermediate grade and B2: ER + low grade (p16 data not shown).

Feature	Grouping	A1 (n=21)	A2 (n=37)	B1 (n=34)	B2 (n=37)	p-value
Age	(mean)	63	67	67	67	0.583
	<50 years	2 (10%)	3 (8%)	0	6 (16%)	0.111
	>50 years	19 (90%)	34 (92%)	34 (100%)	31 (84%)	
Molecular subtype	Luminal A	16 (89%)	15 (42%)	30 (88%)	37 (100%)	<0.001
	Luminal B	2 (11%)	21 (58%)	4 (12%)	0	
Tumor size	(mean)	2.6	2.1	2.2	1.8	0.023
	< 2.0 cm	6 (29%)	21 (58%)	17 (53%)	19 (53%)	0.166
	> 2.0 cm	15 (71%)	15 (42%)	15 (47%)	17 (47%)	
Mitoses/2mm²	(mean)	11	14	12	7	0.004
	0-8 mitoses	7 (33%)	9 (24%)	15 (44%)	27 (73%)	<0.001
	>8 mitoses	14 (67%)	28 (76%)	19 (56%)	10 (27%)	
Grade	I/II	13 (62%)	15 (41%)	22 (65%)	32 (86%)	0.001
	III	8 (38%)	22 (59%)	12 (35%)	5 (14%)	
LN meta	Absent	7 (41%)	10 (36%)	20 (69%)	12 (36%)	0.033
	Present	10 (59%)	18 (64%)	9 (31%)	21 (64%)	
ER	-	5 (24%)	0	0	0	<0.001
	+	16 (76%)	37 (100%)	34 (100%)	37 (100%)	
PR	-	13 (62%)	24 (65%)	4 (12%)	0	<0.001
	+	8 (38%)	13 (35%)	30 (88%)	37 (100%)	
AR	-	11 (52%)	4 (11%)	6 (18%)	1 (3%)	<0.001
	+	10 (48%)	33 (89%)	28 (82%)	36 (97%)	
HER2	-	21 (100%)	33 (89%)	34 (100%)	37 (100%)	0.016
	+	0	4 (11%)	0	0	
Ki67	Low	19 (90%)	19 (51%)	30 (88%)	37 (100%)	<0.001
	High	2 (10%)	18 (49%)	4 (12%)	0	
BRST2	-	11 (52%)	12 (32%)	34 (100%)	0	<0.001
	+	10 (48%)	25 (68%)	0	37 (100%)	
Bcl-2	-	13 (62%)	17 (46%)	3 (9%)	0	<0.001
	+	8 (38%)	20 (54%)	31 (91%)	37 (100%)	
CyclinD1	-	19 (90%)	5 (14%)	0	4 (11%)	<0.001
	+	2 (10%)	32 (86%)	34 (100%)	33 (89%)	
p21	-	21 (100%)	9 (24%)	12 (35%)	25 (68%)	<0.001
	+	0	28 (76%)	22 (65%)	12 (32%)	
p53	-	20 (95%)	25 (68%)	31 (91%)	34 (92%)	0.004
	+	1 (5%)	12 (32%)	3 (9%)	3 (8%)	
EGFR	-	20 (95%)	31 (84%)	34 (100%)	31 (84%)	0.056
	+	1 (5%)	6 (16%)	0	6 (16%)	
CK5/6	-	19 (90%)	31 (84%)	33 (97%)	35 (95%)	0.199
		2 (10%)	6 (16%)	1 (3%)	2 (5%)	

Table 4 Biomarkers and univariate survival rates (log rank).

		5 years survival	
Feature	**Grouping**	**Survival rate**	**p-value**
Tumour size	< 2.0 cm	79%	0.036
	> 2.0 cm	59%	
Mitoses/2mm²	0-8	81%	0.015
	>8	57%	
Grade	I/II	77%	0.027
	III	53%	
Lymph node metastases	absent	82%	0.132
	present	65%	
ER	-	57%	0.633
	+	70%	
PR	-	53%	0.009
	+	77%	
HER2	-	70%	0.046
	+	25%	
Bcl-2	-	60%	0.188
	+	75%	
p53	-	73%	0.042
	+	45%	
Molecular subtype	Luminal A	73%	0.084
	Luminal B	52%	
Cluster analysis	A	55%	0.011
	B	80%	
Cluster analysis	A1	52%	0.062
	A2	57%	
	B1	69%	
	B2	87%	

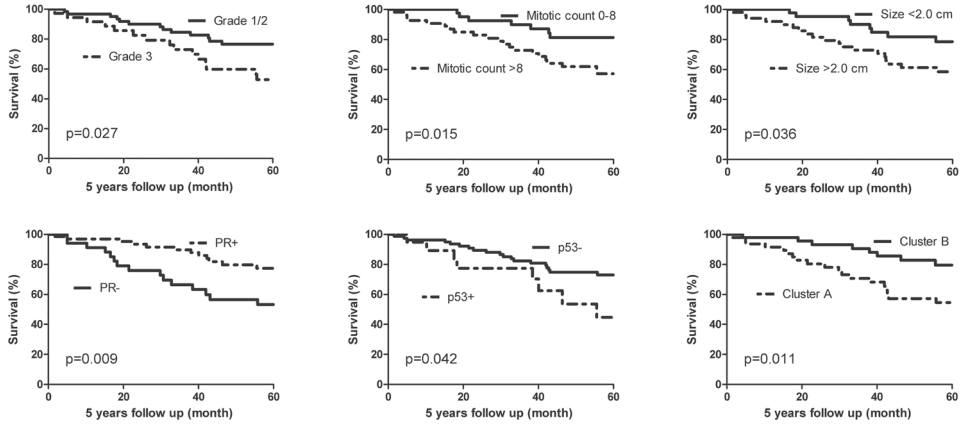

Figure 2 Kaplan Meier survival curves with corresponding p-values (log rank) according to histological grade, mitotic count, tumor size, PR expression p53 accumulation and cluster A and B.

However, cluster A from unsupervised hierarchical clustering had a significantly decreased survival compared to cluster B (p=0.011). In Cox regression, p53 accumulation (p=0.002; hazard ratio 4.1), large tumor size (p=0.008, hazard ratio 3.4), and PR negativity (p=0.020, hazard ratio 2.6) appeared to be independent predictors of poor prognosis. The identified cluster A was an independent prognosticator from histological grade, mitotic count and tumor size (p=0.043; hazard ratio 2.4).

DISCUSSION

Proteins are important in tumor biology and can be routinely assessed by immunohistochemistry in every pathology laboratory. Analysis of proteins such as those involved in regulating the cell cycle can provide important insights into the pathogenesis of cancer and can lead to the identification of biomarkers with therapeutic and prognostic implications. In female breast cancer a variety of biomarkers have been identified, several of which are used in daily practice. Knowledge on male breast cancer is increasing, but still sparse compared to female breast cancer and based on analysis of small single institutional groups. In the present study the immunohistochemical profile of male breast cancer was studied with a panel of 14 widely used markers in a large multi-institutional group of male breast cancer patients.

Like previous studies we found high frequencies of hormone (ER, PR and AR), Bcl-2 and CyclinD1 positive male breast cancer cases[11,23,27,28]. Several biomarkers were correlated with aggressive phenotype. High mitotic count and high grade was more frequent in *HER2*, Ki67, p53 and p21 positive and in PR and Bcl-2 negative tumors. On the other hand no correlation between tumor phenotype and CK5/6 and EGFR positive cases was seen.
In line with our findings, an association of Ki67 and *HER2* with aggressive tumor phenotype and adverse prognosis has been previously reported in MBC[13,14,28,29]. We could not, however, confirm the prognostic relevance of Ki67 in the present group of male breast cancer patients. The prognostic relevance of *HER2* positive tumors should also be interpreted with caution, as only four tumors were *HER2* positive/amplified. No correlations have previously been found between tumor phenotype and p21 expression in male breast cancer[13,14]. However, more recently it was shown that male breast cancer patients with p21 positive tumors have decreased disease free survival[23]. We found a strong correlation between p21 and aggressive tumor phenotype, indicating that this protein is involved in the carcinogenesis of high grade tumors. p21, the most important downstream effector of p53, is a universal Cyclin-CDK inhibitor and inhibits proliferation[30]. Overexpression of this protein is more frequently seen in male breast cancer than in female breast cancer[13,14]. Despite strong correlation with

aggressive tumor phenotype, p21 positive tumors were not correlated with decreased patients' survival. The clinical and prognostic relevance of p21 is also still a matter of debate in female breast cancer[31].

The role of PR and Bcl-2 expression in male breast cancer has not yet been determined. Most studies could not identify clinical relevant correlations, probably due to lack of power because of small series[14,27,32]. In the present study PR negative tumors were strongly correlated with high mitotic count, high grade and decreased survival. Concordant with our findings, a large retrospective epidemiological study recently demonstrated that male breast cancer patients with PR negative tumors had adverse outcome[33]. This is, to the best of our knowledge, the first study demonstrating that Bcl-2, which correlated with low mitotic count, low grade and smaller tumors, should also be regarded as an important biomarker.

However, we did not find a significant difference in 5 years patients' survival between Bcl-2 positive and Bcl-2 negative tumors. In female breast cancer Bcl-2 is, in contrast, one of the most important biomarker in predicting patients' outcome[7].

The presence of lymph node metastases was associated with p53 accumulation and BRST2 positivity. p53 accumulation signals the presence of mutant p53 and is less common in male breast cancer than in female breast cancer[11]. Conflicting results have been published regarding patients' outcome and p53 accumulation[27-29,34]. In the present study p53 accumulation was an independent marker of decreased survival and appeared to be a more powerful prognosticator than classical clinicopathological markers, in Cox regression. BRST2 expression had not been studied in male breast cancer before. BRST2, an antibody against gross cystic disease fluid protein 15, shows uniform expression in cells with apocrine differentiation and has been reported to be expressed in a variable percentage of female breast cancer (23-72%)[35-37]. In the present study BRST2 expression was seen in 56% of the male breast cancer cases and in this group lymph node metastases were twice as common. Apparently in contrast with this finding, BRST2 expression was also more common in tumors with low mitotic count. However, no correlation with patients' outcome was found. Females with BRST2 positive breast cancers seem to have favorable clinicopathological features and better outcome[36-38]. However in one report BRST2 was also correlated with lymph node metastases[36]. The role and prognostic implications of BRST2 in male and female breast cancers have yet to be determined.

Previously we demonstrated that there are differences in the distribution of molecular subtypes between male and female breast cancer[12]. Luminal type breast cancer seems to be much more common in males, whereas HER2 driven, basal-like and unclassifiable triple negative breast cancer is rare. In the present study we further characterized these molecular subtypes in male breast cancer. Luminal type B was characterized by p21 positivity and Bcl-2 negativity. All basal-like breast cancers were negative for AR and CyclinD1, whereas

these markers were positive in the vast majority of the luminal breast cancers. AR is indeed a marker for luminal subtype, but a substantial proportion of the basal-like breast cancers in females seem to be AR positive as well[39]. CyclinD1 and Bcl-2 are known markers for luminal subtype of breast cancer[40]. In our group of luminal B tumors expression of Bcl-2 was less common, despite ER and/or PR positivity, and could be used to differentiate between luminal type A and B in male breast cancer. To the best of our knowledge p21 expression has not been correlated with luminal type B tumors before. Patients with luminal type A tumors had better survival than those with luminal type B tumors (73% versus 52%), but this did not reach significance.

In tackling the tumor heterogeneity of breast cancer, cluster analysis is an attractive approach for predicting clinicopathological features and patients' outcome. Although unsupervised hierarchical cluster analysis has proved its worth in female breast cancer, this method has not been used before to subclassify male breast cancer[15,16]. According to the expression patterns of the 14 analyzed biomarkers, the male breast cancer cases were divided into two major clusters. One group was formed by hormone negative cases and ER+ high grade tumors (cluster A) and in the other group the ER+ intermediate and low grade tumors clustered together (cluster B). The ER+ high grade group (A2) showed, analogously to to the hormone negative group (A1), low frequencies of PR, Bcl-2 and CyclinD1 positivity and had unfavorable histological phenotype with high mitotic count, high grade and common lymph node metastases, which is not surprising in view of the high expression levels of p21, HER2, EGFR and p53. The other two ER+ groups with more indolent features clustered together. In all these tumors Bcl-2 and CyclinD1 expression was common. The main difference was absence of BRST2 reactivity and more often expression of p21 in the ER+ intermediate grade group compared to the ER+ low grade group. However, seemingly in contrast to its low grade phenotype, these cases often showed lymph node metastases.

It is probable that the identified clusters represent distinctive tumor groups with different underlying genetic alterations. This is supported by the fact that luminal type B tumors clustered together in the ER+ high grade group. These tumors have distinctive molecular alterations compared to luminal type A tumors[21,41]. Cluster A was significantly correlated with decreased survival and had additional prognostic value to that provided by histological grade, mitotic count and tumor size.

Unsupervised hierarchical cluster analyses also revealed two protein clusters. Co-expression of most of the proteins in the first group (ER, PR, AR, Bcl-2 and CyclinD1) is a common finding in breast cancer[27,42]. The proteins, that are known markers for aggressive course in female breast cancer (CK5/6, CK14, p53, p16, EGFR, HER2, and Ki67), formed the second cluster (reviewed in[43]).

Unsupervised hierarchical clustering was recently performed on male breast cancer immunohistochemical and gene expression data by Shaaban et al. and Johansson et al.[44,45]. Using this cluster method, both studies found important differences between male and female breast cancer. Johansson et al. described two biological distinctive subgroups of male breast cancer based on gene expression analysis. Even though the majority of male breast cancer cases in both groups were ER positive by immunohistochemistry, the luminal M1 group showed low ER signaling by gene expression analysis[44]. Whether or not our cluster A tumors (ER negative and ER+ high grade) also show differences in ER signaling at gene level remains speculative, but certainly deserves further investigation. In line with our results, Shaaban et al. found high ratios of ER and PR positive tumors and basal-like and HER2 driven male breast cancers were rare. The difference in frequency of luminal type B tumors (none were found in their study) is due to the use of different markers for this classification, as we also used Ki67 for luminal type B classification and did not use HER2 expression alone. Unsupervised hierarchical cluster analysis revealed differences between male and female breast cancers regarding clustering of the hormonal markers[45]. Due to differences in immunohistochemical markers and cutoff criteria, these interesting results are difficult to compare with our present findings.

In the present study we elucidate the clinical relevance of several biomarkers of which their role in male breast cancer was yet to be determined. PR and Bcl-2 positive tumors showed favorable histological features while, on the other hand, HER2, Ki67 and p21 positive tumors were correlated with high grade and high mitotic count. p53 and BRST2 significantly predicted the presence of lymph node metastases. PR negativity and p53 accumulation were independent predictors of adverse patients' outcome. In unsupervised hierarchical clustering analysis, male breast cancer cases were divided into four distinctive groups with differences in clinicopathological features and patients' outcome. Despite heterogeneous expression profiles, this multiple biomarker approach gave more insight into differences in carcinogenesis than relying on single markers and provided prognostic value.

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5

Oncogene amplification in male breast cancer: analysis by multiplex ligation-dependent probe amplification

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ABSTRACT

Introduction

Gene amplification is an important mechanism for oncogene activation, a crucial step in carcinogenesis. Compared to female breast cancer, little is known on the genetic makeup of male breast cancer, because large series are lacking.

Methods

Copy number changes of 21 breast cancer related genes were studied in 110 male breast cancers using multiplex ligation-dependent probe amplification. A ratio of >1.3 was regarded indicative for gene copy number gain and a ratio >2.0 for gene amplification. Data were correlated with clinicopathological features, prognosis and 17 genes were compared with a group of female breast cancers.

Results

Gene copy number gain of *CCND1, TRAF4, CDC6* and *MTDH* was seen in >40% of the male breast cancer cases, with also frequent amplification. The number of genes with copy number gain and several single genes were associated with high grade, but only *CCND1* amplification was an independent predictor of adverse survival in Cox regression (p=0.015; hazard ratio 3.0). In unsupervised hierarchical clustering a distinctive group of male breast cancer with poor prognosis (p=0.009; hazard ratio 3.4) was identified, characterized by frequent *CCND1, MTDH, CDC6, ADAM9, TRAF4* and *MYC* copy number gain. Compared to female breast cancers, *EGFR* (p=0.005) and *CCND1* (p=0.041) copy number gain was more often seen in male breast cancer, while copy number gain of *EMSY* (p=0.004) and *CPD* (p=0.001) and amplification in general was less frequent.

Conclusion

Several female breast cancer genes also seem to be important in male breast carcinogenesis. However, there are also clear differences in copy number changes between male and female breast cancers, pointing toward differences in carcinogenesis between male and female breast cancer and emphasizing the importance of identifying biomarkers and therapeutic agents based on research in male breast cancer. In addition *CCND1* amplification seems to be an independent prognosticator in male breast cancer.

INTRODUCTION

Gene amplification is important in the development and progression of cancer and could serve as a potential biomarker for prognosis or as a target for molecular therapy. In female breast cancer, HER2 is the best described oncogene with frequent amplification. *HER2* amplification is correlated with poor survival and good response to targeted therapy[1,2]. Other genes, like epidermal growth factor receptor (*EGFR*), Fibroblast growth factor receptor 1 (*FGFR1*), topoisomerase IIa (*TOP2A*) and *MYC* are also involved in female breast cancer and have prognostic and therapeutic implications[3-6].

Compared to female breast cancer, there is yet little knowledge regarding the genetic makeup of male breast cancer, because male breast cancer is a rare disease and the few available studies are based on small single institutional series[7]. Treatment of male breast cancer has largely been extrapolated from its female counterpart, while there are important differences between male and female breast cancer, with higher ratios of estrogen receptor (ER) and progesterone receptor (PR) positivity in men[8-10]. Also the distribution of molecular subtypes by immunohistochemical analysis shows important differences. Luminal type A and B are by far the most frequently encountered subtypes and HER2 driven, basal-like and triple-negative tumors are very rare in men[11,12]. The few gene expression studies performed recently in men showed that there might be important differences in molecular profile between male and female breast cancer[13-15]. However, the clinical and prognostic significance of genetic alterations in relevant breast cancer genes still needs to be elucidated in male breast cancer.

Multiplex ligation-dependent probe amplification (MLPA) analysis is a high throughput genomic technique enabling relative quantification of copy number or promoter hypermethylation in a variety of genes in one reaction, based on the simultaneous amplification of specifically hybridized probes on DNA that can be derived from paraffin embedded material[16,17]. We previously showed in female breast cancer that MLPA analysis with a dedicated “breast cancer kit” allows evaluation of copy numbers in 21 important breast cancer genes, providing an overview of the most common amplifications[18]. In the present study, we used MLPA to investigate copy number changes of 21 (female) breast cancer related genes in a large group of male breast cancer and correlate these genomic anomalies with clinicopathological features, patients’ outcome, and with previously obtained MLPA data from female breast cancers.

MATERIALS AND METHODS

Patients: specimens and clinical information

All consecutive cases of surgical breast specimens of invasive male breast cancer from 1986 - 2010 were collected from 4 different pathology labs in The Netherlands (St. Antonius Hospital Nieuwegein, Diakonessenhuis Utrecht, University Medical Center Utrecht, Laboratory for Pathology East Netherlands) as described in more detail previously[12]. Hematoxylin and eosin (HE) slides were reviewed by three experienced observers (PJvD, RK, AM) 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 110 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 0.8 to 5.5 cm (average: 2.2 cm). In 86% lymph node status was known and 55% of these patients had lymph node metastases. The majority of cases were diagnosed (according to the WHO) as invasive ductal carcinoma (90%). The remaining cases were lobular (n=3), mixed type (ductal/lobular) (n=2), invasive cribriform (n=1), papillary (n=1), mucinous (n=2), invasive micropapillary (n=1) or adenoid cystic carcinomas (n=1). According to the modified Bloom and Richardson score[19] most tumors were grade 2 (41%) or grade 3 (36%). Mitotic activity was assessed as before[20] with a mean mitotic index of 11 per 2mm^2 (range 0-56). For all cases hormone receptor and *HER2* status were re-assessed as described previously[12]. Tissue microarray (TMA) slides were used for immunohistochemical stainings for ER, PR and chromogenic in situ hybridization (CISH) for HER2 assessment, the latter showing *HER2* amplification in only 4/110 cases (4%). TMA slides were also stained for E-cadherin. Most tumors were ER positive (102/110, 93%) and PR positivity was also common (71/110; 65%). Only four cases were E-cadherin negative (three lobular carcinomas and one ductal carcinoma).

DNA extraction and MLPA analysis

Representative tumor areas were identified in HE stained slides and corresponding tumor areas (at least 1 cm^2) were dissected with a scalpel from 8 µm paraffin slides[21]. DNA was extracted by overnight incubation in proteinase K (10mg/ml; Roche, Almere, The Netherlands) at 56 °C. After boiling for 10 min and centrifugation, 5 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 P078-B1 kit (MRC Holland), containing 21 breast cancer related genes (Table 1), was used as before[18]. All tests were performed in duplicate. Seven negative references samples (normal breast and blood) were included

in each MLPA run. 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 to 2.0 for gene copy number gain, >2.0 for amplification and <0.7 for lost genes. Values between 0.7 and 1.3 were regarded normal[18,22].

Table 1 Contents of the "breast cancer" MLPA kit P078-B1 (MRC Holland).

Gene	Chrom	Gain (%)	Amp (%)	Loss (%)	Function and clinical relevance
ESR1	06q25.1	6	0	3	Transcription factor; under debate[40-42]
EGFR	07p11.2	22	1	0	Signal transduction; poor survival[4]
FGFR1	08p11.23	29	13	0	Signal transduction; poor survival, tamoxifen resistance[5]
ADAM9	08p11.23	39	11	1	Protein metabolism; promotes invasion[38]
IKBKB	08p11.21	32	6	0	Signal transduction[43]
PRDM14	08q13.3	32	9	0	Transcription regulatory protein; chemoresistance[44]
MTDH	08q22.1	49	12	0	Signal transduction; promoting metastases, chemoresistance, poor survival[36]
MYC	08q24.21	36	10	0	Transcription factor; poor survival[3]
CCND1	11q13.2	46	18	1	Signal transduction; ER positivity, poor survival[35]
EMSY	11q13.5	10	2	3	Transcription regulatory protein; poor survival[45]
CDH1	16q22.1	6	0	9	Cell adhesion[46]
TRAF4	17q11.2	41	4	0	Signal transduction[47]
CPD	17q11.2	9	0	0	Protein metabolism[48]
MED1	17q21.2	23	4	0	Transcriptional coactivator; ER positivity[49]
HER2	17q12	17	4	0	Signal transduction; bad survival; trastuzumab response[2]
CDC6	17q21.2	41	4	0	Signal transduction[50]
TOP2A	17q21.2	26	2	0	Regulation of the topological status of DNA; poor survival, susceptible for certain chemotherapy[6]
MAPT	17q21.31	16	0	0	Microtubule stabilization; chemoresistance (taxanes)[51]
BIRC5	17q25.3	27	2	0	Signal transduction; predict distant recurrence[52]
CCNE1	19q12	2	0	1	Signal transduction; poor survival[53]
AURKA	20q13.31	10	4	12	Signal transduction[54]

For each gene, chromosome location (Chrom), gene copy number gain (Gain; >1.3), amplification (Amp; >2.0), gene loss (Loss; <0.7), function and clinical relevance (in female breast cancer) are shown

Control female breast cancers

A group of female breast cancer described previously was used to study differences in gene copy number change between male and female breast cancer[18]. This group consists of 104 cases with a mean age of 58 years (range 30 to 86 years). Tumor size ranged from 0.2 to 6.5 cm (average 2.1 cm) and 46% of the cases had lymph node metastases. Most cases were diagnosed (according to the WHO) as invasive ductal carcinoma (78%) or invasive lobular carcinoma (11%). Mean mitotic activity was 21 per $2mm^2$ and according to the modified Bloom and Richardson score most tumors were grade 2 (34%) or grade 3 (45%). ER positivity was common (69%, 70/101) and 48% of the tumors were PR positive (48/101). *HER2* amplification defined by immunohistochemistry and CISH was seen in 19% of cases (19/102). The same "breast cancer kit" (P078-A1 kit; MRC Holland) was used, but because the gene content of the kit had been updated by the manufacturer in the meanwhile, only 17 genes could be compared between the groups. In addition, for two genes (*EGFR* and *HER2*) one of the probes was modified and for five genes one probe was deleted. Some reference probes were modified as well (Supplementary Table 1).

Statistics

Statistical calculations were performed using SPSS for Windows v15.0. Correction for multiple comparisons was applied by resetting the 0.05 threshold according to the Holm-Bonferroni method. Differences between gene copy number and clinicopathological characteristics were calculated with ANOVA for continuous variables and with Pearson χ^2 test (or Fisher's exact test when appropriate) for categorical variables. The following clinicopathological features were dichotomized: age (>50 years), tumor size (>2.0cm), mitotic activity (>8 mitoses/2mm2) and histological grade (grade 1/2 vs 3). Correlation between number of gene amplification and clinicopathological features were calculated with Spearman's rho. Unsupervised hierarchical clustering using the statistical program R (www.r-project.org) was performed to identify relevant clusters and co-amplification. We used the maximum distance and Ward's clustering method and calculated the stability of the clusters with pvclust. Logistic regression analysis was performed to compare gene amplification in male and female breast cancer, taking significant differences in clinicopathological features between the two groups into account. Information regarding prognosis and therapy was requested from the Integral Cancer registration The Netherlands (IKNL). Survival data were available for 101 cases with a mean follow up of 5.7 years. Therefore, survival analysis was based on 5 years 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 MLPA

In 4 cases the amount of DNA was insufficient, leaving 106 cases of male breast cancer for further analysis. Gene copy number status of the 21 analyzed genes is presented in Table 1 and Figure 1. All genes analyzed showed copy number alterations with varying frequencies. The average number of genes with copy number gain was four (range 0 to 12), of which one (range 0 to 8) showed amplification. Copy number gain was most frequently seen in the genes *MTDH* (52/106; 49%) and *CCND1* (49/106; 46%), and these genes were also frequently amplified (13/106; 12% and 19/106; 18% respectively). The genes analyzed on chromosome 8 (*FGFR1, ADAM9, IKBKB, PRDM14, MTDH* and *MYC*) were also frequently affected with high rates of copy number gain and amplification. Thirteen cases (12%) showed copy number gain of all genes analyzed on chromosome 8. Also the genes located on chromosome 17 were often affected, particularly *TRAF4, CDC6* and *BIRC5* with copy number gain in 37, 37 and 26% of cases, respectively. However, amplification of these genes was rare (<4%). Amplification of *HER2* was also rare (4/106; 4%). In five cases (5%), all genes analyzed on chromosome 17 showed copy number gain. In 17% of cases (18/106) no gene copy number changes were found. Losses were rare and seen in only seven genes of which *CDH1* (10/106; 9%) and *AURKA* (13/106; 12%) were most frequently affected.

Correlation with clinicopathological features

Tumors with a copy number gain in one or more genes tended to have a more aggressive phenotype with more mitoses (p=0.004) and a higher histological grade (p=0.007) compared to tumors without gene copy number alterations. The number of genes with copy number gain was significantly correlated with a high mitotic count (p=0.001) and a high histological grade (p<0.001). Copy number gain in the genes *MED1* (p<0.001), *BIRC5* (p<0.001), *PRDM14* (p=0.003) and *MTDH* (p=0.003) were significantly correlated with high grade male breast cancer. *MED1* and *HER2* copy number gain were significantly correlated with high mitotic count (p<0.001 and p=0.003, respectively). We found trends for other genes, which did not remain significant after correction for multiple comparisons (Table 2).

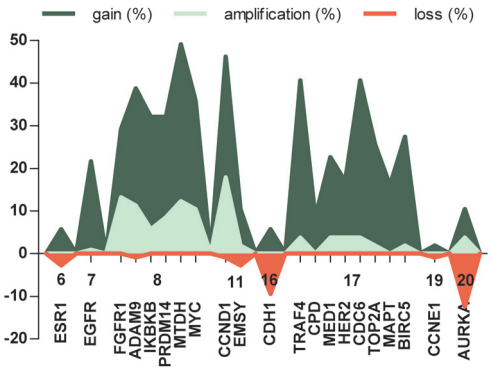

Figure 1 Copy number change of 21 genes with corresponding chromosome in 106 male breast cancer patients. Copy number gain (Gain; >1.3); Amplification (>2.0); Loss (<0.7)

Three out of the four tumors with *HER2* amplification (defined by CISH) also showed *HER2* amplification using MLPA (p<0.001). Loss of *CDH1* was not correlated with any clinicopathological feature and loss of the *CDH1* gene did not correlate with E-cadherin expression.

Comparison with female breast cancer

Because breast cancer is a heterogeneous disease only luminal type male and female breast cancers (defined by ER and/or PR expression) were compared. In this approach mitotic count (11 vs 13 mitoses) and grade (37% vs 33% grade 3 tumors) were quite similar in male and female breast cancers. Only age was significantly different, as male breast cancer patients were significantly older (p<0.001). Figure 2 illustrates gene copy number gain and gene

Table 2 Correlation between gene copy number gain (>1.3) and clinicopathological features.

Gene	Age (mean) Young	Mitoses High (>8)	Mitoses (mean) High	Grade High (3)	LN meta Negative	ER Negative
ESR1						
EGFR						0.038
FGFR1	0.019					
ADAM9	0.043	0.017	0.004			
IKBKB						0.033
PRDM14		0.049		**0.003**		
MTDH		0.019	0.005	**0.003**		
MYC				0.023		
CCND1			0.010			
EMSY				0.016		
CDH1						
TRAF4						
CPD					0.010	
MED1		**0.001**	**<0.001**	**<0.001**		
HER2		0.025	**0.003**	0.014		
CDC6			0.027			
TOP2A		0.045	0.025	0.013		
MAPT						
BIRC5		0.018	0.024	**<0.001**		
CCNE1						
AURKA						

Tumor size and PR were not correlated with any of the studied genes (not shown). p-values were calculated with Pearson χ^2 test or Fisher's exact test when appropriate (number of events <5) for categorical variables and ANOVA for continuous variables. Significant p values after correction for multiple comparison (Holm-Bonferroni method) are depicted in bold. See Supplementary Table 2 for full data. *LN meta* lymph node metastases.

amplification in 101 male and 73 female breast cancer cases. *EGFR* (p=0.005) and *CCND1* (p=0.041) copy number gain were independent predictors of gender in logistic regression, and these genes were more often gained in male breast cancer. *EMSY* (p=0.004) and *CPD* (p=0.001) copy number gain were also independent predictors of gender and these genes were more frequently gained in female breast cancer. Two genes, *TRAF4* (p=0.024) and *EMSY* (p=0.041) were more often amplified in female breast cancer. None of the studied genes was significantly more frequently amplified in men.

Cluster analysis

Unsupervised hierarchical clustering revealed a separate gene cluster, consisting of *FGFR1*, *ADAM9, HER2, MED1, EMSY* and *CCND1* (Figure 3). One small sub-cluster was formed by *FGFR1* and *ADAM9* which showed simultaneously copy number gain in 29% of all cases (31/106). Gains in both genes was correlated with younger age (62 vs 68 years; p=0.019). No associations with other clinicopathological features were found.

Reasoning from the cases, two major clusters were found (Figure 3). These clusters were stable according to the approximately unbiased p-values calculated with pvclust (p<0.001). Cluster A consisted of 55 cases and was characterized by a low rate of gene copy number gain and gene amplification. Cluster B consisted of 51 cases and was characterized by CCND1 (73%), *MTDH* (69%), *CDC6* (63%), *ADAM9* (57%), *TRAF4* (57%) and *MYC* (53%) copy number gain. The male breast cancers in cluster B showed significantly more mitosis compared to the tumors in cluster A (8 vs 14 mitosis; p<0.001). Cluster B tumors were also more often grade 3 (p=0.020) and were larger (2.4 vs 2.0 cm; p=0.036) compared to cluster A tumors.

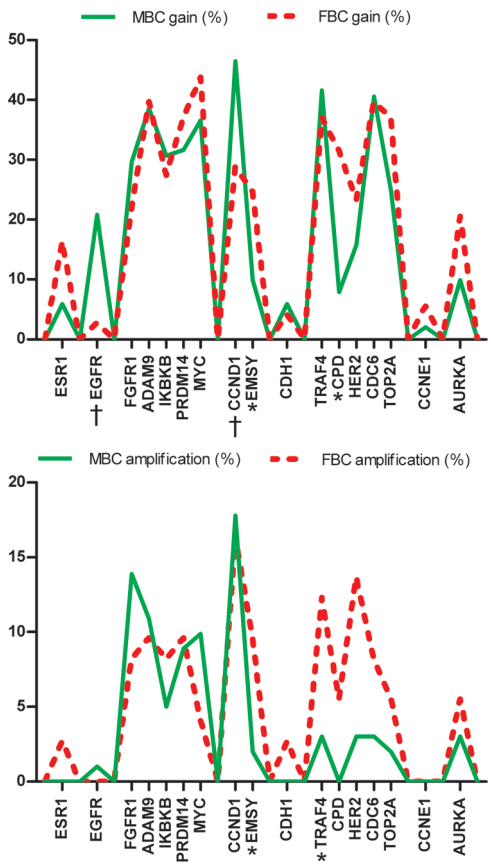

Figure 2 Comparison of frequency of copy number gain (>1.3; upper graph) and amplification (>2.0; lower graph) of 17 genes between luminal type male and female breast cancer

MBC: Male breast cancer; FBC: Female breast cancer; Amp: amplification. † Genes significantly more affected in men, * genes significantly more affected in women.

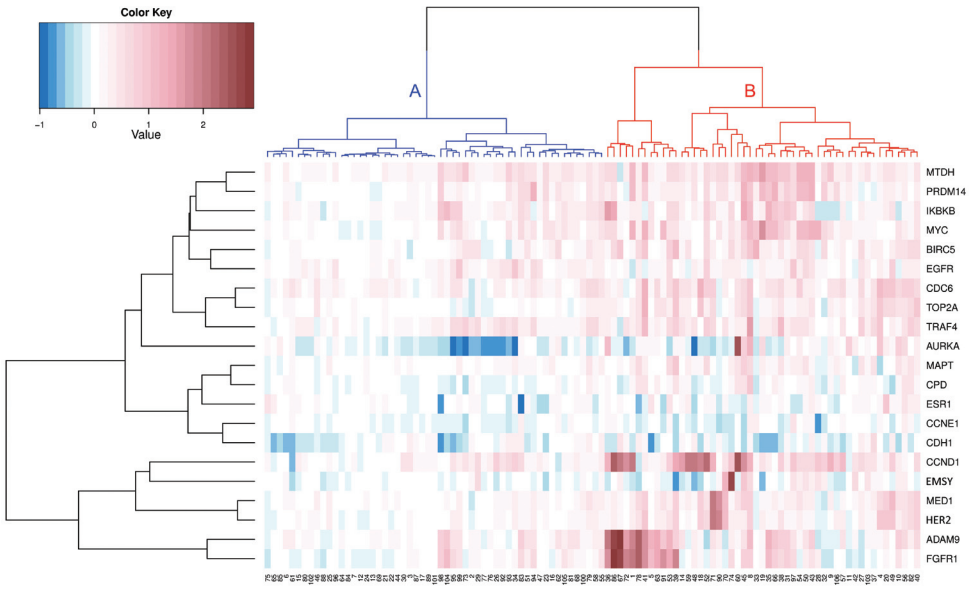

Figure 3 Unsupervised hierarchical clustering of copy number changes in 21 breast cancer related genes in 106 male breast cancer patients. The identified clusters of patients (horizontal) are depicted in different colors

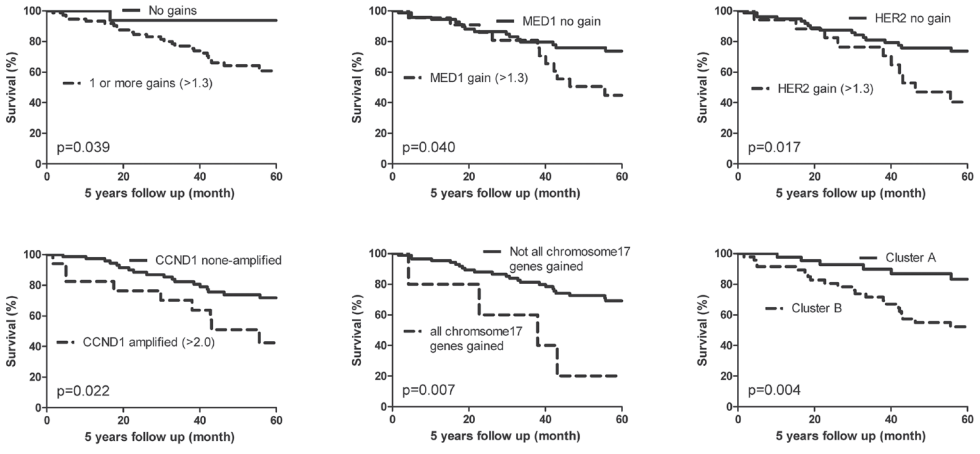

Figure 4 Kaplan-Meier survival curves with corresponding p-values (log rank) according to 1 or more gained genes, *MED1* (>1.3), *HER2* (>1.3), *CCND1* amplification (>2.0), copy number gain of all analyzed genes located on chromosome 17 and cluster A vs cluster B

Survival analysis

Grade 3 (p=0.027), high mitotic count (>8; p=0.015) and large tumor size (>2.0 cm; p=0.036) were correlated with a decreased 5 years survival. Chemotherapy was given in 14% of the cases and 40% received hormone therapy. Both treatment regimes did not correlate with patients' survival (p=0.700 and p=0.140, respectively). Univariate survival analysis is presented in Figure 4. Tumors with one or more gains had a poorer outcome compared with tumors without gains (p=0.039). *MED1* and *HER2* copy number gain also seem to correlate with poor survival (p=0.040 and p=0.017 respectively). In case amplification was analyzed the genes *CCND1* (p=0.022) and *EMSY* (p=0.040) were correlated with decreased survival. However, for *EMSY* only two cases were amplified. In case correction for multiple comparisons was performed, no single prognostic factor remained significant. On the other hand, tumors with a copy number gain of all genes on chromosome 17 had a poorer survival (p=0.007). Cluster B from unsupervised hierarchical clustering had adverse patients' outcome (p=0.004).

Using a Cox regression analysis, *CCND1* amplification appeared to be the only single gene which was a predictor of survival aside from grade, mitotic count and tumor size (p=0.015; hazard ratio 3.0). When chemotherapy and hormone therapy were included in Cox regression, *CCND1* was retained as an independent prognosticator. However, hormone therapy was an independent prognostic factor as well and was correlated with a favorable prognosis (p=0.004; hazard ratio 0.225). Cluster B tumors (p=0.009; hazard ratio 3.4) and tumors with copy number gain of all analyzed genes on chromosome 17 (p=0.005; hazard ratio 4.8) were also independent predictors of poor survival. The multivariate models are supplied in supplementary format (Supplementary Table 3).

DISCUSSION

Gene amplification is an important mechanism of oncogene activation and is crucial for the development and progression of cancer. The identification of frequent copy number change in certain chromosomal regions can lead to identification of functional important genes in carcinogenesis, reveal distinctive groups of breast cancer and can be used as prognostic markers. Knowledge of gene profiling in male breast cancer is sparse, because male breast cancer is a rare disease and most studies are based on small single institutional series. In the present study we used the high throughput technique MLPA to study gene copy number alterations of 21 breast cancer related genes in a large multi-institutional cohort of 106 male breast cancer patients.

The average amount of genes that showed copy number gain was four (range 0 to 12), of which one (range 0 to 8) was amplified. 18 cases (17%) did not show any copy number change

in the studied genes. These 18 cases tended to be low grade cancers with few mitoses and seem to have favorable prognosis compared to male breast cancers with gene copy number gain. The number of genes with copy number gain was correlated with high grade and a high mitotic count. This is in line with female breast cancers, as the genome in high grade female breast cancers is also more rearranged and these patients have a poor outcome[23,24]. Simultaneous copy number gain of all analyzed genes on chromosome 8, 11 and 17 was seen in 12, 10 and 5% of the cases respectively. This points to polysomy or gain of whole chromosome arms, a finding often seen in male breast cancer[14]. This is interesting, as polysomy of e.g., chromosome 17 has been refuted in female breast cancer[25-30]. In our group of male breast cancer copy number gain of all genes located on chromosome 17 was an independent predictor of adverse prognosis.

Using unsupervised hierarchical cluster analysis a small sub-cluster was formed by *FGFR1* and *ADAM9*. In female breast cancer, co-amplification of these chromosomal regions is also a common finding[24,31]. In addition, two stable clusters of male breast cancer patients were identified with additional prognostic value to classical clinicopathological prognosticators.

HER2 amplification defined by MLPA in the present study strongly correlated with *HER2* amplification status defined by CISH on TMA slides. Small differences found could be due to heterogeneity of tumors which could be missed or overrepresented in TMA slides. We have also previously validated MPLA against CISH and FISH[16].

CCND1 and *MTDH* were the genes which most frequently showed copy number gain (49 and 46% respectively), and often had amplification, indicating that these genes probably play an important role in male breast carcinogenesis. *CCND1* encodes for cyclin D1, which is a cell cycle protein driving cell cycle progression through the G1 phase. It also enhances ER-mediated gene transcription and is especially overexpressed in ER positive female breast cancer[32]. *CCND1* amplification has been linked to ER positive tumors as well, although some did not find such a correlation[18,24,33]. In the present study, we could not identify a correlation between *CCND1* copy number gain or amplification and ER status. A clear cut association between *CCND1* amplification and patients' outcome in female breast cancer is lacking, but *CCND1* amplification may be associated with a poor prognosis, particularly in ER positive tumors[23,24,33-35]. In the present group of male breast cancers, tumors with *CCND1* copy number gain tended to have a higher mean mitotic count compared to tumors without CCND1 amplification, a finding which is in line with the encoding protein function. More importantly, amplification of *CCND1* was the only single gene which correlated with poor survival and had additional prognostic value aside from tumor size, mitotic count and histological grade using a Cox regression analysis.

MTDH is involved in several signaling pathways and amplification of *MTDH* promotes

metastases, enhances chemo-resistance and is associated with poor outcome in female breast cancer patients[36]. In line with these findings in females, we demonstrated that male breast cancer with *MTDH* copy number gain showed a more aggressive phenotype with a high mitotic count and a high histological grade. However, no correlation with prognosis and *MTDH* copy number change was found in our group of male breast cancer and no correlation with lymph node metastasis was found either.

The genes located on different amplicons on chromosome 8p11 (*FGFR1*; 29%, *ADAM*9; 39% and *IKBKB*; 32%) were also often gained. These genes have been correlated with ER positive female breast cancers[18]. Since most male breast cancer cases are ER positive (93% in the present group), frequent copy number gain of these genes can be explained by the high ratio of ER positive tumors[8,9]. Nevertheless, we could not confirm the correlation between copy number gain or amplification of these genes and ER positive tumors in male breast cancer. However, in view of the low rate of ER negative male breast cancers in the present study, these results need to be interpreted with care. It is important to note that *FGFR1* amplification enhances tamoxifen resistance, which is particularly clinically relevant in male breast cancer, as endocrine therapy is often indicated in these patients[5]. Since *FGFR1* copy number gain and amplification seems to be common in male breast cancer and is suitable for targeted therapy, this gene could be of further interest in male breast cancer[37]. ADAM9, which is important in cell adhesion and tumor cell invasion, has potential in male breast cancer as well, since this gene is often affected and could be used for targeted therapy[38,39].
Among the other genes studied, copy number gain of *MED1*, *PRDM14* and *BIRC5* were associated with a high grade phenotype, indicating that these genes play a role in the development or progression of aggressive male breast cancer. Indeed MED1 copy number gain tends to correlate with poor survival. We could not confirm the prognostic relevance of the other genes in male breast cancer patients.

Comparison with 103 female breast cancers revealed differences in copy number change between male and female breast cancer in a variety of genes, pointing toward differences in carcinogenesis. In male breast cancer *CCND1* and *EGFR* were more often gained than in the female breast cancer group. In the group of female breast cancers *EMSY* and *CPD* copy number gain were seen more often than in males. In line with a previous comparative genomic hybridization study, female breast cancer showed more frequent amplification in a variety of genes, particularly in *TRAF4* and *EMSY*[14]. None of the genes studied were significantly more often amplified in male breast cancer. Alongside gender specific differences between male and female breast cancers, differences in genetic predisposition may also influence the genetic profile of these tumors. Approximately 10% of men with breast cancer are known to have a

genetic predisposition, and especially BRCA2 mutations seem to be important[7]. Differences in BRCA mutations status between male and female breast cancers would have implications for the genetic makeup of these tumors and deserves further investigation.

In conclusion, copy number gain of the genes *CCND1* (11q13), *TRAF4* (17q11), *CDC6* (17q21) and *MTDH* (8q22) is very common in male breast cancer (>40%) and these genes probably play a role in male breast carcinogenesis. Tumors with copy number gain of one or more genes showed a highly malignant phenotype. Also *MED1*, *PRDM14*, *MTDH* and *BIRC5* seem to be important in the development or progression of high grade male breast cancer. Amplification of *CCND1* was the most important single gene as it correlated with poor survival and had prognostic value in addition to the classical clinicopathological prognostic factors. Using unsupervised hierarchical clustering a distinctive group of male breast cancer tumors was identified with poor survival. Compared to female breast cancer *CCND1* and *EGFR* were found to be more frequently amplified in male breast cancer, while in females *EMSY* and *CPD* were more often involved and more frequent amplifications of *TRAF4* and *EMSY* were found. Our results point toward important differences in carcinogenesis between male and female breast cancer, emphasizing the importance in identifying specific biomarkers and therapeutic targets for male breast cancer.

Supplemental Table 1 Contents of the "breast cancer" MLPA kit P078-B1 breast tumor (MRC Holland; lot 0109) and modification compared to MLPA kit P078-A1.

Table 1a Contents of the MLPA kit P078-B1. Modified probes are depicted bold. New genes are depicted italic.

Gene	Probe	Chromosome posistion	Map View	Length (nt)
ESR1	11998-L12826	06q25.1	06-152.457215	232
ESR1	11996-L12824	06q25.1	06-152.423838	214
EGFR	05969-L05386	07p11.2	07-055.233957	265
EGFR	**02063-L03283**	**07p11.2**	**07-055.191055**	**427**
FGFR1	01046-L00624	08p11.23	08-038.434092	373
FGFR1	04440-L03826	08p11.23	08-038.391533	400
ADAM9	11992-L12820	08p11.23	08-038.998319	136
IKBKB	11993-L12821	08p11.21	08-042.292902	148
IKBKB	12003-L12831	08p11.21	08-042.302676	454
PRDM14	12002-L12830	08q13.3	08-071.130073	445
MTDH	*04151-L03506*	*08q22.1*	*08-098.742504*	*281*
MTDH	*04152-L03507*	*08q22.1*	*08-098.788082*	*337*
MYC	S0247-L08464	08q24.21	08-128.821796	118
MYC	00580-L00625	08q24.21	08-128.822151	157
MYC	00672-L00169	08q24.21	08-128.822001	238
CCND1	00583-L00148	11q13.2	11-069.175089	292
CCND1	05402-L04808	11q13.2	11-069.167779	463
EMSY	09173-L09347	11q13.5	11-075.902087	132
EMSY	09175-L09349	11q13.5	11-075.926543	256
CDH1	02410-L02237	16q22.1	16-067.404826	178
CDH1	02860-L01849	16q22.1	16-067.328716	355
TRAF4	09176-L09350	17q11.2	17-024.098403	124
CPD	09628-L09913	17q11.2	17-025.795018	226
MED1	*09963-L13205*	*17q21.2*	*17-034.840858*	*346*
HER2	*S0393-L12911*	*17q12*	*17-035.133169*	*113*
HER2	*00675-L00146*	*17q12*	*17-035.118101*	*142*
HER2	***12048-L12913***	***17q12***	***17-035.136344***	***244***
HER2	*00986-L00406*	*17q12*	*17-035.127183*	*310*
CDC6	*08611-L13204*	*17q21.2*	*17-035.699283*	*196*
TOP2A	*11994-L12822*	*17q21.2*	*17-035.818297*	*172*
TOP2A	*11999-L13177*	*17q21.2*	*17-035.812698*	*329*
TOP2A	*12000-L12828*	*17q21.2*	*17-035.816651*	*364*
MAPT	*08358-L08211*	*17q21.31*	*17-041.423085*	*416*
BIRC5	*03717-L02410*	*17q25.3*	*17-073.722036*	*316*
BIRC5	*03025-L14708*	*17q25.3*	*17-073.724340*	*383*
BIRC5	*03189-L02540*	*17q25.3*	*17-073.722396*	*436*
CCNE1	02881-L02348	19q12	19-035.005214	166
CCNE1	09170-L09344	19q12	19-035.000150	190
AURKA	10236-L10717	20q13.31	20-054.389980	481

Table 1b Two probes in the P078-A1 breast kit that were modified in the updated P078-B1kit (see table 1a, depicted bold).

Gene	Probe	Chromosome posistion	Map View	Length (nt)
EGFR	05959-L05376	07p11	07-055.196767	247
HER2	00717-L00390	17q12	17-035.136627	337

Table 1c Five probes in the P078-A1 breast kit, not present anymore in the updated P078-B1 kit.

Gene	Probe	Chromosome position	Map View	Length (nt)
CCND1	00601-L00162	11q13	06-152.307247	184
HER2	12044-L12908	17q12	07-055.196767	268
CCNE1	05782-L05724	19q12	11-069.165399	391
CDH1	02414-L01860	16q22	17-035.122165	283
ESR1	12001-L12829	06q25	16-067.419579	436

Table 1d Reference probes in the P078-B1 breast kit. Modified genes are depicted bold. Three probes were not used as reference probes (depicted in italic), because of common copy number change in these probes.

Gene	Probe	Chromosome position	Map View	Length (nt)
PLA2G6	09570-L10024	22q13	22-036.865968	220
IDH3A	00978-L00565	15q25	15-076.239491	274
ANXA7	00971-L09490	10q22	10-074.828036	301
RTN4	00963-L00550	02p16	02-055.068269	408
VWF	**11350-L12075**	**12p13**	**12-006.015847**	**184**
SLITRK3	**10223-L10704**	**03q26**	**03-166.390414**	**208**
TRPM3	**10224-L10705**	**09q21**	**09-072.566364**	**490**
RELN	**10218-L14675**	**07q22**	**07-102.864424**	**500**
ZNF198	*05730-L06767*	*13q11*	*13-019.465729*	*202*
GLRA1	*08964-L09059*	*05q33*	*05-151.214886*	*391*
LPIN2	*09205-L09581*	*18p11*	*18-002.950707*	*472*

Table 1e Reference probes in the P078-A1 kit. Modified genes are depicted bold (not present in the updated P078-B1 kit).

Gene	Probe	Chromosome position	Map View	Length (nt)
PLA2G6	09570-L10024	22q13	22-036.865968	220
IDH3A	00978-L00565	15q25	15-076.239491	274
ANXA7	00971-L09490	10q22	10-074.828036	301
RTN4	00963-L00550	02p16	02-055.068269	408
LPIN2	09205-L09581	18p11	18-002.950707	472
NP220	**00992-L00552**	**02p13**	**02-071.430713**	**202**
CASP2	**02051-L01583**	**07q35**	**07-142.699658**	**319**
TSPAN15	**00973-L00560**	**10q22**	**10-070.936627**	**382**

Supplemental Table 2 Frequencies of gene copy number gain (>1.3; Table 2a), gene amplification (>2.0; Table 2b) and gene loss (<0.7; Table 2c) according to clinicopathological features. For the continues variables (age and mitoses) mean values are depicted for each gene.

Table 2a Copy number gain

Gene	age (mean)	Mitoses High (>8)	Mitoses (mean)	Grade High (3)	LN meta negative	ER negative
ESR1	63	5 (83%)	14	4 (67%)	3 (60%)	0
EGFR	67	13 (57%)	11	10 (44%)	9 (45%)	4 (17%)
FGFR1	62	20 (65%)	12	14 (45%)	16 (57%)	1 (3%)
ADAM9	63	28 (68%)	14	19 (46%)	19 (54%)	2 (5%)
IKBKB	65	19 (56%)	11	13 (38%)	17 (59%)	5 (15%)
PRDM14	67	23 (68%)	12	19 (56%)	15 (54%)	3 (9%)
MTDH	65	34 (65%)	13	26 (50%)	21 (49%)	4 (8%)
MYC	64	24 (63%)	12	19 (50%)	17 (53%)	1 (3%)
CCND1	66	29 (59%)	13	21 (43%)	20 (49%)	3 (6%)
EMSY	67	8 (73%)	14	8 (73%)	3 (30%)	1 (9%)
CDH1	69	5 (83%)	16	3 (50%)	3 (60%)	0
TRAF4	66	28 (65%)	12	20 (47%)	15 (42%)	3 (7%)
CPD	62	5 (56%)	10	4 (44%)	8 (89%)	1 (11%)
MED1	64	20 (83%)	16	16 (67%)	11 (52%)	3 (13%)
HER2	65	14 (78%)	16	11 (61%)	10 (63%)	2 (11%)
CDC6	67	28 (65%)	13	20 (47%)	21 (54%)	3 (7%)
TOP2A	65	19 (70%)	14	15 (56%)	12 (52%)	2 (7%)
MAPT	68	10 (59%)	12	9 (53%)	9 (60%)	1 (6%)
BIRC5	65	21 (72%)	14	20 (69%)	11 (46%)	1 (3%)
CCNE1	70	2 (100%)	17	1 (50%)	2 (100%)	0
AURKA	61	6 (55%)	11	3 (27%)	7 (70%)	1 (9%)

Table 2b Gene amplification

Gene	age (mean)	Mitoses High (>8)	Mitoses (mean)	Grade High (3)	LN meta negative	ER negative
ESR1						
EGFR	79	0	1	0	1 (100%)	0
FGFR1	57	8 (57%)	11	6 (43%)	9 (69%)	0
ADAM9	57	6 (50%)	10	4 (33%)	9 (82%)	1 (8%)
IKBKB	53	2 (33%)	8	2 (33%)	5 (83%)	1 (17%)
PRDM14	65	7 (78%)	13	6 (67%)	4 (44%)	0
MTDH	64	10 (77%)	14	7 (54%)	7 (58%)	1 (8%)
MYC	61	8 (73%)	12	7 (64%)	5 (46%)	1 (9%)
CCND1	63	13 (68%)	17	8 (42%)	9 (60%)	2 (11%)
EMSY	65	2 (100%)	18	2 (100%)	0	0
CDH1						
TRAF4	60	2 (50%)	8	1 (25%)	2 (50%)	1 (25%)
CPD						
MED1	60	4 (100%)	30	4 (100%)	1 (33%)	1 (25%)
HER2	60	4 (100%)	30	4 (100%)	1 (33%)	1 (25%)
CDC6	63	3 (75%)	11	1 (25%)	3 (75%)	1 (25%)
TOP2A	62	1 (50%)	10	1 (50%)	2 (100%)	0
MAPT						
BIRC5	74	1 (50%)	10	1 (50%)	1 (50%)	0
CCNE1						
AURKA	66	1 (25%)	11	0	2 (50%)	1 (25%)

Table 2c Gene loss.

Gene	age (mean)	Mitoses High (>8)	Mitoses (mean)	Grade High (3)	LN meta negative	ER negative
ESR1	63	2 (67%)	14	0	3 (100%)	0
EGFR						
FGFR1						
ADAM9	63	1 (100%)	14	1 (100%)	0	0
IKBKB						
PRDM14						
MTDH						
MYC						
CCND1	66	0	13	0	0	0
EMSY	67	0	14	0	2 (100%)	1 (33%)
CDH1	69	3 (30%)	16	1 (10%)	5 (50%)	0
TRAF4						
CPD						
MED1						
HER2						
CDC6						
TOP2A						
MAPT						
BIRC5						
CCNE1	70	1 (100%)	17	1 (100%)	0	0
AURKA	61	6 (46%)	11	5 (39%)	3 (30%)	1 (8%)

Supplemental Table 3 Multivariate analysis (Cox regression).

Table 3a Cox regression model with mitoses (>8), tumor size (>2.0 cm), grade (1/2 versus 3), *CCND1* amplification, *EMSY* amplification, *HER2* copy number gain, *MED1* copy number gain, Hormone therapy and chemotherapy.

Significant features	B	SE	Wald	df	Sig.	Exp(B)
Mitoses	,810	,450	3,236	1	,072	2,247
Size	1,285	,475	7,313	1	,007	3,615
Hormone therapy	-1,490	,510	8,520	1	,004	,225
CCND1 amplification	,955	,443	4,649	1	,031	2,599

Table 3b Cox regression model with mitoses (>8), tumor size (>2.0 cm), grade (1/2 versus 3), CCND1 amplification, EMSY amplification, HER2 copy number gain and MED1 copy number gain.

Significant features	B	SE	Wald	df	Sig.	Exp(B)
Grade	,972	,409	5,657	1	,017	2,643
Size	1,146	,463	6,130	1	,013	3,147
CCDN1 amplification	1,095	,452	5,877	1	,015	2,990

Table 3c Cox regression model with mitoses (>8), tumor size (>2.0 cm), grade (1/2 versus 3) and Cluster B.

Significant features	B	SE	Wald	df	Sig.	Exp(B)
Grade	,723	,398	3,309	1	,069	2,061
Size	1,074	,448	5,749	1	,016	2,928
Cluster B	1,227	,470	6,814	1	,009	3,412

Table 3d Cox regression model with mitoses (>8), tumor size (>2.0 cm), grade (1/2 versus 3) and copy number gain of all analyzed chromosome 17 genes

Significant features	B	SE	Wald	df	Sig.	Exp(B)
Mitoses	,927	,445	4,347	1	,037	2,528
Size	,896	,449	3,985	1	,046	2,450
Copy number gain all chromosome 17 genes	1,559	,556	7,851	1	,005	4,753

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6

Promoter hypermethylation in male breast cancer: analysis by multiplex ligation-dependent probe amplification

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ABSTRACT

Introduction

Epigenetic events are along with genetic alteration important in the development and progression of cancer. Promoter hypermethylation causes gene silencing and is thought to be an early event in carcinogenesis. The role of promoter hypermethylation in male breast cancer has not yet been studied.

Methods

In a group of 108 male breast cancers, methylation status of 25 genes was studied using methylation specific multiplex ligation-dependent probe amplification. Methylation of more than 15% was regarded indicative for promoter hypermethylation. Methylation status was correlated with clinicopathological features, patients' outcome and with 28 female breast cancer cases.

Results

Promoter hypermethylation of the genes *MSH6, WT1, PAX5, CDH13, GATA5* and *PAX6* was seen in more than 50% of the cases, while uncommon or absent in normal male breast tissue. High overall methylation status was correlated with high grade (p=0.003) and was an independent predictor of poor survival (p=0.048; hazard ratio 2.5). *ESR1* and *GSTP1* hypermethylation were associated with high mitotic count (p=0.037 and p=0.002, respectively) and high grade (both p=0.001). No correlation with survival was seen for individual genes. Compared to female breast cancers (logistic regression), promoter hypermethylation was less common in a variety of genes, particularly *ESR1* (p=0.005), *BRCA1* (p=0.010), *BRCA2* (p<0.001). The most frequently hypermethylated genes (*MSH6, CDH13, PAX5, PAX6* and *WT1*) were similar for male and female breast cancer.

Conclusion

Promoter hypermethylation is common in male breast cancer and high methylation status correlates with aggressive phenotype and poor survival. *ESR1* and *GSTP1* promoter hypermethylation seem to be involved in development and/or progression of high grade male breast cancer. Although female and male breast cancer share a set of commonly methylated genes, many of the studied genes are less frequently methylated in male breast cancer, pointing towards possible differences between male and female breast carcinogenesis.

INTRODUCTION

Along with genetic alterations, epigenetic events are important in cancer development and progression. Hypermethylation of CpG islands in promoter regions (further denoted "methylation") is the most well characterized epigenetic change and is a common mechanism for silencing tumor suppressor genes[1]. Methylation is reversible and therefore an attractive therapeutic target, and can serve as a marker for therapy response and prognosis[2].

Methylation is very common in virtually all cancer types, but can also be a physiological event, as in genomic imprinting[3]. Methylation is involved in the development of female breast cancer with frequent methylation of *PAX6, BRCA2, PAX5, WT1, CDH13* and *MSH6* in ductal carcinoma *in situ* and invasive ductal cancer[4]. On the contrary, methylation was less common in estrogen receptor (ER) negative, lymph node negative and *BRCA1*-associated female breast cancer[5]. Methylation is thought to be an early event in carcinogenesis of female breast cancer and methylation status of specific genes may therefore be useful as a potential screening target in clinical practice[4,6].

Most of the diagnostic and therapeutic algorithms for male breast cancer have been extrapolated from female breast cancer although we and others already demonstrated that there seem to be important differences between the two. Male breast cancers are more often hormone positive while HER2 amplified and basal-like breast cancers are rare in men[7-10]. Different genes and mechanisms of oncogene activation also play a role in the carcinogenesis of male breast cancer: high level amplification is less common, but whole chromosome arm gains are more often seen in male breast cancer[11]. Because of its general importance in carcinogenesis, methylation is probably also important in the development of male breast cancer, but this has not been studied yet.

Several techniques are available to assess methylation. The methylation specific multiplex ligation-dependent probe amplification (MS-MLPA) technique allows simultaneous evaluation of methylation status of a variety of genes in one PCR. With this high throughput approach, which shows good correlations with other methylation specific techniques, a reliable general view of methylation in several important tumor suppressor genes can be obtained[12,13].

In this study we investigated the role of methylation of several important tumor suppressor genes in male breast cancer using MS-MLPA. We correlated methylation patterns with clinicopathological features and prognosis. The results were also compared to a group of female breast cancers.

METHODS

Patients: specimens and clinical information

One hundred and ten consecutive cases of surgical breast specimens of invasive male breast cancer from 1986 - 2010 were collected from 4 different pathology labs in The Netherlands (St. Antonius Hospital Nieuwegein, Diakonessenhuis Utrecht, University Medical Center Utrecht, Laboratory for Pathology East Netherlands) as described in more detail before[10]. Hematoxylin and eosin (HE) slides were reviewed by three experienced observers (PJvD, RK, AM) 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. Mean age of these patients was 66 years (range: 32 - 89 years). Tumor size ranged from 0.8 to 5.5 cm (average: 2.2 cm). In 86% lymph node status was known and 55% of these patients had lymph node metastases. The majority of cases were diagnosed (according to the WHO) as invasive ductal carcinoma (90%). The remaining cases were lobular (n=3), mixed type (ductal/lobular) (n=2), invasive cribriform (n=1), papillary (n=1), mucinous (n=2), invasive micropapillary (n=1) or adenoid cystic carcinomas (n=1). According to the modified Bloom and Richardson score[14] most tumors were grade 2 (41%) or grade 3 (36%). Mitotic activity was assessed as before[15] with a mean mitotic index per 2 mm^2 of 11 (range 0-56). For all cases hormone receptor and HER2 status were re-assessed as described previously[10]. TMA slides were used for immunohistochemical stainings for ER, progesterone receptor (PR) and chromogenic *in situ* hybridization (CISH) for HER 2 assessment. Most tumors were ER (102/110, 93%) and PR positive (71/110; 65%), and *HER2* amplification was rare (4/110, 4%).

In addition normal male breast tissue was obtained from 10 autopsies. These patients had no history of a breast tumor. The subareolar region was resected and after fixation in 4% formalin, dissected and embedded in paraffin. From these blocks 4 µm sections were cut and stained for HE and if ducts were present, the areas richest in ducts were dissected for DNA isolation. Anonymous use of redundant tissue for research purposes is part of the standard treatment agreement with patients in our hospital[16]. Ethical approval was not required.

DNA extraction and MS-MLPA analysis

Representative tumor areas were identified on HE stained slides and corresponding areas (at least 1 cm^2) were dissected from 8 µm paraffin slides using a scalpel. DNA was extracted by overnight incubation in proteinase K (10mg/ml; Roche, Almere, The Netherlands) at 56°C. MS-MLPA was performed according to the manufacturers' instructions (MRC Holland, Amsterdam, The Netherlands), using a Veriti® 96-well thermal cycler (Applied Biosystems, Foster City, CA, USA). The ME002-B1 kit (MRC Holland), containing 25 tumor suppressor genes (Table 1), was used as before[4]. For the genes *MGMT* and *RB1* two different CpG probes were

available. The principle of MS-MLPA has been described elsewhere in more detail[17]. In short, MS-MLPA kits contain probes for methylation quantification, which are similar to those in conventional MLPA, except that the sequence detected by the MS-MLPA probes contains a restriction site for the methylation sensitive HhaI enzyme. After DNA denaturation and overnight incubation with the probe mix, the samples are divided into two tubes one of which is incubated with HhaI. In this tube, unmethylated DNA is digested and not exponentially amplified by PCR. Because methylated DNA is prevented from being digested by Hha1 these probes are ligated and therefore amplified by PCR. The ratio between probes incubated with and without Hha1 gives an estimation of the methylation status.

Table 1 Genes contained in the ME002-B1 MS-MLPA kit and frequencies of promoter hypermethylation (>15%) in 108 male breast cancer patients.

Gene	Hypermethylation	Chromosome	Gene name
MSH6	104 (96%)	02p16.3	mutS homologue 6
WT1	91 (84%)	11p13	Wilms tumour 1
PAX5	85 (79%)	09p13.2	Paired box 5
CDH13	83 (77%)	16q24.1	Cadherin 13, H-cadherin
GATA5	60 (56%)	20q13.33	GATA binding protein 5
PAX6	57 (53%)	11p13	Paired box 6
GSTP1	47 (44%)	11q13.1	Glutathione S-transferase p1
THBS1	21 (19%)	15q14	Thrombospondin 1
BRCA2	18 (17%)	13q13.1	Breast cancer gene 2
CD44	17 (16%)	11p13	CD44 molecule (Indian blood group)
TP73	14 (13%)	01p36.32	Tumour protein p73
TP53	12 (11%)	17p13.1	Tumour protein p53
ESR1	9 (8%)	06q25.1	Oestrogen receptor 1
CADM1	9 (8%)	11q23.2	Cell adhesion molecule 1
MGMT	8 (7%)	10q26.3	O-6-Methylguanine–DNA Methyltransferase
STK11	8 (7%)	19p13.3	Serine/threonine kinase 11
RARB	5 (5%)	03p24.2	Retinoic acid receptor beta
PTEN	5 (5%)	10q23.31	Phosphatase and tensin homologue
PYCARD	5 (5%)	16p11.2	PYD and CARD domain containing (TMS1)
RB1	3 (3%)	13q14.2	Retinoblastoma 1
BRCA1	2 (2%)	17q21.31	Breast cancer gene 1
CDKN2A	2 (2%)	09p21.3	Cyclin-dependent kinase inhibitor 2A (p14–ARF)
VHL	2 (2%)	03p25.3	von Hippel-Lindau
ATM	1 (1%)	11q22.3	Ataxia telangiectasia mutated
CHFR	1 (1%)	12q24.33	Checkpoint with forkhead and ring finger domains

Appropriate negative and positive (Sssi methylated DNA) controls were taken along with each MS-MLPA run. The PCR products were separated by electrophoresis on an ABI 3730 capillary sequencer (Applied Biosystems). Methylation analysis was carried out with Genescan v4.1 (Applied Biosystems) and Coffalyser v9.4 (MRC-Holland) software. First relative probe peaks were calculated by dividing the signal of each probe by the signal of every reference probe in one sample (intra sample normalisation). For final methylation status the ratio of relative probe peaks of the undigested sample (without Hha1) and the corresponding digested sample (with Hha1) were calculated for each probe. In case two CpG loci were present for one gene (*MGMT* and *RB1*), the mean methylation status was calculated for further analysis.
Promoter methylation ratio >0.15 (corresponding to >15% methylation) was regarded as indicative for promoter hypermethylation, based on cell line experiments and previous experiences[4,18]. The cumulative methylation index (CMI) was calculated as the sum of the methylation percentage of all genes[5].

Comparison to female breast cancer

A previously described group of female breast cancers was used to compare promoter hypermethylation in male and female breast cancer[4]. This group consists of 33 patients with invasive ductal carcinoma and a mean age of 55 years (range: 32 - 81 years). Tumor size ranged from 0.5 to 6.5 cm (average: 2.1 cm). Mean mitotic activity was 14 per $2mm^2$ and according to the modified Bloom and Richardson score most tumors were grade 2 (10/33; 30%) or grade 3 (17/33; 52%). ER positivity was common (27/31; 87%) and 71% of the tumors were PR positive (22/31). HER2 amplification was seen in 2 cases (2/31; 6%). The same tumor suppressor kit (ME002-B1 kit; MRC Holland) was used.

Statistics

Statistical calculations were performed using SPSS for Windows v15.0, regarding two-sided p-values <0.05 as significant. Correlations between promoter hypermethylation (>15% methylation) and clinicopathological characteristics were calculated with ANOVA for continuous variables and with Pearson χ^2 test (or Fisher's exact test when appropriate) for categorical variables. The following clinicopathological features were dichotomized: age (>50 years), tumor size (>2.0 cm), mitotic count (>8) and histological grade (1/2 vs. 3). Mann-Whitney test was used to calculate differences in CMI and clinicopathological features. Correlation between number of methylated genes and clinicopathological features was calculated with Spearman's rho. Unsupervised hierarchical clustering using the statistical program R (www.r-project.org) was performed to analyze relevant clusters and co-methylation. Absolute methylation percentages were used and all cases with methylation <5% were pooled together. Logistic regression analysis was performed to compare methylation in male and female breast cancer,

taking significant clinicopathological differences between the two groups into account. Backward stepwise method was used until the most predictive variables remained.
Survival data were obtained from the Integral Cancer registration The Netherlands (IKNL). Outcome data were available for 101 cases with a mean follow up of 5.7 years. Therefore, survival analysis were based on 5 years survival rates. For univariate survival analysis, Kaplan–Meier curves were plotted and analyzed with the logrank test. Multivariate survival analysis was done with Cox regression (enter and remove limits 0.10). CMI and number of methylated genes were dichotomized for survival analysis according to the most predictive threshold.

RESULTS

Methylation status by MS-MLPA

In two male breast cancer cases the amount of DNA was insufficient, leaving 108 cases for further analysis. Methylation status of the 25 analyzed tumor suppressor genes is presented in Table 1. All cases except one showed methylation (15% cutoff) of at least one gene with an average of 6 genes (range 0-26). Methylation was very common for *MSH6* (96%), *WT1* (83%), *PAX5* (79%) and *CDH13* (77%). On the contrary, methylation was very rare in *RB1* (3%), *BRCA1* (2%), *CDKN2A* (2%), *VHL* (2%), *ATM* (1%) and *CHFR* (1%). Mean CMI was 364 (range 129 - 904).
In male breast tissue derived from autopsies, gynecomastia was seen in three cases. The other seven cases harbored normal male breast ducts. Methylation was seen in the genes *MSH6* (4/10; 40%), *ESR1* (2/10; 20%), *PAX5* (1/10; 10%) and *CDH13* (1/10; 10%). No methylation was found in all of the other genes. Mean CMI in these cases was also low (16; range 11-27).

Correlation with clinicopathological features

Higher CMI was correlated with high mitotic count (p=0.046) and high grade (p=0.003). The number of methylated genes was significantly higher in grade 3 cancers (p=0.034), and correlated with high mean mitotic count (p=0.021). Two individual genes were associated with a more aggressive phenotype: mean mitotic count was higher in tumors with *ESR1* (10 versus 16; p=0.037) and *GSTP1* (8 versus 14; p=0.002) methylation. Both genes were also associated with high grade (both p=0.001). For *ESR1* 8/9 methylated tumors were grade 3 and for *GSTP1* 25/47 methylated tumors were grade 3. Finally, tumors with *MGMT* methylation had a mean tumor size of 3.2 cm, which was significantly larger compared to tumors without *MGMT* methylation (2.1 cm; p=0.002). No association was seen between any gene on the one hand and age, lymph node, PR and *HER2* status on the other.

Cluster analysis

Hierarchical cluster analysis revealed three groups of clustered genes (Figure 1). One group consisted of the genes *WT1*, *CDH13*, *MSH6*, *PAX5*, *GSTP1*, *GATA5*, and *PAX6*, seven genes in which methylation was very common. Indeed, in 15% of cases all these genes showed methylation. The second cluster was formed by genes with intermediate methylation rates (5-19%). In the third group the remaining genes clustered together. Methylation was rare in these genes (<8%). Reasoning from the patients, male breast cancer cases were not divided into clear distinctive clusters. At least four different groups could be identified and these clusters displayed no distinct clinicopathological features. One case did not fit into any of the groups. This grade 3 male breast cancer case showed a high methylation ratio in nearly all genes.

Comparison with female breast cancer

Because breast cancer is a heterogeneous disease only luminal type male breast cancer and luminal type female breast cancer (defined by ER and/or PR expression) were compared. In this approach age was the only clinicopathological features that was significantly different

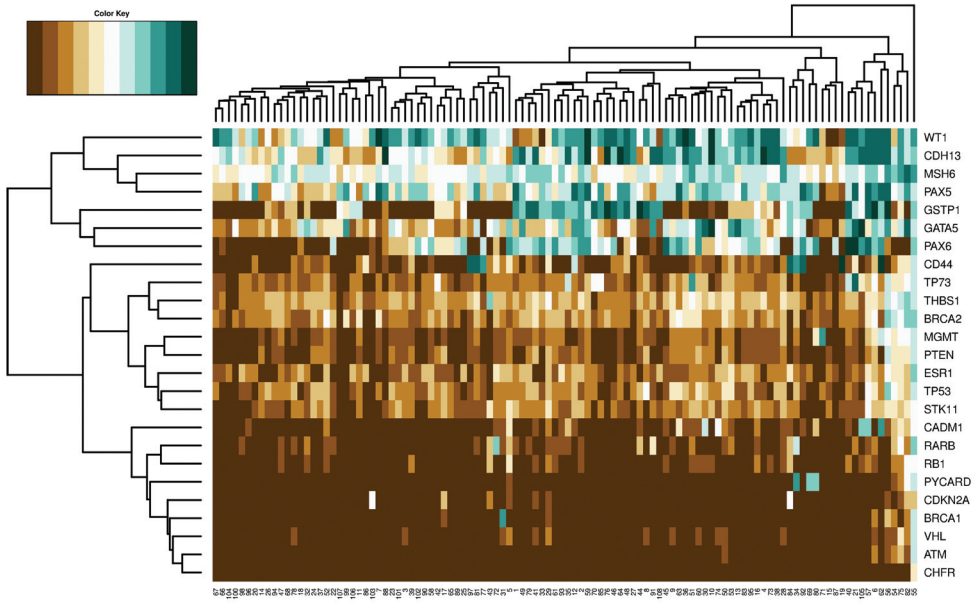

Figure 1 Unsupervised hierarchical clustering of absolute methylation percentages in 25 genes in 108 male breast cancer patients. One gene cluster consisted of WT1, CDH13, MSH6, PAX5, GSTP1, GATA5, and PAX6, seven genes in which methylation was very common. The second cluster was formed by genes with intermediate methylation rates (5-19%), and in the third cluster the remaining genes with little methylation (<8%) grouped together. No clear distinctive clusters of male breast cancer cases were found.

between the two groups. Male breast cancer patients were significantly older (66 versus 54 years; p<0.001). Figure 2 illustrates methylation status of the 25 studied genes in luminal type male (n=95) and luminal type female (n=28) breast cancers. Methylation was much less frequent in male breast cancer in a variety of genes. Particularly *ESR1*, *BRCA1* and *BRCA2* were less often methylated compared to female breast cancer and were strong independent predictors of gender in logistic regression analysis (p=0.005, p=0.010 and p<0.001, respectively). The genes *CD44* (p=0.050), *RARB* (p=0.026), *ATM* (p=0.017), and *STK11* (p=0.040) showed also less frequent methylation in male breast cancer. On the other hand, the high frequency of methylation in *MSH6*, *PAX5*, *PAX6*, and *CDH13* was shared between male and female breast cancer. Only age was taken into account during logistic regression analysis using gender as determinant, as no other clinicopathological feature was significantly different between the two groups. When leaving out age, using the Pearson χ^2 test, methylation in *PTEN* and *VHL* were also significantly less common in male breast cancer (p=0.029 and p=0.025, respectively). None of the studied genes was more frequently methylated in male breast cancer.

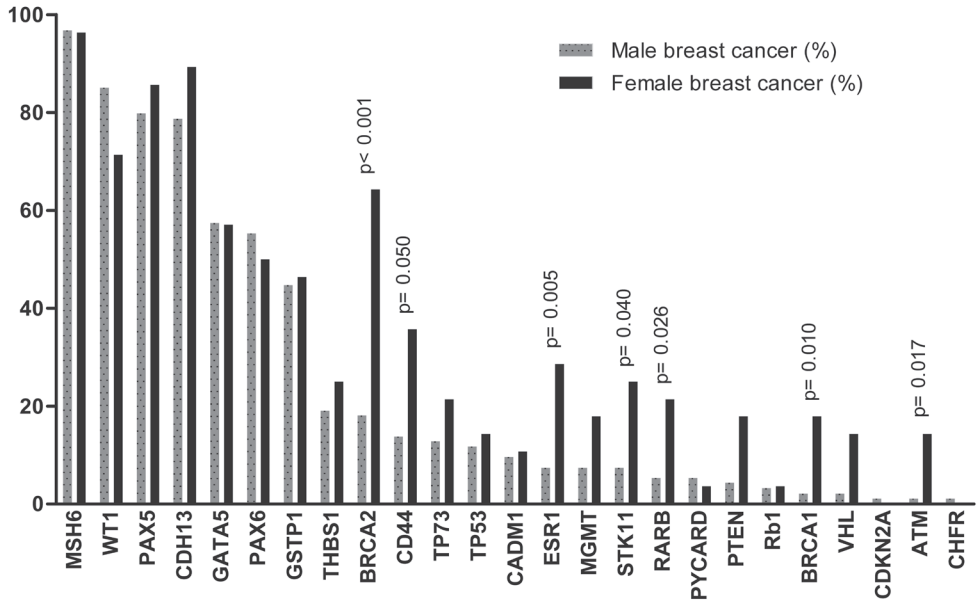

Figure 2 Promoter hypermethylation (>15% methylation) of the 25 studied genes in luminal type male (n=95) and female (n=28) breast cancers.

Survival analysis

Grade 3 (p=0.027), high mitotic count (>8; p=0.015) and large tumor size (>2.0 cm; p=0.036) were correlated with decreased 5 years survival as expected. No individual methylated gene was significantly correlated with patients' outcome, although tumors with *GATA5* methylation showed a trend towards decreased 5 years survival (64% versus 82%, p=0.083). However, when the number of methylated genes was dichotomized using a threshold of 6 methylated genes, the group with 6 or more methylated genes had significantly decreased survival compared to tumors with less than 6 methylated genes (p=0.022; Figure 3), but was not a significant independent prognostic factor in Cox regression (p=0.057). Tumors with high CMI (>350) had also poorer survival (p=0.033; Figure 3) and high CMI was an independent prognosticator in Cox regression (p=0.048; hazard ratio: 2.5).

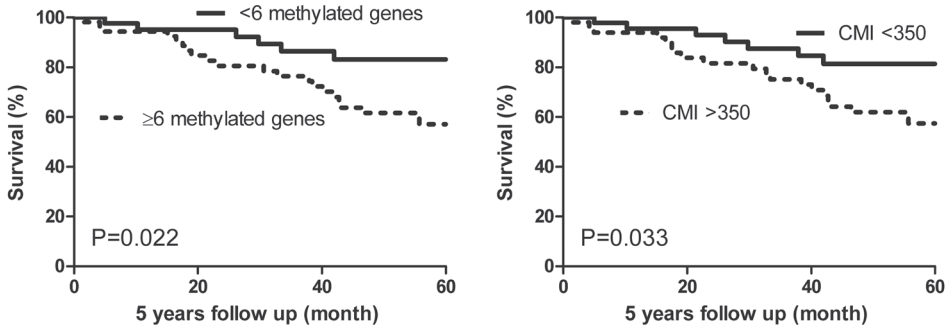

Figure 3 Five years survival with corresponding p-values (log rank) according to high number of methylated genes (≥6) and high cumulative methylation index (>350).

DISCUSSION

Promoter hypermethylation is an important gene silencing mechanism thought to be an early event in carcinogenesis[1]. Understanding the epigenetic role in male breast cancer is important to gain further insight in male breast carcinogenesis and for the identification of potential biomarkers for diagnosis and treatment[19,20]. Epigenetic changes in male breast cancer had not yet been studied and therefore we investigated promoter hypermethylation in a large group of 108 patients with this rare disease using the high throughput approach MS-MLPA, enabling evaluation of methylation status of a variety of genes in one PCR.

Not surprisingly, methylation does occur in male breast cancer. The genes *MSH6*, *WT1*, *PAX5*, *CDH13*, *GATA5* and *PAX6* showed promoter hypermethylation in more than 50% of

cases, indicating that these genes are probably often involved in male breast carcinogenesis. These genes are required for normal development of several organ systems and/or play a role in DNA repair, cell adhesion, cell growth and migration, although the function of some of these genes is still poorly understood[21-25]. Loss of function of both alleles leads to complete knock down of these genes which may facilitate malignant transformation. Methylation, with aberrant silencing of one of these alleles, could be the initiating event, the second hit or both[26]. *MSH6* methylation was also quite common in the normal male breast, although at a lower frequency than our group of male breast cancer cases. The other commonly methylated genes in male breast cancer were not found to be methylated in our 10 cases of normal male breast tissue, confirming the important role of methylation in the development of male breast cancer.

In male breast cancer methylation was very rare in *BRCA1*, *CDKN2A*, *VHL*, *ATM* and *CHFR* (<2%), indicating that methylation of these genes does not seem to play a prominent role in male breast carcinogenesis.

Male breast cancer with an aggressive phenotype harbored an increased number of methylated genes and had a higher CMI. In addition, tumors with 6 or more methylated genes or high CMI had a worse outcome. High CMI was even an independent predictor of poor survival when corrected for grade, mitotic count and tumor size. This indicates that accumulation of methylated genes and an overall higher methylation status seem to be important in the development of more aggressive male breast cancer with poor survival. The hallmark of high grade breast cancer is genetic instability[27], which in male breast cancer seems to include accumulation of methylated genes. A similar trend was noted in female breast cancer and female breast cancer patients with increasing number of methylated genes have a worse outcome as well[4,28,29].

Two single genes were identified in which methylation was correlated with high mitotic count and high grade: *ESR1* and *GSTP1*. High grade breast cancer is believed to arise from high grade precursor lesions by gaining different genetic and epigenetic changes compared to low grade breast cancer[30,31]. *ESR1* and *GSTP1* methylation could be important in the development of these high grade male breast cancers. *GSTP1* belongs to a family of metabolic enzymes and is involved in the detoxification of carcinogens and chemotherapeutic agents by conjugating them with glutathione[32]. In female breast cancer *GSTP1* hypermethylation is correlated with high grade ductal carcinoma in situ and high grade invasive breast cancer, presence of lymph node metastasis and poor outcome[4,29,33,34]. ER, encoded by *ESR1*, is an important factor in breast cancer, because studies in females have shown that patients with hormone negative tumors do not benefit from endocrine therapy[35]. In the present study we could not

demonstrate a relation between ESR1 methylation and ER expression, although this needs to be interpreted with caution since only 7 out of 108 cases were ER negative in the present study. Another recent study also concluded that the relation between *ESR1* methylation and protein expression is weak and unlikely to represent a predominant mechanism of ER silencing[36]. There was also no relation between methylation and expression of TWIST as shown by us, so this may not be unusual[37]. Larger series of ER negative male breast cancer cases will be needed to further explore this relationship. Similar to female breast cancer methylation of *ESR1* seems to be a biomarker for high malignant male breast cancer. Indeed in female breast cancer *ESR1* promoter hypermethylation has been correlated with poor prognosis[38]. *ESR1* and *GSTP1* methylation were not significantly correlated with poor survival in our group of male breast cancer and therefore do not seem to be useful prognostic biomarkers in male breast cancer.

Compared to female breast cancer, methylation was less common in male breast cancer in several of the studied genes, particularly *ESR1, BRCA1* and *BRCA2. BRCA1* and *BRCA2* promoter hypermethylation was encountered in respectively 2% and 18% in male breast cancer, but was seen in 18% and 64% in female breast cancer, using the same approach and similar cutoff criteria. These results points towards possible important differences between female and male breast carcinogenesis with regard to methylation. *BRCA1* methylation is more common in relatively young, premenopausal women[39], which could explain the higher incidence in female breast cancer, since the male breast cancer patients were significantly older than the female breast cancer patients. However, in the present study we corrected for age in logistic regression, so gender specific differences also seem to play a role here. Differences in genetic predisposition may also influence the epigenetic profile of these tumors and could be responsible for some of the differences found in promoter hypermethylation between male and female breast cancer. Approximately 10% of men with breast cancer are known to have a genetic predisposition, and especially BRCA2 mutations seem to be important[40]. Unfortunately no data regarding BRCA germline mutations were available for both cohorts, but it seems quite probable that there is a higher rate of hidden BRCA2 mutations carriers in the male breast cancer group. This may well explain the lower rate of BRCA2 promoter hypermethylation in the male breast cancer group compared to female breast cancers[41]. Interestingly, genes with frequent methylation in male breast cancer (*MSH6, CDH13, PAX5, PAX6* and *WT1*) were also very commonly methylated in female breast cancer.
The methylation status of both groups was obtained using the same technique. However, the male breast cancer cases were microdissected by a scalpel and the female breast cancer cases by laser microdissection. Although the latter method is more precise we do not think this may have influenced our results. The male breast cancer tumors were quite large and rich in

tumor cells and could therefore be well harvested for DNA isolation based on scalpel dissection. Besides, MLPA is relatively insensitive to tumor cell content[42].

CONCLUSIONS

Methylation seems to be important in de development of male breast cancer. More than 50% of the tumors showed methylation in *MSH6, WT1, PAX5, CDH13, GATA5* and *PAX6*. The accumulation of methylated genes and an overall high methylation status was correlated with a more aggressive phenotype and poor survival. *ESR1* and *GSTP1* were the only single genes associated with mitotically active and high grade male breast cancers. Compared to female breast cancer, methylation occurred less often in male breast cancer. On the other hand, the most frequently methylated genes were shared between male and female breast cancer. Our results point towards differences in carcinogenesis between male and female breast cancer, hidden behind similarities.

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The 3-layered Ductal Epithelium in Gynecomastia

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ABSTRACT

Introduction

Gynecomastia is the most common abnormality in the male breast and has been associated with male breast cancer, but whether there is an etiological role remains unknown. In the present study we conducted an immunohistochemical investigation to further characterize gynecomastia.

Methods

A total of 46 cases of gynecomastia were immunohistochemically stained on tissue microarrays for estrogen receptor (ER), progesterone receptor, HER2, androgen receptor, cytokeratins (CK5, CK14, CK7, CK8/18), p63, E-cadherin, BRST2, CyclinD1, Bcl-2, p53, p16, p21 and Ki67. In addition, 8 cases of male ductal carcinoma *in situ* and normal breast tissue obtained from autopsies (n=10) and adjacent to male breast cancer (n=5) were studied.

Results

Normal ductal male breast epithelial cells were very often ER and Bcl-2 positive (> 69%) and progesterone receptor and androgen receptor expression was also common (> 39%). Gynecomastia showed a consistent 3-layered pattern: 1 myoepithelial and 2 epithelial cell layers with a distinctive immunohistochemical staining pattern. The intermediate luminal layer, consisting of vertically oriented cuboidal-to-columnar cells, is hormone receptor positive and expresses Bcl-2 and CyclinD1. The inner luminal layer is composed of smaller cells expressing CK5 and often CK14, but is usually negative for hormone receptors and Bcl-2. Male ductal carcinoma *in situ* was consistently ER positive and CK5/CK14 negative.

Conclusion

For the first time we describe the 3-layered ductal epithelium in gynecomastia, which has a distinctive immunohistochemical profile. These results indicate that different cellular compartments exist in gynecomastia and therefore gynecomastia does not seem to be an obligate precursor lesion of male breast cancer.

INTRODUCTION

The male breast is composed of rudimentary ducts, without lobule formation, unless it is exposed to high levels of endogenous or exogenous estrogen[1]. Gynecomastia is the most common disease of the male breast and is often seen in newborn male infants, adolescent and elderly men. Hormonal imbalance between stimulation of estrogens and inhibition of androgens is the key factor in the pathogenesis of gynecomastia[2]. In neonates, estrogen levels are high due to exposure to maternal estrogens. In puberty there is a relative increase in estrogen production and in aging men there is an imbalance in increasing estrogen levels and decreased testosterone levels. In addition to these physiological alterations, drugs and several diseases, such as Klinefelter's syndrome, obesity and adrenal and testicular neoplasms, can lead to hormonal imbalances and cause gynecomastia[3,4].

The main clinical challenge is to discriminate gynecomastia from male breast cancer. Mammography and high resolution ultrasound can be helpful in distinguishing between these breast lesions[5]. The role of gynecomastia in the development of male breast cancer remains uncertain. Gynecomastia is often seen alongside invasive breast cancer, but gynecomastia is also frequently encountered in healthy men[2,6,7]. However, several studies found a significant correlation between gynecomastia and male breast cancer[8,9]. Further, atypical ductal hyperplasia and ductal carcinoma *in situ* (DCIS), 2 known precursor lesions for breast cancer in females, have been reported in male breasts with gynecomastia[10-12]. Histologically, gynecomastia can be divided into 3 groups. The florid type, reflecting recent onset, the fibrous (quiescent or inactive) type, probably the end stage of gynecomastia (>6-12 mo), and the intermediate type with features of both the florid and fibrous type.

Only a few studies have been published on the immunohistochemical profile of gynecomastia[13-15] and expression of biomarkers, such as cytokeratins, has not well been described. Therefore, in this study, we have used a broad panel of immunohistochemical markers to further characterize gynecomastia.

METHODS

Patient material

A total of 46 gynecomastia cases from 2000 to 2010 were retrieved from the archives of the Department of Pathology of the University Medical Centre Utrecht. Only surgical resections were used. Original hematoxylin and eosin stained (H&E) slides were reviewed by 2 experienced observers (R.K., P.J.v.D.) to confirm the diagnosis and to subtype gynecomastia. The florid type showed intraductal epithelial proliferation with a flat or micropapillary

appearance or both. The surrounding stroma had increased cellularity, accompanied by edema and prominent vessels. Some degree of fibrosis could be seen and often pseudoangiomatous stromal hyperplasia (PASH) was present. The fibrous type showed prominent periductal or confluent fibrosis, without high cellularity or edema and less conspicuous or absent intraductal epithelial proliferation. The third type is the intermediate type, with features of both florid and fibrous type[16]. The presence of PASH was scored separately.

Male breast tissue was also obtained from 10 autopsies. These patients had no history of breast tumors. The subareolar region was resected, fixed in 4% formalin, dissected and embedded in paraffin. From these blocks 4 µm serial sections were cut and stained with H&E, and used for immunohistochemical stainings, in case ducts were present. The H&E-stained slides were carefully examined by 3 observers (R.K., A.H.J.V.-M., P.J.v.D.) to evaluate the ductal and stromal appearances. Further, the presence of gynecomastia was noted. We also reviewed original H&E-stained slides from a large group of male breast cancer patients to identify "normal" male breast epithelium adjacent to invasive carcinoma[17]. Finally, 8 cases with DCIS in men were retrieved from the archives of the Department of Pathology of the University Medical Centre Utrecht.

Immunohistochemistry

Immunohistochemical staining on gynecomastia cases was performed using tissue microarray (TMA) blocks. H&E-stained slides were used to identify representative areas of gynecomastia with a high density of ducts. From these areas, 1 large 2.0-mm punch biopsy from the formaldehyde-fixed and paraffin-embedded tissue blocks was taken and embedded in a recipient paraffin block, using a precision tissue array instrument (Beecher Instruments, Sun Prairie, WI, USA). Sections of 4 µm thickness were cut and immunohistochemistry for estrogen receptor (ER), progesterone receptor (PR), HER2, androgen receptor (AR), cytokeratin 5 (CK5), cytokeratin 14 (CK14), cytokeratin 7 (CK7), cytokeratin 8/18 (CAM5.2), p63, E-cadherin, BRST2, CyclinD1, Bcl-2, p53, p16, p21 and Ki67 (Table 1) was performed using a Bond-Max autostainer (Leica Microsystems, Wetzlar, Germany) with the Bond polymer refine detection kit (Leica Microsystems, DS9800). Epidermal growth factor receptor (EGFR) staining was carried out manually as described previously[18]. In addition, triple stainings were performed for CK5-ER-p63 and CK5-AR-p63, respectively. For final identification of the 3 antibodies in 1 slide, they were consecutively developed with permanent red chromogen (DAKO, Glostrup, Denmark) for CK5, Vector blue alkaline phosphatase substrate kit III (Vector laboratories; Burlingame, CA, USA) for p63 and diaminobenzidine for ER and AR. Normal breast tissue derived from autopsy was also stained for ER, PR, CK5, CK14, Bcl-2 and CyclinD1, using whole tissue sections.

The DCIS cases and normal male breast tissue adjacent to invasive male breast cancer were stained for ER, CK14 and CK5 on whole tissue sections. Appropriate positive and negative controls were used throughout.

Scoring and statistics

The immunohistochemical stainings were scored by consensus of 2 experienced observers (R.K., P.J.v.D.). The percentages of positively stained cells were estimated and averaged and used for a semiquantitative approach. Expression levels were divided into 4 groups: no reactivity (-), faint expression in < 10% of the cells (+/-), moderate expression in 10 to 50% of the cells (+) and strong expression in > 50% of the cells (++). Luminal and myoepithelial cell layers were scored separately.

For correlations between categorical variables Pearson χ^2 test (or Fisher's exact test when appropriate), was calculated using SPSS for Windows version 15.0. P-values of < 0.05 were regarded as significant.

Table 1 Antibodies used for immunohistochemical characterization of gynecomastia and normal male breast.

Antibody	Source	Clone	Dilution	Antigen retrieval
ER	DAKO	1D5	1:200	EDTA
PR	DAKO	PgR636	1:100	Citrate buffer
HER2	Neomarkers	SP3	1:100	EDTA
Ki67	DAKO	MIB-1	1:100	Citrate buffer
AR	Novocastra	AR27	1:20	Citrate buffer
BCL-2	DAKO	124	1:200	Citrate buffer
CyclinD1	Neomarkers	SP4	1:40	EDTA
BRST2	Signet	D6	1:400	none
p21	DAKO	SX118	1:40	EDTA
p53	Biogenex	BP53-12	1:100	Citrate buffer
CK5	Novocastra	XM26	1:100	EDTA
CK14	Neomarkers	LL002	1:400	EDTA
EGFR	Zymed	31G7	1:30	Prot K
p63	Neomarkers	4A4	1:400	EDTA
CK7	Biogenex	OV-TL 12/3	1:800	Citrate buffer
CK8/18	BD Bioscience	CAM5.2	1:40	Pepsine
E-cadherin	Novacastra	36B5	1:40	EDTA

DAKO, Glostrup, Denmark; Neomarkers, Fremont, CA, USA; Novocastra, Newcastle, United Kingdom; Signet laboratories, Dedham, MA, USA; Biogenex, San Ramon, CA, USA; Zymed, Carlsbad, CA, USA; BD Bioscience, Franklin Lakes, NJ, USA.

RESULTS

In male breast tissue derived from autopsies, gynecomastia was seen in 2 cases (2/10; 20%). In 1 case no ducts were found in the tissue blocks. In 7 cases (age: 57; range 38-72) "normal" male breast tissue was successfully identified, and this showed a relatively consistent pattern (Table 2 and Figure 1) with sparse ducts and no lobule formation. The outer layer of the ducts was formed by a single layer of myoepithelial cells that were positive for CK5 and CK14. The inner luminal cells were almost always arranged in a crowded single layer, had a cuboidal shape, and showed some apical snout formation. Most luminal cells showed strong expression for ER (69%). PR and AR staining was also relatively common, with 39% and 48% positive luminal cells, respectively. There was a large variability in CK5 and CK14 expression between the ducts (mean: 15% and 6% respectively). Often only a few single cells were positive, but there were also ducts with large groups of mainly CK5 positive and sometimes CK14 positive luminal cells. Bcl-2 was strongly expressed in nearly all luminal cells (81%) and very few cells showed nuclear staining for CyclinD1 (< 1%). The "normal" ducts adjacent to invasive carcinoma (n=5) showed a staining pattern similar to that of "normal" male breast tissue derived from autopsy with high expression of ER and only sporadic positive luminal cells in the CK5 and CK14 staining. All 8 DCIS cases were of grade 2 and consistently showed CK5 and CK14 staining in the myoepithelial cells, whereas all luminal cells expressed ER, but were negative for CK5 and CK14.

The 46 gynecomastia cases consisted of 21 florid type (46%), 11 fibrous type (24%) and 14 (30%) intermediate type. Patients' ages ranged from 14 to 77 years (mean age: 49 y).

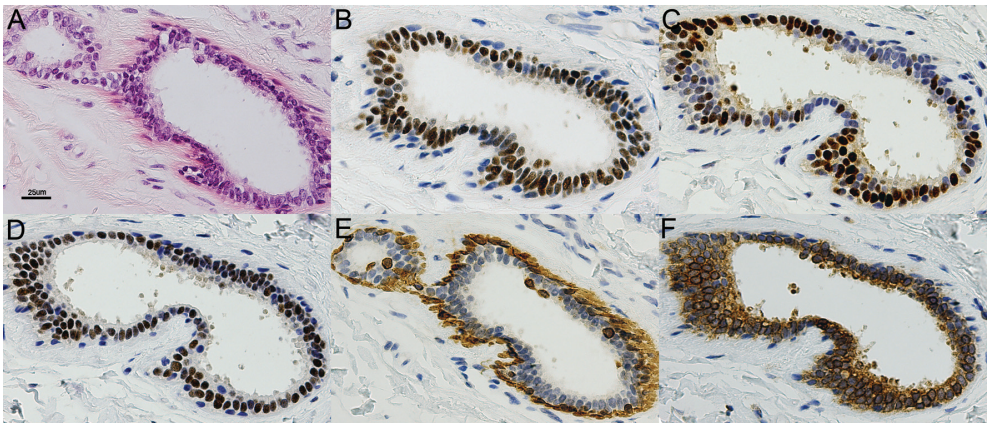

Figure 1 Normal male breast showing a single layer of crowded luminal cells in the H&E staining (A) with high expression of ER (B), PR (C) and AR (D). Only single luminal cells are positive in the CK5 staining along with strong staining of the myoepithelial cells (E). Bcl-2 is also strongly positive (F)

Two peak ages were seen: 14 to 28 years (n=17), reflecting gynecomastia in adolescents/ young men, and 54 to 77 years (n=29), reflecting gynecomastia in aging men. PASH was present in 50% of the cases (23/46) and was seen in 81% (17/21) of the florid type gynecomastia, whereas only 1 case (1/11; 9%) of the fibrous type gynecomastia showed PASH ($p<0.001$). Secreted fluid in the lumen was common.

Biomarker profile of gynecomastia

The immunohistochemical profile of the gynecomastia cases is presented in Table 3 and Figure 2. No differences were seen in morphology or immunohistochemical expression patterns between younger and older patients. All ducts were surrounded by an outer layer of myoeptihelial cells with expression of CK5, CK14 and p63. Interestingly, in most cases of gynecomastia the ducts were lined by 2 epithelial cell layers with a distinctive immunohistochemical staining pattern, and these layers could also be identified in the H&E staining. The intermediate luminal layer consisted of cuboidal-to- columnar vertically oriented cells. The nuclei had a regular oval shape with occasional small nucleoli. The cytoplasm was eosinophilic with vague cell borders and these cells often showed luminal tufts. The vast majority of these cells expressed ER, PR and AR. The inner luminal layer is composed of slightly smaller and more horizontally oriented cells. Most often these cells were arranged in a single layer, but more layers and micropapillary formations were also present. These cells had regular to slightly irregular small nuclei with only a sporadic small nucleolus.

Using immunohistochemistry, this inner luminal layer was easily identified because of consistent expression of CK5 in 67% of cells. Also CK14 was common (21%), but expression of hormone receptors (ER, PR and AR) was rare and p63 was absent. In both layers nearly all cells showed strong expression of CAM5.2. In the triple stainings this stratification was visualized more readily, as the expression patterns of different markers were demonstrated in the same cell (Figure 2G, H). In 5 cases, no evident 3-layered architecture was identified.

Table 2 Immunophenotype of luminal epithelium in ducts from normal male breasts (n=7).

Antibody	luminal layer (mean % positive cells)	SD	Range
CK5	+ (15%)	14	3-40
CK14	+/- (6%)	5	1-15
ER	++ (69%)	16	50-95
PR	+ (39%)	15	20-60
AR	+ (48%)	19	33-80
Bcl-2	++ (81%)	11	60-90
CyclinD1	- (<1%)	<1	0-1

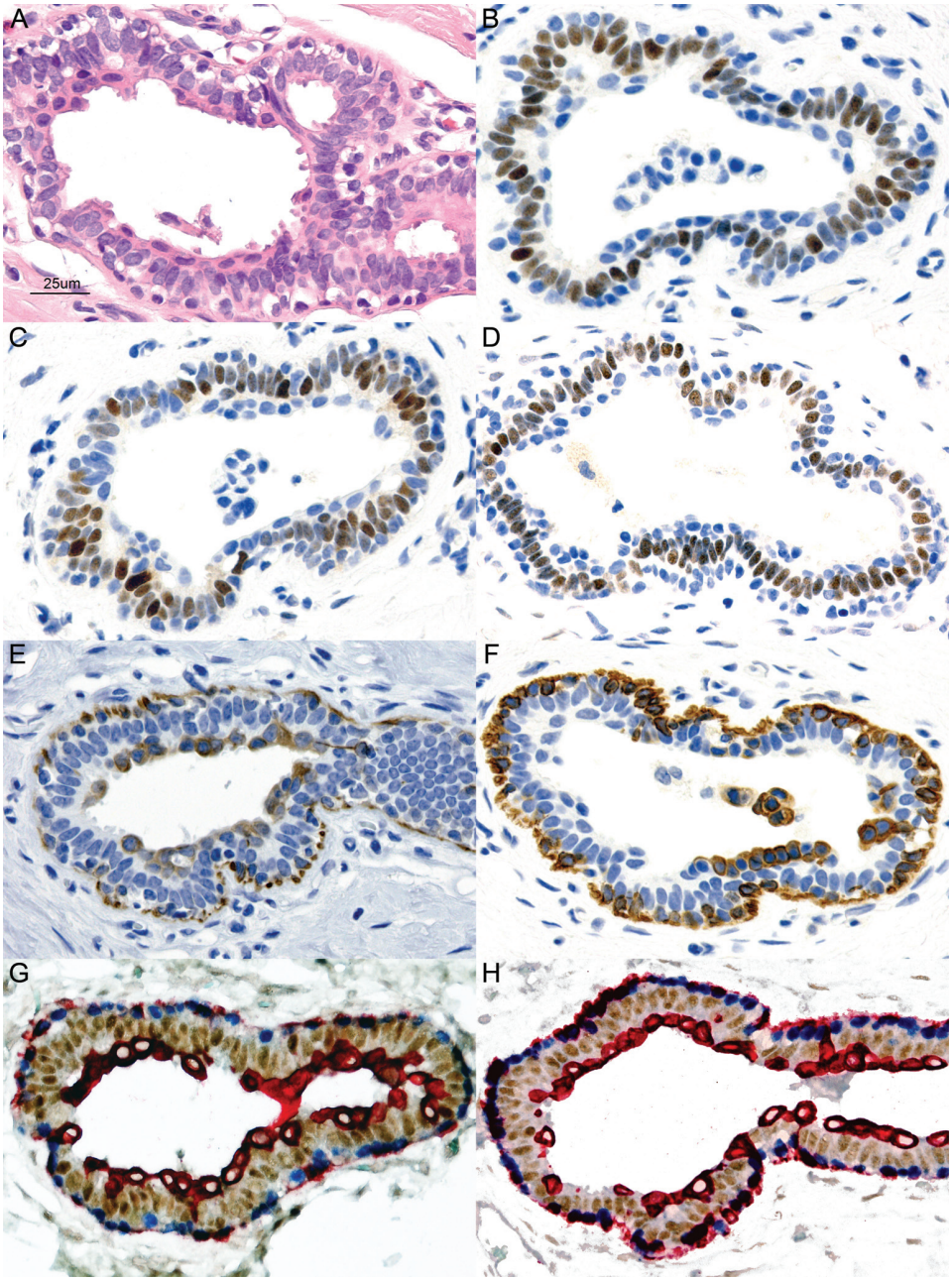

Figure 2 The 3-layered ductal epithelium in gynecomastia, which can be identified in the H&E staining (A). The cells in the intermediate luminal layer show high expression of ER (B), PR (C) and AR (D). The inner luminal layer shows high ratios of CK5-positive cells alongside with staining of the myoepithelial cells (E). Some inner luminal cells also show expression for CK14 (F). The 3 layers are also illustrated in 2 triple stainings (G: CK5-ER-p63 and H: CK5-AR-p63). CK5 is developed in red, p63 in blue and ER and AR in brown.

Table 3 Expression patterns for the three layers of gynecomastia

Antibody	Inner luminal layer (mean % positive cells)	SD	Intermediate luminal layer (mean % positive cells)	SD	Myoepithelial (mean % positive cells)	SD
CK5	++ (67%)	23	+/- (6%)	10	++ (84%)	15
CK14	+ (21%)	25	+/- (1%)	2	++ (99%)	7
ER	+/- (6%)	6	++ (83%)	18	- (0%)	0
PR	+/- (4%)	5	++ (77%)	14	- (0%)	0
AR	+/- (2%)	2	++ (75%)	18	- (0%)	0
Bcl-2	+ (23%)	21	++ (91%)	12	- (0%)	0
CyclinD1	+/- (1%)	1	+/- (9%)	16	- (0%)	0

No significant association was found between type of gynecomastia and the presence or absence of these 3 layers.

The 3-layered composition of the ductal epithelium in gynecomastia was also seen in the other immunohistochemical markers. Bcl-2 showed strong staining in 91% of the cells in the intermediate luminal layer, whereas the cells in the inner luminal layer were usually negative or only weakly positive (23%). In most cases moderate-to-strong Bcl-2 staining was seen in stromal cells (74%) and this was strongly correlated with the type of gynecomastia ($p<0.001$), with all cases of gynecomastia florid type and 55% of gynecomastia intermediate type showing Bcl-2 staining in stromal cells. Only 1 case of gynecomastia fibrous type showed moderate-to-strong Bcl-2 staining in stromal cells. CyclinD1 showed nuclear staining confined to the intermediate luminal cells with a variable frequency (range, 0 to 60%; mean 9%). Ki67 positive cells were rare (< 2%) and were seen in the intermediate and inner luminal layers. All epithelial cells showed strong staining in the CK7 and E-cadherin. HER2 staining was completely negative and p21 showed sporadic positive cells both in the luminal layers and in the surrounding stroma. In the p53 staining, only wild type expression of single cells in ducts or stroma was seen. In most cases ducts were negative for BRST2 and EGFR. Interestingly, in 74% of the gynecomastia cases, prominent stromal staining was seen for EGFR, and this was correlated with the presence of PASH ($p=0.002$)

DISCUSSION

The male breast is composed of rudimentary ducts ending in terminal buds, generally without lobule formation. In comparison with the female breast, relatively little is known about the male breast and its diseases. Gynecomastia is by far the most common disease in male breasts

and is often seen in association with male breast cancer; however it is also frequently encountered in healthy men[2,6,7]. In the present study, the morphology and immunohistochemical marker profile of gynecomastia were studied and compared with findings in normal male breast tissue. The most important finding of our study was a consistent 3-layered composition of ducts in gynecomastia, composed of a myoepithelial, an intermediate luminal and a, to our knowledge, not previously described inner luminal cell layer.

The outer myoepithelial cell layer surrounds the ducts and expresses CK5, CK14 and p63, and is negative for hormone receptors (ER, PR and AR), Bcl-2 and CyclinD1, similar to the female breast. The 2 luminal cell layers can be observed on H&E staining, but are more easily identified by immunohistochemistry. The intermediate luminal layer, consisting of vertically oriented cuboidal-to-columnar cells, is hormone receptor positive (ER, PR and AR) and expresses Bcl-2 and CyclinD1. These cells seem to be responsible for luminal tuft formation and are probably responsible for the secretion of protein-rich fluid in the lumen, which is commonly seen. The inner luminal layer is composed of slightly smaller and more horizontally oriented cells and shows expression of CK5 and often CK14, but is usually negative for hormone receptors, Bcl-2 and CyclinD1. All types of gynecomastia showed these 3 layers. Only single sporadic inner luminal cells were seen in normal male breast epithelium obtained from autopsies and adjacent to invasive male breast carcinoma. Autopsy-derived cases with gynecomastia also showed this 3-layered pattern.

In usual ductal hyperplasia in female patients there is a proliferation of 2 cell types showing a heterogeneous expression for CK5 and ER, which are intermingled in a “polyclonal” mosaic-type pattern and more or less fill up the lumen[19,20]. These proliferating cells belong to intermediary glandular cells (CK5+ and CK8+) and differentiated glandular cells (CK5- and CK8+) according to a previously described progenitor cell concept of breast epithelium[21]. In gynecomastia, proliferation of these 2 cell types is also seen, but interestingly these cells are arranged in a characteristic pattern with CK5- and CK8/18+ cells in an intermediate luminal layer and CK5+ and CK8/18+ cells arranged in an inner luminal layer. The ER-positive cells are also arranged in this characteristic pattern and represent the intermediate luminal layer of gynecomastia. In most cases of usual ductal hyperplasia in female patients, intraductal zones of contiguous ER positivity of variable size can be seen. However, in many areas the ER-positive cells are mixed with and separated by ER-negative cells and sometimes a higher proportion of ER-positive cells at the periphery can be noted[22]. In gynecomastia there is an ER-positive and ER-negative cell compartment as well. In light of these findings, gynecomastia does not seem to be a likely precursor of breast cancer, because there is a proliferation of 2 cell types such as seen in usual ductal hyperplasia in female patients, which is considered to be a carcinogenetic cul-de-sac. Why these cells are arranged in different layers is yet to be determined. The hyperplastic nature of these cells is further supported by the fact that the

8 DCIS cases in men, analyzed in the present study, were negative for CK5 and CK14. This is in line with the immunohistochemical profile of DCIS in women and confirms the fact that usual ductal hyperplasia and gynecomastia, on the one hand, and atypical ductal hyperplasia and DCIS on the other, represent phenotypically distinct lesions[19,20]. Nevertheless, CK5/6 and CK14 expression has been reported in some cases of DCIS in men[23], which is in line with the fact that a small percentage of male breast cancer shows CK5 and/or CK14 expression[17,23].

The high ratio of ER-positive, PR-positive and AR-positive cells in the intermediate luminal layer could reflect hormonal imbalance, which is important in the pathogenesis of gynecomastia[2]. There was also strong expression of Bcl-2 in these epithelial cells and stromal cells. Bcl-2 expression of stromal cells was seen in all cases of gynecomastia florid type, but in only 1 case of gynecomastia fibrous type, indicating that stromal cells seem to be important in the initiation and development of gynecomastia. Bcl-2 and ER are strongly correlated in breast epithelium and Bcl-2 is probably upregulated because of high levels of estrogens, by mechanisms that are still not clearly defined[24,25]. Also, ducts in normal male breasts show high levels of ER, PR and AR expression, as described before[22]. Similar to postmenopausal women, there are low levels of circulating estrogens in men. Most of these estrogens are synthesized in the peripheral tissue and have local effects[26,27]. These locally produced estrogens seem to be very potent in stimulating the growth of ER-positive cells and are important in the development of ER-positive breast cancers in postmenopausal females[28]. In fact, postmenopausal women have higher ratios of ER-positive cells in normal ducts compared with premenopausal women, but do not reach the ER expression level of the male breast[19,22]. As most ductal epithelial cells in the male breast are ER positive, the high ratio of Bcl-2-positive cells is not unexpected.

PASH was seen frequently in the cases with gynecomastia, in line with a previous report[29]. In the present study, we demonstrated that PASH was particularly seen in the florid type gynecomastia. The high incidence of PASH in the present study is probably due to the relatively high frequency of the florid type of gynecomastia. PASH was associated with stromal staining for EGFR, which is an interesting finding deserving further investigation.

In conclusion, for the first time we report that the ductal epithelium in gynecomastia consistently shows a 3-layered pattern: an outer myoepithelial layer, an intermediate luminal layer expressing hormone receptors, Bcl-2 and CyclinD1, and an inner luminal layer with expression of CK5 and often CK14, but without expression of hormone receptors, Bcl-2 and CyclinD1. In light of these findings, gynecomastia does not appear to be a precursor lesion of male breast cancer.

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8

Summary and future perspectives

Male breast cancer is a rare disease and most of the knowledge has been extrapolated from females. However, it had already been demonstrated that there are differences between male and female breast cancer and experts agree that male breast cancer should be considered a unique disease, rather than a male variant of female cancer[1]. Male breast cancer is more frequently estrogen receptor (ER) and progesterone receptor (PR) positive, while female breast cancers more frequently (over)express *HER2* and accumulate p53[2-4]. Like postmenopausal women, locally (in the breast itself) produced estrogens seem to be important in the development of ER positive male breast cancer[5,6]. A better understanding of male breast carcinogenesis is crucial for developing novel targets suitable for personalized and effective treatment and perhaps imaging. A major problem in studying male breast cancer is the fact that large series of male breast cancer patients are lacking, because it is a rare disease. Most published studies are therefore based on small single institutional series with a maximum of 50 patients.
For the present thesis we collected a large multi-institutional cohort of male breast cancer patients to study immunophenotype and genotype of male breast cancer. These male breast cancer patients were collected from four different pathology labs in The Netherlands (St. Antonius Hospital Nieuwegein, Diakonessenhuis Utrecht, University Medical Center Utrecht, Laboratory for Pathology East Netherlands) and two hospitals in Germany (Paderborn and Cologne). A total 134 male breast cancer patients were collected, which represents one of the largest group of male breast cancers published until now. The results of the different studies from this thesis are summarized in this chapter and future perspectives are discussed as well.

Tremendous efforts have been made to subdivide female breast cancer patients into clinical and prognostic relevant subgroups beyond classical clinicopathological features. Molecular studies have become very popular in the last decade and based on gene expression profiles several distinctive "molecular" subtypes have been identified[7-9]. Immunohistochemical surrogates have been developed for this classification, since gene expression profiling is not yet routinely feasible[10,11]. Only a few studies based on small series have tried to classify male breast cancer using immunohistochemistry[12,13]. In **Chapter 2** we therefore studied the molecular subtypes of male breast cancer defined by immunohistochemistry. Luminal type A (ER+ and/or PR+ and *HER2*- and Ki67 low) was by far the most frequently encountered subtype of male breast cancer, representing 75% of the cases. Luminal type B (ER+ and/or PR+, and *HER2*+ or Ki67 high) was seen in 21% and the remaining 4% of cases were classified as basal-like (ER-, PR-, *HER2*-, CK5/6+ and/or CK14+ and/or EGFR+) and unclassifiable triple-negative (negative for all 6 markers). No *HER2* driven (ER-, PR-, *HER2+*) cases were identified. Luminal type B seem to represent a subtype with an aggressive phenotype with a high mitotic

count and high grade. The distribution of molecular subtypes in male breast cancer is clearly different compared to female breast cancers, with more luminal type and rare *HER2* driven, basal like and unclassifiable triple negative male breast cancers, pointing to possible important differences in carcinogenesis.

In order to identify novel clinical and prognostic relevant markers for male breast cancer patients, fibrotic focus (FF) and the expression of hypoxia related markers, hypoxia inducible factor-1α (HIF-1α), carbonic anhydrase IX (CAIX) and Glut-1, were studied in **Chapter 3**. These markers are correlated with aggressive tumors and adverse patients' outcome in female breast cancer patients[14-20]. FF was seen in 25% of the cases and correlated with high grade (p=0.005), high mitotic activity (p=0.005) and presence of lymph node metastases (p=0.037). Hypoxia is thought to be the crucial link between FF and tumour phenotype and progression, which is confirmed by the significant correlation between FF and HIF-1α overexpression (p=0.023). The presence of a large FF (>8mm) correlated with poor survival (p=0.035), but was not an independent prognostic marker in Cox regression. HIF-1α positive tumours were more often high grade (p=0.003) and *HER2* amplified (p=0.005). Glut-1 expression was also more common in grade 3 tumours (p=0.038), but no associations were found for CAIX. HIF-1α overexpression was correlated with decreased patients' outcome (p=0.008) and was an independent and even the most powerful predictor of survival in Cox regression analysis (p=0.029; hazard ratio 2.5). The presence of a FF as well as HIF-1α overexpression should be regarded as markers for aggressive behaviour and seem to have similar clinical and prognostic importance as reported in female breast cancer.

Proteins are important in tumor biology and can be evaluated in every pathology laboratory. The proteins can be used as indicators of therapy response and/or as prognosticators. These biomarkers can also be used in unsupervised hierarchical clustering, which seem to be a potent tool in subdividing female breast cancer patients in novel clinically relevant groups[21,22]. The clinical and prognostic implications of several immunohistochemical markers still need to be determined in male breast cancer. In **Chapter 4** the expression patterns of 14 widely used biomarkers were studied and correlated with clinicopathological features and prognosis. High mitotic count and high grade were correlated with high Ki67, *HER2* amplification/ overexpression, p53 accumulation, high p21, low PR and Bcl-2 expression. PR and p53 were the most promising individual prognostic markers, as they were independent markers for poor survival. Using unsupervised hierarchical clustering, four distinctive and prognostic relevant male breast cancer groups were identified, indicating that protein expression profiling may be clinically useful in male breast cancer.

Gene amplification is important in the development and progression of cancer and could serve as a potential biomarker for prognosis or as a target for molecular therapy. Compared to female breast cancer, there is yet little knowledge on the genetic makeup of male breast cancer and most of the few available studies are based on small single institutional series. These few recently performed gene expression studies in men showed that there might be important differences in molecular profile between male and female breast cancer[23-25]. However, the clinical and prognostic significance of genetic alterations in relevant breast cancer genes still need to be elucidated in male breast cancer. Therefore in **Chapter 5** copy number changes of 21 (female) breast cancer related genes were studied using the high throughput genomic technique multiplex ligation-dependent probe amplification (MLPA). Gene copy number gain of *CCND1, TRAF4, CDC6* and *MTDH* was seen in >40%, with also frequent amplification, indicating that these genes probably play an important role in male breast carcinogenesis. The number of amplified genes and several single amplified genes were associated with high grade, but only *CCND1* amplification was an independent predictor of adverse survival in Cox regression (p=0.015; hazard ratio 3.0). Using unsupervised hierarchical clustering a distinctive group of male breast cancer with poor prognosis (p=0.009; hazard ratio 3.4) was identified, characterized by frequent *CCND1*, *MTDH, CDC6, ADAM9, TRAF4* and *MYC* copy number gain. All luminal type male breast cancers (n=101) were compared to a group of 73 cases of luminal type female breast cancer. *EGFR* (p=0.005) and *CCND1* (p=0.041) copy number gain were more often seen in male breast cancer, while gain of *EMSY* (p=0.004) and *CPD* (p=0.001) and amplification of *TRAF4* (p=0.024) and *EMSY* (p=0.041) were less frequent, pointing towards differences in carcinogenesis between male and female breast cancer and emphasizing the importance of identifying biomarkers and therapeutic agents based on research in male breast cancer itself.

Along with genetic alterations, epigenetic events are important in the development and progression of cancer. Hypermethylation of CpG islands in promoter regions is the best characterized epigenetic change and is an important gene silencing mechanism thought to be an early event in carcinogenesis[26]. Because of its importance in carcinogenesis, methylation is probably also important in the development of male breast cancer, but this had not been studied yet. Therefore, promoter hyerpmethylation of 25 tumor suppressor genes was studied in male breast cancer using methylation specific MLPA (MS-MLPA), as described in **Chapter 6**. Promoter hypermethylation of the genes *MSH6, WT1, PAX5, CDH13, GATA5* and *PAX6* was seen in >50% of the cases, while uncommon or absent in normal male breast tissue, indicating that promoter hypermethylation of these genes are important in the carcinogenesis of male breast cancer. High overall methylation status was correlated with high grade (p=0.003) and was an independent predictor of poor survival (p=0.048; hazard ratio 2.5). *ESR1* and *GTSP1*

were the only individual genes for which promoter hypermethylation correlated with tumor phenotype, both with a high mitotic count (p=0.037 and p=0.002) and high grade (both p=0.001), indicating that promoter hypermethylation of these genes are involved in development and/or progression of high grade male breast cancer. However no correlation with survival was seen for these or other individual genes. Compared to luminal type female breast cancers, promoter hypermethylation was less common in a variety of genes, particularly *ESR1* (p=0.005), *BRCA1* (p=0.010), *BRCA2* (p<0.001). The most frequently hypermethylated genes (*MSH6, CDH13, PAX5, PAX6* and *WT1*) were similar for luminal type male and female breast cancer. Although male and female breast cancer apparently share a set of commonly methylated genes, many of the studied genes are less frequently methylated in male breast cancer, pointing towards possible differences between male and female breast carcinogenesis.

Gynecomastia is the most common disease of the male breast and occurs due to hormonal imbalance between stimulation of estrogens and inhibition of androgens. Whether or not gynecomastia has an etiological role in development of male breast cancer is still debated. Gynecomastia is often seen alongside male breast cancer and atypical ductal hyperplasia and ductal carcinoma *in situ* (DCIS), two known precursor lesions for breast cancer in females, have been reported in male breasts with gynecomastia[27-30]. However, gynecomastia is also frequently encountered in healthy men and is from an epidemiological point of view not a likely or at least a very rare precursor[31,32]. Since the expression of biomarkers, like cytokeratins, has not well been described in gynecomastia a broad panel of immunohistochemical markers was used to further characterize gynecomastia, as described in **Chapter 7**. For the first time we described the three layers of gynecomastia, which can be appreciated in a HE staining, but are more easily identified by immunohistochemistry. The outer myoepithelial cell layer surrounds the ducts and expresses CK5, CK14 and p63. The intermediate luminal layer, consisting of vertically oriented cuboidal to columnar cells, is hormone receptor positive (ER, PR and AR) and expresses Bcl-2 and CyclinD1. The inner luminal layer is composed of slightly smaller and more horizontally orientated cells and shows expression of CK5 and often CK14, but is usually negative for hormone receptors, Bcl-2 and CyclinD1. These results indicate that different cellular compartments exist in gynecomastia. In light of these findings, gynecomastia does not seem to be a precursor lesion of male breast cancer, because there is a proliferation of two cell types such as seen in usual ductal hyperplasia in females, which is considered to be a carcinogenetic cul-de-sac.

FUTURE PERSPECTIVES

Although male breast cancer incidence is rising, it remains a rare disease with approximately 100 cases each year in The Netherlands[33-36]. Acquiring sufficient numbers of male breast cancer patients to allow robust translational and fundamental research is one of the major challenges in studying male breast cancer. Probably in the (near) future there is a need for even larger series, particularly in case different therapy regimes and novel agents are tested in men with breast cancer. Collaborative efforts are therefore essential in moving forward and to study more complex issues[1]. Several international male breast cancer consortia have been set up and also in The Netherlands there are collaborative efforts and of course we will share our material and participate within these projects[1,37].

Until now most of the treatment regimes are extrapolated from female breast cancers. However, in the present thesis we demonstrated that there are important differences between male and female breast cancer, supporting the idea that male and female breast cancers are distinctive and unique diseases. The oncologist should be aware of this and realize that male breast cancer patients could respond differently to standard treatment and may actually benefit from different treatment regimes. Furthermore, the differences between male and female breast cancer described in the present thesis emphasizes the importance of testing (novel) treatment regimes in male breast cancer patients themselves. Endocrine therapy is the keystone in adjuvant treatment of male breast cancer, as the vast majority is hormone positive[2,3,38]. Aromatase inhibitors are currently recommended in postmenopausal women with hormone positive breast cancer during adjuvant treatment[39]. Like in postmenopausal women, most of the estrogens are synthesized in the peripheral tissue, by aromatization of precursors. These peripherally produced estrogens have only local effects[5,6]. Aromatase inhibitors prevent the conversion to estrogen and may therefore also be a potent tool in the treatment of male breast cancer patients. However, in men 20% of the circulating estrogens are produced in the testis which is an aromatase independent process. Therefore aromatase inhibitors could be less effective in male breast cancer and these drugs need to be studied in more detail, before implementing them in the treatment of male breast cancer[40].
Because of its rarity, treatment of male breast cancer patients should probably be focused in specialized facilities. These hospitals, like the University Medical Center Utrecht where we have opened a special outpatient clinic for male breast cancer, are able to work with state of the art treatment regimes, collaborate in prospective studies and are able to facilitate fundamental and translational research. In this way, knowledge and experience is built up much more quickly, resulting in more standardized treatment. Collecting fresh frozen tumor tissue is also a standard procedure in our laboratory.

In the present thesis we studied the genetic makeup of a variety of breast cancer related genes by using MLPA. With this high throughput approach several genes were identified which are involved in the development and progression of male breast cancer. We also found high rates of FGFR1 and ADAM9 gene copy number gain, two genes suitable for targeted therapy[41,42]. Novel molecular based treatment regimes targeting these genes could have great potential in male breast cancers with gains or amplification of these genes. Still the knowledge of the genetic makeup of male breast cancer is limited and our results should be confirmed by others.

In our MLPA study we found several cases with copy number gains in all genes located on chromosome 17, indicating polysomy of this chromosome. This is interesting, as polysomy of chromosome 17 has been denied in female breast cancers, which could indicate different mechanisms of oncogene activation in a subset of male breast cancers[43-48]. Therefore we will use a dedicated chromosome 17 MLPA kit to study the presence of chromosome 17 polysomy in male breast cancer. Chromosome 16 also seems to be an interesting chromosomal region in male breast cancer. Chromosome 16q loss is associated with ER positive and luminal type of female breast cancer[49]. Because most male breast cancer cases are ER positive and of luminal type, this chromosomal region is probably often involved in male breast cancer. Nowadays new methods are available to study chromosomal regions. Next generation sequencing is a recently developed technique which is able to analyze high quality nuclear acid sequence data of the whole genome in a relative short period and with relative low costs [50]. We already successfully sequenced eight cases of male breast cancer with this next generation approach and want to extend this group in the near future.

For the first time we studied epigenetic alterations in male breast cancer and described the role of promoter hypermethylation in several important tumor suppressor genes in male breast cancer by using MS-MLPA. With this technique we identified several genes with high methylation ratios in male breast cancer, while being unmethylated in normal male breast tissue. These genes probably play a role in carcinogenesis of male breast cancer. It will be of interest to study epigenetic changes in several other tumor suppressor genes as well. A (novel) platform based approach could be helpful to study methylation patterns of a large amount of genes at once. Methylation is reversible and therefore an attractive therapeutic target and several demethylating agents have been developed recently targeting these processes[51]. However, first our results need to be confirmed by others and several clinical trials need to be done before these agents can be used in daily practise.

In chapter 7 we described the three layers of gynecomastia. We identified an inner luminal layer with a distinctive immunohistochemical profile. To further study these different epithelial

cell compartments, cell lines could be produced to further characterize these cells and study the biology of these cell compartments *in vitro*. Pseudoangiomatous stromal hyperplasia (PASH) was often seen in gynecomastia specimens. The pseudo-vascular luminal areas seen in PASH did show high expression of EGFR, indicating that EGFR plays a role in PASH and / or gynecomastia formation. To further elucidate the role of EGFR in PASH and gynecomastia several EGFR ligands, like EGF and TGFα, could be stained to evaluate whether EGFR is functional or not. Also cell lines of fibroblast isolated from gynecomastia specimens could be produced to study these cells *in vitro*.

Mammary stem cells play a role in repopulating the breast. In females these cells facilitate rapid expansion and regression during pregnancy and each menstrual cycle. Several markers have been used to identify human (cancer) stem cells, of which Aldehydedehydrogenase 1 (ALDH1) is among the most widely used[52]. The exact role of ALDH1 in the mammary stem cells is largely unknown, but expression has been correlated with ER, PR negative and *BRCA1* related breast cancer[52,53]. ALDH1 expression have not yet been studied in normal male breast, gynecomastia or male breast cancer. The male breast is, in contrast to the female breast, a non functional rudimentary organ and therefore stem cells seem to be less important in the male breast.

In conclusion, data presented in this thesis provide a better understanding of male breast cancer development and progression. Several biomarkers were identified and differences with female breast cancer were highlighted. Finally the present thesis provides proof that gynecomastia is a hyperplastic lesion and should not be regarded as a precursor lesion of male breast cancer.

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Nederlandse samenvatting

NEDERLANDSE SAMENVATTING

(ook voor niet medici)

Borstkanker wordt gezien als een aandoening specifiek voor vrouwen. Toch zijn er jaarlijks ongeveer 100 mannen in Nederland bij wie de diagnose borstkanker wordt gesteld en dit aantal neemt toe. Omdat borstkanker bij mannen een relatief zeldzame diagnose is, waardoor het lastig is om (voldoende) grote groepen te verzamelen, is er nauwelijks gefundeerd onderzoek verricht naar deze aandoening. Behandelingsprincipes van borstkanker bij mannen worden, bij gebrek aan kennis, overgenomen van behandelingsprincipes bij vrouwen. Het is echter al langer bekend dat er belangrijke verschillen zijn tussen borstkanker bij mannen en vrouwen.

De mannelijke borst is een rudimentair / niet functioneel orgaan en is opgebouwd uit vet, steunweefsel en verspreid spaarzame klierbuizen, zonder dat er lobjes (klierweefsel) worden gevormd. Borstkanker bij mannen is in het overgrote deel (en vaker dan borstkanker bij vrouwen) hormoongevoelig, doordat er een groot aantal hormoonreceptoren op deze tumorcellen aanwezig zijn. Dit komt doordat er, net als bij vrouwen na de overgang, slechts een geringe hoeveelheid in het bloed circulerende oestrogenen (belangrijk hormoon) aanwezig is. De oestrogenen worden voornamelijk lokaal in het borstweefsel geproduceerd en hebben een direct effect op de omliggende cellen. Het effect van deze lokaal geproduceerde oestrogenen is krachtig en waarschijnlijk verantwoordelijk voor het hoge aantal oestrogeen- en progesteronreceptor positieve tumoren bij mannen en vrouwen na de overgang. Verder zijn mannelijke borstkanker patiënten gemiddeld ouder dan vrouwelijke. Erfelijke factoren spelen in ca. 15-20% van de gevallen een rol en nader genetisch onderzoek lijkt gewenst indien borstkanker bij een man is gediagnosticeerd. Voornamelijk het BRCA2 gen speelt een prominente rol, terwijl het BRCA1 gen bij mannen minder belangrijk lijkt, in tegenstelling tot borstkanker bij vrouwen. Hiernaast lijken er ook verschillen te bestaan op eiwit niveau en moleculair niveau tussen borstkanker bij mannen en vrouwen.

Deze verschillen tussen borstkanker bij mannen en vrouwen onderstrepen het belang om borstkanker bij mannen te beschouwen als een unieke ziekte. Het is dus essentieel om wetenschappelijk onderzoek te verrichten naar borstkanker bij mannen, om zo kennis te vergaren over de ontstaanswijze van deze ziekte en wetenschappelijk gefundeerde therapiekeuzes te kunnen maken. Nu deze klinische relevantie is doorgedrongen, moeten voldoende grote onderzoeksgroepen gevormd worden om betrouwbaar onderzoek te kunnen doen. Samenwerking met verschillende instituten is hiervoor noodzakelijk. Het materiaal van de in dit proefschrift beschreven onderzoekspopulatie is afkomstig uit 4 ziekenhuizen in Nederland (UMC Utrecht, Diakonessenhuis Utrecht, Antonius Ziekenhuis Nieuwegein en het laboratorium Oost Nederland) en tevens uit 2 instituten in Duitsland (Paderborn en Keulen). Door

deze samenwerking hebben we materiaal en patiëntgegevens van 134 mannen met borstkanker kunnen verzamelen, een van de grootste groepen tot nog toe beschreven in de literatuur. Van deze patiënten werden belangrijke tumoreigenschappen geëvalueerd (delingsactiviteit, tumorgraad, tumorgrootte, lymfklier metastase) en er werden overlevingsdata opgevraagd bij het IKNL om uiteindelijk de prognose van deze patiënten te analyseren. Van elke patiënt was een weefselblokje beschikbaar, waarmee tissue microarray weefselblokken werden gemaakt. Tissue microarray weefselblokken bevatten van elke tumor 3 kleine biopten (diameter 0.6 mm) genomen uit de oorspronkelijke weefselblokjes. Met deze methode kunnen op een snelle en goedkope manier eiwitexpressie patronen, met behulp van immunohistochemische kleuringen, onderzocht worden van alle 134 tumoren. Tevens werd er DNA geïsoleerd voor moleculair onderzoek.

In **hoofdstuk 2** worden, aan de hand van immunohistochemische kleuringen, de 134 borstkanker tumoren bij mannen ingedeeld in 5 verschillende groepen: (moleculaire subtypes) luminal type A, luminal type B, *HER2* gedreven, basal like en niet classificeerbaar tripel negatief. Deze verschillende groepen hebben specifieke genetische veranderingen en hebben bovendien therapeutisch en prognostische voorspellende waarde. Het blijkt dat bijna alle borstkanker tumoren bij mannen geclassificeerd dienen te worden als luminal type A (75%) en luminal type B (21%). Er worden slechts 4 tumoren gevonden die tot de basal like tumoren behoren en 1 tumor die niet classificeerbaar tripel negatief is. Er worden geen *HER2* gedreven tumoren aangetroffen. De luminal type B tumoren zijn gecorreleerd met ongunstige tumorkenmerken en vertegenwoordigen de oestrogeenreceptor positieve tumoren met een agressief gedrag. Deze verdeling van moleculaire subtypes is duidelijk anders in vergelijking met vrouwen, waar bijvoorbeeld ca. 17% van de tumoren basal like zijn, hetgeen wijst op fundamentele verschillen in borstkanker bij mannen en vrouwen.

Op zoek naar nieuwe en niet eerder beschreven kenmerken van agressieve tumoren en voorspellers van een ongunstige prognose bij mannelijke borstkankerpatiënten hebben we in **hoofdstuk 3** de rol van fibrotic focus en de expressie van hypoxie (zuurstof gebrek) gerelateerde markers geanalyseerd. Een fibrotic focus is een littekenachtig gebied middenin een tumor bestaande uit bindweefsel en onregelmatige gerangschikte bloedvaten. Een fibrotic focus is omgeven door celrijke tumorgebieden. Wij hebben voor het eerst aangetoond dat de aanwezigheid van een fibrotic focus, net als bij borstkanker bij vrouwen, gecorreleerd is met agressieve borstkanker tumoren bij mannen. Indien een fibrotic focus groter is dan 8 mm hebben mannelijke borstkanker patiënten een significant slechtere prognose. Hypoxie is waarschijnlijk de verbindende factor tussen fibrotic focus en agressieve tumoren. Er bestaan verschillende hypoxie gerelateerde markers, die bij borstkanker bij vrouwen gerelateerd zijn

aan een slechte prognose. Wij beschrijven in hoofdstuk 3 onder andere de rol van Hif-1a (één van de hypoxie gerelateerde markers) in borstkanker bij mannen. Overexpressie van Hif-1a is gecorreleerd met agressieve tumoren, de aanwezigheid van een fibrotic focus en een slechte prognose. Hif-1a heeft additionele prognostische waarde aan de in de dagelijkse praktijk gebruikte tumorkenmerken.

Om het eiwit expressieprofiel van borstkanker bij mannen te onderzoeken hebben we 14 veel gebruikte immunohistochemische markers getest op alle 134 tumoren. De resultaten staan beschreven in **hoofdstuk 4**. Tumoren met overexpressie van ki67, HER2, p21, p53 en tumoren negatief voor progesteronreceptor en bcl-2 tonen agressieve/ongunstige tumorkernmerken. Bovendien kunnen de markers p53 en progesteronreceptor gebruikt worden als prognostische markers, aangezien patiënten met tumoren waarin p53 overexpressie en patiënten met tumoren negatief voor progesteronreceptor een significant slechtere prognose hebben, bovenop de in de dagelijkse praktijk gebruikte tumorkenmerken. Door al deze markers met een rekenkundig model (unsupervised hierarchical clustering) te combineren kunnen 4 groepen worden onderscheiden. Deze clusters hebben unieke tumorkenmerken en ook significante verschillen in overleving. Met deze methode kunnen borstkanker tumoren bij mannen ingedeeld worden in klinisch en prognostisch relevante groepen.

In hoofdstuk 5 en 6 wordt op DNA niveau onderzoek gedaan naar borstkanker bij mannen. Hiervoor werd gebruik gemaakt van de techniek multiplex probe-ligation amplification (MLPA) waarmee in één run tientallen genen tegelijk kunnen worden geanalyseerd.

In **hoofdstuk 5** wordt de rol van genkopie toename in 25 borstkanker gerelateerde (onco) genen onderzocht. Toename van kopieën van bepaalde (onco)genen spelen een belangrijke rol in het ontstaan van kanker. Indien de toename van het aantal genkopieën hoog is (>10 kopieën) spreekt men van amplificatie. Toename van kopieën in de genen *CCND1*, *TRAF4* en *CDC6* en de genen op chromosoom 8 (voornamelijk *MTDH*) wordt aangetroffen in >40% van de tumoren, waarbij vaak sprake is van amplificatie. Deze genen spelen waarschijnlijk een belangrijke rol in het ontstaan en / of progressie van borstkanker bij mannen. Toename van kopieën in verschillende genen is gecorreleerd met agressievere tumoren, maar alleen amplificatie van *CCND1* is een onafhankelijke prognostische factor. De resultaten hebben we ook vergeleken met 73 borstkanker tumoren van vrouwen met een vergelijkbaar eiwitexpressie profiel, die onderzocht zijn met behulp van dezelfde methode. Er zijn een aantal duidelijke verschillen zichtbaar in de toename van aantal kopieën van de verschillende genen. Genkopie toename van *EGFR* en *CCND1* wordt significant vaker gezien in borstkanker bij mannen en toename van aantal kopieën van de genen *EMSY* and *CPD* wordt vaker aangetroffen bij

vrouwen. Ook gen amplificatie van verschillende genen wordt vaker gezien in borstkanker bij vrouwen. Deze resultaten duiden op moleculaire verschillen tussen mannen en vrouwen met borstkanker.

Naast genetische afwijkingen spelen ook epigenetische veranderingen een belangrijke rol in het ontstaan en progressie van kanker. Met epigenetische veranderingen worden veranderingen in genfunctie bedoeld zonder dat hier structurele afwijkingen aan het genetisch materiaal aan ten grond slag ligt. Promoter hypermethylatie geldt als een van de belangrijkste epigenetische veranderingen en kan resulteren in het stilleggen van een gen. De functie van dit gen gaat dan verloren en indien dit een tumorsuppressorgen betreft kunnen cellen kwaadaardig ontaarden (tumorsuppressorgenen remmen de groei en ontwikkeling van tumoren). De rol van promoter hypermethylatie in borstkanker bij mannen werd voor het eerst door ons onderzocht en de resultaten staan beschreven in **hoofdstuk 6**. Promoter hypermethylatie in de genen *MSH6, WT1, PAX5, CDH13, GATA5* and *PAX6* wordt gezien in >50% van de gevallen, terwijl dit niet of nauwelijks wordt aangetroffen in het genetisch materiaal van normaal borstweefsel van de man. Promoter hypermethylatie van deze genen speelt dus waarschijnlijk een belangrijke rol in het ontstaan en / of progressie van borstkanker bij mannen. Promoter hypermethylatie in de genen *ESR1* en *GTSP1* wordt vaker gezien in tumoren met agressieve kenmerken, er wordt echter geen relatie met overleving gevonden. Patiënten met tumoren waarin meerdere gemethyleerde genen en een gemiddeld hoge methylatie status hebben vaak agressievere tumoren en een slechtere prognose. De resultaten werden ook vergeleken met 33 borstkankertumoren van vrouwen die onderzocht zijn met dezelfde methode. Hierbij komt naar voren dat de meest voorkomende genen met promoter hypermethylatie van vrouwen en mannen met borstkanker identiek zijn. Echter er zijn ook vele genen die vaker promoter hypermethylatie tonen in borstkanker tumoren bij vrouwen in vergelijking met tumoren bij mannen. Dus ondanks overeenkomsten zijn er ook op epigenetisch niveau duidelijke verschillen tussen tumoren van mannen en vrouwen met borstkanker.

De rol van gynaecomastie (borstvorming bij mannen) in het ontstaan van borstkanker bij mannen staat nog steeds ter discussie. **Hoofdstuk 7** beschrijft een morfologische en immunohistochemische studie van gynaecomastie. In deze studie beschrijven we de 3 lagen van de klierbuizen in gynaecomastie. Rondom de klierbuizen bevindt zich, net als rond alle goedaardige klierbuizen in de borst bij de man en de vrouw, een myoepitheliale cellaag. Voorts werden 2 luminale (aan de binnenzijde van de klierbuis) cellagen aangetroffen, waarvan de binnenste nog niet eerder in deze setting in de literatuur beschreven is. Deze 2 luminale lagen zijn met behulp van immunohistochemische kleuringen makkelijk te herkennen,

aangezien de intermediaire luminale laag positief is voor hormoonreceptor kleuringen, maar negatief voor cytokeratine 5 en 14, terwijl de binnenste luminale cellaag negatief is voor de hormoonreceptor kleuringen en positief voor cytokeratine 5 en 14. Het feit dat gynaecomastie ontstaat uit deze 2 celcompartimenten pleit ervoor, analoog aan intraductale hyperplasie bij vrouwen waarin ook 2 celcompartimenten aanwezig zijn, dat gynaecomstie geen voorloper laesie is van borstkanker bij mannen.

Samenvattend, het huidige promotieonderzoek geeft inzicht in de factoren die een rol spelen bij het ontstaan en de progressie van borstkanker bij mannen. Deze determinanten worden beschreven op histologisch niveau, eiwit niveau, genetisch en epigenetisch niveau. Voorts worden meerdere biomarkers beschreven die van prognostische waarde zijn en waarvan sommigen een rol kunnen spelen in de (toekomstige) behandeling. Dit promotieonderzoek levert bewijs dat er belangrijke verschillen zijn tussen borstkanker bij mannen en vrouwen, hetgeen het belang van het doen van onderzoek in grote groepen mannelijke borstkanker patiënten benadrukt. Bovendien hebben we voor het eerst beschreven dat de klierbuizen in gynaecomastie bekleed zijn met 3 cellagen. Deze gelaagdheid pleit, analoog aan intraductale hyperplasie bij vrouwen, voor het feit dat gynaecomastie geen voorloper laesie is van borstkanker bij mannen.

Dankwoord

Curriculum Vitae

List of Publications

DANKWOORD

Het huidige promotieonderzoek had niet tot stand kunnen komen zonder hulp van een groot aantal mensen. Hierbij dien ik feitelijk de hele afdeling Pathologie in het UMC Utrecht te bedanken, maar natuurlijk zijn er ook een aantal mensen, die ik graag in het bijzonder wil noemen.

Prof P.J. van Diest, beste Paul. Aan jou ben ik de meeste dank verschuldigd. Zonder jou zou dit project er niet zijn geweest en zonder jouw onuitputtelijke werklust, inspiratie en optimisme evenmin. Ik heb onze samenwerking altijd erg gewaardeerd en zonder deze stimulans was het project nooit zo'n succes geworden. We hebben een duidelijke klik, wat tot een prettige en efficiënte samenwerking heeft geleid. Naast het werkgerelateerde contact, kijk ik ook met plezier terug op de avonden waarop we als AIOS groep bij je thuis waren uitgenodigd en, zeker niet minder, de paar potjes die ik tegen je heb getennist. "Sir yes sir" of is "ernstig" hier meer op zijn plaats...

Alle AIOS in het UMC Utrecht bedankt! We hebben een geweldige groep met veel werklust, maar ook veel humor en gezelligheid. Succes met jullie opleidingen en tot ziens, misschien wel als directe collega's. Anoek, jou wil ik in het bijzonder bedanken, want mede dankzij jou ben ik in dit avontuur gestapt. Jij was voor mij het voorbeeld hoe je een PhD project kan combineren met het AIOS-schap en ouderschap. Hoe jij dit hebt gecombineerd verdient bewondering.

Alle pathologen werkzaam binnen het UMC Utrecht en Gelre ziekenhuis Apeldoorn bedankt. Jullie hebben mijn opleiding vormgegeven. De kennis die ik vergaard heb, was nuttig voor mijn promotieonderzoek en essentieel voor mijn verdere carrière als patholoog.

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Cathy, zonder jou had ik de MLPA nooit zo snel onder de knie gekregen. Bedankt dat ik deze techniek van "de MLPA-specialiste" heb mogen leren.
Jeroen, ook wij hebben samen een leuk project lopen. Molecular imaging in male breast cancer, dat is nog eens fancy stuff. Bedankt voor het prettige contact en succes met je verdere geneeskundig georiënteerde toekomst.

Mitko, together we are also working on male breast cancer and we did a lot of manual and automatic nuclear segmentations. Good luck with your PhD and hopefully your computer based approach will not replace the pathologist, but instead can be used additionally.

Alle analisten van de histologie bedankt. Bedankt voor het fijne contact, de humor en de laagdrempelige hulp. Vergeet niet dat jullie de ruggengraat van de afdeling pathologie zijn. Grada, jou ben ik in het bijzonder dank verschuldigd. Wat een prachtige TMA's kun jij maken met grote en kleine stansen. Ik denk dat maar weinig mensen dit precisiewerk met zoveel kwaliteit voor elkaar krijgen.

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Moleculair analisten, jullie gaan een steeds prominentere rol spelen binnen de afdeling pathologie en binnen het ziekenhuis. Jullie kunnen dat zeker aan. Met veel plezier heb ik vele MLPA runs bij jullie op het lab verricht. Ik heb het prettige contact en de laagdrempelige hulp erg gewaardeerd. Remco en Joyce, bedankt dat ik deel mocht uitmaken van jullie huwelijksfeest en veel geluk samen. Erwin bedankt voor het isoleren van DNA uit tumor en normaal borstweefsel van de man.

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Het aantal mannelijke borstkanker patiënten, geïncludeerd in dit promotieonderzoek, zou nooit zo groot zijn geweest zonder de hulp van het Diakonessenhuis Utrecht, Antonius Ziekenhuis Nieuwegein en Pathologie Oost Nederland. Joost, Peter en Marieke bedankt. Hiernaast heeft onze serie een international tintje gekregen, doordat we ook materiaal uit Duitsland (Paderborn en Keulen) hebben mogen gebruiken. Dear Horst and Bernd, thanks a lot for the tissue blocks with male breast cancer tissue. They were of great use. Horst you were also very important in solving our CK5/6 staining problems. Also your constructive comments on the manuscripts and all the new ideas I very much appreciated.

Al mijn vrienden, familie en schoonfamilie ben ik dank verschuldigd. Door jullie kan ik mijn werk en onderzoeksbevindingen relativeren en praten / lachen om niet geneeskundige zaken.

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En tenslotte het belangrijkste van allemaal.

Lieve Marijke, bedankt voor alle liefde die je me geeft. Jij bent mijn grote liefde en zonder jouw steun was dit nooit mogelijk geweest. En natuurlijk bedankt voor het geven van onze geweldige zoon Siem. Niets is heerlijker dan met jullie genieten en dat zullen we nog heel veel gaan doen. Jullie maken me zo trots….

CURRICULUM VITAE

Robert Kornegoor werd op 9 augustus geboren in Vierakker. Na het behalen van zijn atheneum diploma (Baudartius College, Zutphen), begon hij in 1999 aan de studie biomedische gezondheidswetenschappen aan de St. Radboud Universiteit Nijmegen (RUN). Hij liep 3 maanden stage bij Prof. Dr. M.T.E. Hopman op de afdeling fysiologie (RUN) waar hij onderzoek deed naar de invloed van veneuze occlusie op de centraal veneuze druk bij dwarslaesie patiënten en gezonde personen. Tevens liep hij gedurende 6 maanden stage bij Prof. Dr. B. Hillen op de afdeling anatomie en embryologie (RUN) waar hij onderzoek deed naar de korte en lange termijn effecten van een a. carotis interna occlusie op de macro en microvascularisatie van het rattenbrein. Na afronding van deze stage is hij nog 4 maanden als junior onderzoeker betrokken gebleven bij dit onderzoek. Na het behalen van zijn doctoraal begon hij in 2003 aan de studie geneeskunde eveneens aan de RUN. Tijdens zijn co-schappen ging zijn interesse uit naar de pathologie waarin hij ook een keuze co-schap heeft gevolgd bij Prof. Dr. van Krieken. In 2007 begon hij als arts in opleiding tot specialist op de afdeling pathologie aan het universitair medisch centrum Utrecht (opleider Prof. Dr. J.G. van den Tweel en Drs. R.J. Leguit). In het kader van de B-opleiding is hij twee jaar werkzaam geweest in het Gelre ziekenhuis Apeldoorn (opleider E.F. Weltevreden). Gedurende 2 jaar zat hij in het bestuur van de landelijke assistenten vereniging pathologie (LPAV) en namens de LPAV in het bestuur van het consilium pathologicum. In 2010 startte hij zijn promotieonderzoek bij Prof. Dr. P.J. van Diest, dat uiteindelijk leidde tot dit proefschrift.

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