Modern Pathology (2012) 25, 398–404

Molecular subtyping of male breast cancer by immunohistochemistry

Robert Kornegoor[1], Anoek HJ Verschuur-Maes[1], Horst Buerger[2], Marieke CH Hogenes[3], Peter C de Bruin[4], Joost J Oudejans[5], Petra van der Groep[1], Bernd Hinrichs[6] and Paul J van Diest[1]

[1]*Department of Pathology, University Medical Center Utrecht, Utrecht, The Netherlands;* [2]*Institute of Pathology, Paderborn, Germany;* [3]*Laboratory for Pathology East Netherlands, Enschede, The Netherlands;* [4]*Department of Pathology, St Antonius Hospital, Nieuwegein, The Netherlands;* [5]*Department of Pathology, Diakonessenhuis, Utrecht, The Netherlands and* [6]*Centre for Pathology and Cytology, Koeln, Germany*

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. 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). Luminal type A was by far the most encountered type of male breast cancers, 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. In 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.
Modern Pathology (2012) **25,** 398–404; doi:10.1038/modpathol.2011.174; published online 4 November 2011

Keywords: breast cancer; immunohistochemistry; male; molecular typing

Male breast cancer is a relatively uncommon disease accounting for <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, 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

Correspondence: PJ van Diest, MD, PhD, Department of Pathology, University Medical Center Utrecht, PO Box 85500, 3508 GA Utrecht, The Netherlands.
E-mail: p.j.vandiest@umcutrecht.nl
Received 11 July 2011; revised 4 September 2011; accepted 6 September 2011; published online 4 November 2011

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), Her2neu, 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 of a large series of male breast cancer 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 Koeln). 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, AHJV-M) 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, Westburg). 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) 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.

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

Scoring of the immunohistochemical staining 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.

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's χ^2 (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 cm

(average: 2.13 cm). 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 4 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 (three *HER2* 3+ and CISH amplified, one *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.

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), years	66	67	65	54[a]	59
<50	12 (9%)	9 (9%)	2 (7%)	1 (25%)	0
>50	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), cm	2.13	2.09	2.24	2.43	2.00
<2.0	63 (50%)	47 (50%)	14 (52%)	2 (50%)	0
>2.0	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%)[a]	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%)[a]	1 (25%)	0
Mitotic activity index/2 mm²	11.0	9.1	18.3 [a]	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%)[a]	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%)[a]	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

[a]Significantly different compared with luminal type A breast cancer.

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 4 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 with 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 also showed ER and/or PR positivity.

Molecular Sybtyping Using the 1% ER/PR Threshold

Using the 1% ER/PR threshold, a minor shift of male breast cancer cases toward 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.

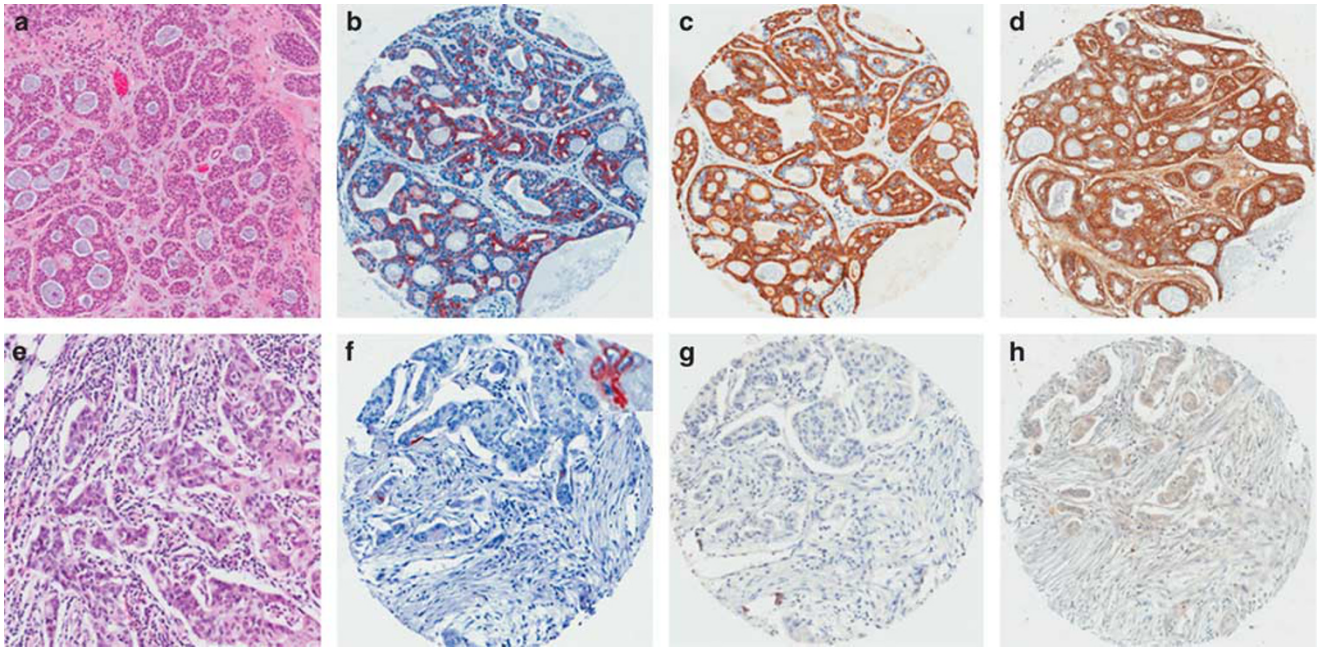

Figure 1 Two cases of basal-like breast cancer. One case, an adenoid cystic carcinoma (**a**; HE) showed CK5/6 (**b**), CK14 (**c**) and EGFR (**d**) reactivity. The other case, a high-grade basal-like breast cancer (**e**; HE) showed single-positive tumor cells in the CK5/6 staining (**f**), but no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

Discussion

In female breast cancers, molecular subtyping has 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 manner, 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 with 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*.,[19] identified four basal-like breast cancers in a small group of male breast cancer ($n = 28$), representing 14% of their studied cases. 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 in 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 makers 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 cancers subtypes in men is different compared with females, pointing to possible important differences in carcinogenesis.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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