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Expression of markers of stem cell characteristics, epithelial-mesenchymal transition, basal-like phenotype, proliferation and androgen receptor in metaplastic breast cancer and their prognostic impact

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Background: Metaplastic breast cancer (MpBC) is a heterogeneous subtype of invasive mammary carcinoma associated with epithelial-mesenchymal transition (EMT) and cancer stem cell characteristics. Data regarding prognostic markers and potentially actionable targets for therapy are still limited. The aims of the present study were to characterize the immunohistochemical landscape of this rare malignancy and to identify potential prognostic factors and targets for therapy.

Material and methods: A total of 75 patients diagnosed with MpBC over a 15-year period were included in the study. We performed immunohistochemical analyses for Ki-67 (MIB-1), epidermal growth factor receptor (EGFR), cytokeratin 5/6, vimentin, CD44, and androgen receptor (AR) and correlated their expression with clinicopathologic features and clinical outcome. The p-values for survival analyses were corrected for multiple testing (threshold 0.01).

Results: Most tumors expressed CK5/6 (73 %), EGFR (59 %), CD44 (81 %), and vimentin (87 %). Eighty-nine percent had a high Ki-67 index. Eighty-four percent were classified as basal-like (CK 5/6 or EGFR positive). AR was expressed in 21 % of the tumors. The basal-like phenotype was significantly ($p=0.009$) associated to inferior disease-free (DFS) and to breast-cancer specific overall survival (BCOS) with borderline significance ($p=0.01$). In addition, a low Ki-67 index was associated to improved DFS ($p = 0.033$) and BCOS ($p = 0.03$).

Conclusion: Most MpBCs express basal markers (CK5/6, EGFR), epithelial-mesenchymal transition marker vimentin and the stem cell marker CD44. Expression of basal-like markers was significantly related to inferior DFS. All the 11 patients with lack of expression of basal markers survived without relapse.

Background

MpBC is morphologically and clinically distinctive type of breast cancer characterized with aggressive behaviour and poor outcome. Patients with MpBC usually present with larger, higher-grade tumors and have worse 5-year overall survival (OS) compared to other types of invasive carcinoma [1-4] . MpBC comprises tumours with entirely epithelial as well as mixed epithelial and mesenchymal components. In addition, it can also include areas with conventional types of breast cancer [5]. Previous studies have revealed that MpBCs often overexpress markers of EMT and cancer stem cells. EMT is a process by which epithelial cells acquire a mesenchymal cell phenotype and gain migratory and invasive potential. Loss of E-cadherin and upregulation of mesenchymal proteins such as vimentin and smooth muscle actin are crucial steps in EMT. E-cadherin is repressed by specific transcription factors including Snail, Slug, Twist and the zinc-finger binding protein (ZEB1). MpBCs may show elevated CD44/CD24 ratios, which are thought to identify breast cancer stem cells. These features may contribute to the aggressive phenotype and chemoresistance of MpBCs. [5-8].

Most MpBCs express markers associated with basal-like tumours e.g. EGFR and cytokeratins 5/6. Weigelt et al. reported that 95 % of 20 MpBCs displayed a basal like genetic profile. [9]. Thus, it has been suggested that MpBCs are part of the spectrum of basal-like breast carcinomas (BLBC). However, BLBCs often respond favourably to neo-adjuvant chemotherapy, whereas MpBCs are known to be chemoresistant [5]. Hennessy et al., however, showed that MpBC is genetically heterogeneous, and represents an independent subtype distinct from the most common subtypes, including BLBC [6]. Moreover, their transcriptional profiles are most closely related to claudin-low tumors [6]. Claudin-low tumors also express high levels of EMT and cancer stem cell markers. Unlike claudin-low tumors, however, MpBCs also show a high frequency

(53 %) of PI3K/AKT pathway mutations. The PI3K signalling pathway affects cell growth, survival and metabolism. Inhibition of this pathway can reduce cell proliferation and promote cell death, which makes PI3K/AKT pathway a potentially attractive therapy target [6].

Most MpBCs are triple-negative [1-3]. A subset of triple negative breast cancers (TNBCs) express AR and may benefit from AR-targeted therapies. The percentage of nuclear AR-expression in TNBC detected by immunohistochemistry have ranged from 12 – 55 % in different studies [10]. Data on AR-expression in MpBC is scarce; in one study of 34 MpBC patients 5.9 % were AR-positive [11]. The prognostic significance of AR in TNBC is controversial, as some initial studies have suggested AR to be a potential negative prognostic factor, while a growing body of evidence indicates AR to be associated with favourable prognosis. Three recent meta-analyses have shown longer DFS in AR-positive breast cancer patients. In the neo-adjuvant chemotherapy setting AR was associated with a lower pCR rate, but nonetheless AR-positive women had better OS and DFS [12]

Only a few previous studies have tested the prognostic impact of tumor markers in MpBC [2, 13-15]. In the largest of these studies high EGFR-expression (n = 139) was related to a higher risk of cancer death in addition to large tumor size (n = 146) and mixed histological subtype (n = 131) [14]. Most other studies have been small with number of patients varying from 13 to 63, and the results diverse. A poor prognosis of MpBC has been attributed to high proliferation in one study [13], a combination of stem-cell and EMT markers in one [2] or the EMT-marker ZEB1 in one small study, n=13 [15].

Previously we have shown that most patients with MpBC (n=78) had grade III (83%), T2 (46%), N0 (82%), and triple negative disease (85%). Large tumor size and mixed subtype were associated with worse outcome [16].

The aims of the present study were to explore the expression of immunohistochemical markers and their potential prognostic impact in MpBC including markers for stem cell characteristics (CD44), basal-like phenotype (CK 5/6 and EGFR), EMT (vimentin), proliferation (Ki-67), and AR.

Material and Methods

The study was approved by the local Ethics committee. The study comprised 78 patients diagnosed with MpBC at the Helsinki University Comprehensive Cancer Centre during 2002 – 2016. All cases were reviewed by a breast pathologist in order to verify the diagnosis and histological subtype classification. Ki-67 was considered high if ≥ 20 %. Due to the limited availability of tissue, staining was performed in 75 tumors in this cohort. One further case lacked tissue material for analysis of CK5/6. Ki-67 analyses were conducted on 78 patients. Tumor tissue was subclassified according to WHO recommendation into low-grade adenosquamous carcinoma, fibromatosis-like metaplastic carcinoma, squamous cell carcinoma, spindle cell carcinoma and carcinoma with mesenchymal differentiation [17]. MpBCs having different metaplastic components were classified as mixed metaplastic carcinomas and MpBCs including also a conventional type of breast cancer as mixed type.

Ki-67 (MIB-1) was scored from hot spots in whole-tumor sections or from biopsy material obtained as part of routine diagnostic work-up and scored as percentages of positive nuclei with 5% accuracy. Estrogen and progesterone receptor and HER2 scores were collected from routine diagnostic reports.

The other markers (CK 5/6, EGFR, CD44 and AR) were predominantly assessed from TMAs. Hematoxylin and eosin-stained (H&E) slides were reviewed and representative areas were marked out on the matching formalin-fixed, paraffin embedded tissue blocks. Either one- (when tumor tissue was scarce) or two-millimetre cores from paraffin blocks were used to construct TMAs (four cores per case). Whole tissue sections were used if the cores were dislodged or failed to contain tumor tissue. Nuclear staining for AR (Dako, M3562, 1:50, Ventana), membranous staining for EGFR (Zymed, 31G7, 1:25, Autostainer) and CD44 (Dako, M7082, 1:50, Autostainer) and cytoplasmic staining for CK5/6 (Dako, M7237, 1:40, Ventana) and vimentin (Dako, M0725, 1:1000, Ventana) were evaluated by an experienced breast pathologist. AR nuclear staining was considered positive if ≥ 1 % of the tumour cells were stained. Staining intensity (0, 1+, 2+ and 3+) and percentage of positively stained tumour cells were analysed for EGFR, CD44, CK5/6 and vimentin and considered positive if ≥ 10 % of the tumor cells had +2 or +3 staining. The Basal-like subtype was defined as either EGFR or CK5/6 –positivity.

The association of expression of markers to other clinicopathological characteristics was tested with the $\chi 2$ or Fisher's exact test for dichotomous variables. A Mann-Whitney U-test was used for ordinal-scale variables and the independent sample t-test for continuous variables. The association between markers and histological subtype was tested with the $\chi 2$ test. All the tests were 2-sided.

Kaplan-Meier analysis was performed to evaluate the prognostic impact of these biomarkers on BCOS and DFS, and the log-rank test was used for statistical testing. Survival probabilities at 5 years were estimated using the life-table method. Patients with metastasis at diagnoses (n=2) were excluded from the analysis of DFS and BCOS. A Bonferroni adjustment for multiple testing was used in assessing the prognostic

impacts of marker expression, and the significance level was set to 0.01 in these analyses.

The analyses were performed with SPSS statistical package version 25. The study was carried out by the reporting recommendations for tumor marker prognostic studies (REMARK) and the relevant checklist can be found in the supplementary data [18].

Results

Seventy-five patients were included in the analyses of CD44, AR and Vimentin. Seventy-four patients were included in the analysis of the basal markers (CK 5/6, EGFR) due to inadequate histological material in one patient.

Expression of markers and clinicopathologic characteristics

Immunohistochemical results of biomarkers are summarized in Table 1 A and B. Eighty-nine percent of tumors had a high Ki-67 index (≥20 %). The majority of the tumors were enriched for basal epithelial markers 73% for CK 5/6, 59% for EGFR (84% for CK5/6 or EGFR), 87% for vimentin and 81% for the cancer stem cell marker CD44. Twenty-one percent were AR-positive. There was a significant association between the expression of basal markers and CD44 ($p = 0.028$). All basal-like tumors were PR-negative ($p=0.024$).

Furthermore, vimentin expression was significantly associated to lower nodal stage ($p=0.043$) and PR-negativity ($p=0.016$). CD44 expression was associated with a more advanced T-stage ($p=0.04$). All AR-negative tumors were PR-negative ($p=0.043$).

Biomarker expression across different histological subtypes is summarized in Table 2. There was a significant association between subtype and vimentin as well as CD44 expression (both $p=0.034$), while Ki-67, AR expression and basal-like phenotype,

were not significantly associated to histological subtype, p= 0.17, 0.20 and 0.60, respectively. All mixed metaplastic tumors were basal-like and expressed CD44. The majority also expressed vimentin. All carcinomas with mesenchymal differentiation and all spindle cell tumors expressed vimentin.

Expression of markers and prognosis

Two patients with distant metastases at diagnosis were excluded from prognostic analyses.

The association of CD44, basal-like phenotype, vimentin, Ki-67 and AR to DFS and BCOS is shown in Table 3. We found a significant association between basal tumor cell characteristics and DFS (p = 0.009) and of borderline nature for BCOS (p = 0.01). In ER-positive cases 5 years DFS in the basal-like group was 43 % (p = 0.167) and BCOS 41 % (p = 0.167), whereas in ER-negative cases the corresponding figures were 57 % (p = 0.026) and 55 % (p = 0.029). A high Ki-67 index was associated to lower DFS (p = 0.033) and BCOS (p = 0.03) but the p-value did not reach the Bonferroni-corrected threshold (p = 0.01). All the 9 patients with low Ki-67 index and the 11 patients with lack of expression of basal markers survived without relapse. Furthermore, these observations were independent, since none of the 11 patients with non-basal-like tumors had low Ki-67 index (p=0.6, Fisher's exact test). The other markers were not significantly associated to DFS or BCOS.

Kaplan-Meier plots of DFS and BCOS according to basal-like phenotype (CK5/6 (cytokeratin 5/6) or EGFR (epidermal growth factor receptor) positivity) and Ki-67 low (< 20 %) vs. high (≥ 20 %) are shown in Figure 1.

Discussion

MpBC is a rare type of invasive breast cancer but due to its aggressiveness and chemoresistance it accounts for a significant proportion of breast cancer mortality. Probably reflecting the morphological and molecular heterogeneity of this tumor, there is no pathognomonic pattern on immunohistochemistry to diagnose MpBC.

The most striking finding of the present study was the strong prognostic effect of the expression of a basal-like phenotype according to cytokeratin 5/6 or EGFR-expression. The prognosis was excellent in the 15% of cases not expressing this phenotype, since no patients experienced a relapse in this group. The result is supported by a recent study by McCart Reed et al. where EGFR-expression, large tumor size and mixed MpBC histological subtype were significant indicators of poor prognosis [14]. However, expression of CK 5/6 was not prognostic in this study. The results of other biomarker studies in MpBC have been variable. A small study of EMT-markers in MpBc (n=13), including vimentin, and the EMT-inducer ZEB1 found ZEB1 to be an independent prognostic factor for poor DFS [15]. In a study of 55 cases Song et al. showed that high Ki-67 (>14%) and clinical stage was significantly associated to disease-free survival in MpBC [2]. The prognostic implication of proliferation rate was confirmed in the present study, although the p-value did not reach the pre-defined significance level due to correction for multiple testing.

A study by Oon et al. of 63 patients with MpBC suggested that expression of EMT- and stem cell markers was significantly associated to high risk of relapse and death [13]. Basal-like phenotype was associated to expression of stem cell markers as in the present study. However, we could not verify the prognostic impact of stem cell and EMT markers. It is of note, however, that the present study used a different marker (vimentin) for EMT than the Oon et al. study (E-cadherin, Twist), which might explain this discrepancy.

The basal-like breast cancer subtype was originally defined according to gene expression analysis [19]. Subsequently attempts to define this subtype according to immunohistochemical markers have been made. Nielsen et al. showed that the basal-like subtype was characterized by expression of basal-type cytokeratins 5/6 and EGFR and negative expression of ER and HER2 [20].

Accordingly, basal-like breast cancer is often defined as triple negative phenotype with either positive EGFR or CK5/6 –expression [21]. However, 8-29% of TNBCs do not have a basal-like phenotype in gene expression analysis, and vice versa, 18-40% of basal-like cancers are not triple negative [22]. In a large collaborative study comprising 10 159 breast cancer patients from 12 studies expression of basal IH markers were prognostic not only within the TNBC group, but also in the ER-positive subset [23]. In the present study, we chose to test the prognostic impact of basal marker irrespective of ER-expression, and indeed also in the subset of ER-positive cases all relapses and deaths occurred in the basal-like group.

In our study, most of the patients expressed vimentin, CD44 and the basal markers CK5/6 or EGFR. In a review by Rakha et al., a wide range of immunohistochemical biomarkers were evaluated in 172 local MpBC patients as well as 730 patients published in 61 studies. Seventy-four percent of the tumors expressed CK5/6, 76 % EGFR, and 85 % vimentin, which is similar to our results, 73%, 59% and 87%, respectively. However, no consistent immunophenotype was identified and no individual marker was positive in all tumors [24].

In our series AR-positivity was more common (21 %) than ER- (12 %) or PR-positivity (3 %). In previous studies, there has been a large range of AR-expression in TNBC-patients, which can partly be due to different criteria for positive AR-expression (1 or 10 % positivity). For example in a study by Min Kim et al., only 5.9 % of the

patients with MpBC were AR-positive. However, in that study AR was considered to be positive when > 10 % of tumor cells were stained. There is no standardized way to evaluate AR-expression, however, in a previous review a combination of AR-expression by IHC ≥ 1 % and genomic biomarker expression was recommended in order to best identify AR+ patients who may benefit form AR-targeted therapy [25].

A recent phase II study of enzalutamide in patients with locally advanced or metastatic AR-positive TNBC has shown promising results. The clinical benefit rate was 25 % and median OS of 12.7 months in patients with AR staining > 0 %. The number of objective responses, however, was low, 6%. Fatigue was the only treatment-related grade 3 or higher adverse event with an incidence of > 2 % [26]. Thus, anti-androgen treatment targeting AR might be a novel therapeutic option in MpBC. AR enriched TNBC cell lines often carry PI3KCA-mutations, which makes them sensitive to PI3K/mTOR inhibition [12]. Therefore, the PI3K/mTOR pathway has been studied in order to boost anti-AR endocrine therapy [27]. Previous studies have also shown an association with AR and EGFR activity in TNBC [25]. There are currently several ongoing trials testing AR antagonists alone and in combination with other drugs. EGFR-targeted therapies such as tyrosine kinase inhibitors and monoclonal antibodies have also been tested in metastatic breast cancer patients in phase II trials but have shown low efficacy. In one of these trials of cetuximab alone or in combination with carboplatin in metastatic TNBC patients, responses were seen in only 6 % and 17 % of patients, respectively [28-30].

A considerable strength of the present study is the confirmation of the diagnosis by an expert breast cancer pathologist. On the other hand, limitations include a relatively small sample size and retrospective nature. Another limitation is the use of

TMAs instead of full tissue sections for the evaluation of immunohistochemical markers, especially since many MpBCs are heterogeneous.

In conclusion, our study demonstrated that MpBCs frequently express cancer stem cell, basal and epithelial-mesenchymal transition markers. Basal-like phenotype was significantly associated with shorter DFS and with borderline significance with worse BCOS.

Disclosure statement

The authors report no conflicts of interest.

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Figure 1. Disease free survival and breast cancer specific overall survival by basal-like phenotype and Ki-67 (low vs. high).

Table 1A. Clinicopathologic characteristics and immunohistochemical expression of biomarkers.

Features		Basal-like (n=62) n (%)	Non-basal-like (n = 12) n (%)	*P*	All (n = 75) n (%)
Age at primary diagnosis (years)	Median	66	68	0.856[a]	67
Histological grade	1	2 (3)	0 (0)	1.000[b]	2 (3)
	2	8 (13)	2 (17)		10 (13)
	3	52 (84)	10 (83)		63 (84)
Pathological tumor stage	T1	18 (30)	4 (33)	0.524[b]	22 (30)
	T2	27 (44)	6 (50)		33 (45)
	T3	11 (18)	2 (17)		13 (18)
	T4	5 (8)	0 (0)		5 (7)
	TX	1			2
Pathological nodal stage	N0	46 (82)	10 (83)	0.952[b]	56 (82)
	N1	7 (13)	0 (0)		7 (10)
	N2	1 (2)	1 (8)		2 (3)
	N3	2 (4)	1 (8)		3 (4)
	NX	6	0		7
Metastasis at diagnoses	No	62	11		73
	Yes	0	1		2
ER	Positive	6 (10)	3 (25)	0.156[d]	9 (12)
	Negative	56 (90)	9 (75)		66 (88)
PR	Positive	0 (0)	2 (17)	**0.024[d]**	2 (3)
	Negative	62 (100)	10 (83)		73 (97)
Her2	Positive	2 (3)	1 (8)	0.417[d]	3 (4)
	Negative	60 (97)	11 (92)		72 (96)
AR	Positive	11 (18)	5 (42)	0.065[c]	16 (21)
	Negative	51 (82)	7 (58)		59 (79)
Ki-67	≥ 20 %	54 (87)	12 (100)	0.339[d]	67 (89)
	< 20 %	8 (13)	0 (0)		8 (11)
Vimentin	Positive	54 (87)	10 (83)	0.661[d]	65 (87)
	Negative	8 (13)	2 (17)		10 (13)
CD44	Positive	53 (86)	7 (58)	**0.028[c]**	61 (81)
	Negative	9 (15)	5 (42)		14 (19)

ER (estrogen receptor), PR (progesterone receptor), HER2 (human epidermal growth factor receptor 2), AR (androgen receptor).

[a] The independent samples T-test

[b] Mann-Whitney test

[c] $\chi 2$ test

[d] Fisher's exact test

Table 2. Expression of biomarkers across different histological subtypes.

	AR % (n) n=75	CD44 % (n) n=75 *	Basal-like % (n) N = 74	Vimentin % (n) n=75 *	Ki-67 high % (n) n=78
Low grade adenosquamous	0/1	0/1	1/1	1/1	1/2
Squamous	22 % (4/18)	94 % (17/18)	89 % (16/18)	83 % (15/18)	90 % (18/20)
Spindle	6 % (1/17)	77 % (13/17)	82 % (14/17)	100 % (17/17)	77 % (13/17)
Mesenchymal differentiation	23 % (3/13)	62 % (8/13)	77 % (10/13)	100 % (13/13)	92 % (12/13)
Mixed metaplastic	11 % (1/9)	100 % (9/9)	100 % (9/9)	89 % (8/9)	89 % (8/9)
Mixed type	41 % (7/17)	82 % (14/17)	75 % (12/16)	65 % (11/17)	100 % (17/17)

AR (androgen receptor), Basal-like: CK5/6 (cytokeratin 5/6) or EGFR (epidermal growth factor receptor) positivity
* statistically significant difference (p=0.034)

Table 3. Actuarial 5 years DFS and BCOS according to marker expression.

Marker expression	5 years DFS Low	5 years DFS High	*P*	5 years BCOS Low	5 years BCOS High	*P*
Ki-67	100 %	58 %	0.033	100 %	56 %	0.030
Basal-like	100 %	56 %	0.009	100 %	53 %	0.01
CD 44	70 %	61 %	0.343	68 %	59 %	0.474
Vimentin	60 %	63 %	0.993	65 %	61 %	0.503
AR	61 %	71 %	0.562	59 %	71 %	0.558

DFS (disease free survival), BCOS (breast cancer specific overall survival), Basal-like: CK5/6 (cytokeratin 5/6) or EGFR (epidermal growth factor receptor) positivity, AR (androgen receptor)