Characteristics of the clinicopathology and immune microenvironment of the basal-like phenotype in HR+/HER2-breast cancer

He Jiehua (hejh@sysucc.org.cn)
State Key Laboratory of Oncology in South China; Collaborative Innovation Center for Cancer Medicine

Jinhui Zhang
State Key Laboratory of Oncology in South China; Collaborative Innovation Center for Cancer Medicine

Lu Pan
State Key Laboratory of Oncology in South China; Collaborative Innovation Center for Cancer Medicine

Zhishan Zhou
Guangdong Provincial Hospital of Chinese Medicine

Keming Chen
State Key Laboratory of Oncology in South China; Collaborative Innovation Center for Cancer Medicine

Jiabin Lu
State Key Laboratory of Oncology in South China; Collaborative Innovation Center for Cancer Medicine

Mei Li
State Key Laboratory of Oncology in South China; Collaborative Innovation Center for Cancer Medicine

Xue Chao
State Key Laboratory of Oncology in South China; Collaborative Innovation Center for Cancer Medicine

Rongzhen Luo
State Key Laboratory of Oncology in South China; Collaborative Innovation Center for Cancer Medicine

Peng Sun
State Key Laboratory of Oncology in South China; Collaborative Innovation Center for Cancer Medicine

Research Article

Keywords: HR+/HER2- breast cancer, Basal-like marker, Immune characteristics, Prognosis

Posted Date: February 14th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1338624/v1

Abstract

Background Expression of basal-like markers (BMs) is usually observed in triple-negative breast cancer (TNBC) and HER2-overexpressing breast cancer and is related to poor prognosis. Recently, some have reported that BM is expressed in 7.8%-27.1% of HR+/HER2- breast cancers (basal-like phenotype, BM+ BCs), but their biological behaviours remain controversial. This study aimed to compare the differences in clinicopathologic features, clinical outcomes and benefit from adjuvant regimens between BM+ BCs and nonbasal-like phenotype breast cancer (BM- BCs) and evaluate their tumour immune microenvironment.

Methods BM+ BCs were defined as EGFR- and/or CK5/6-positive (n=150). Overall, 180 BM- BCs were selected as the control cohort. Clinicopathological characteristics were retrospectively collected. Univariate and multivariate analyses were used to assess the relationship between disease-free survival (DFS), overall survival (OS) and BM status. Stromal tumour-infiltrating lymphocytes (sTILs) were evaluated on haematoxylin & eosin-stained slides, and CD3, CD8, FOPX3, chemokine (C-X-C motif) ligand 13 (CXCL13), CD68, PD-1 and PD-L1 were stained by IHC.

Results Compared to BM- BCs, BM+ BCs were more frequently diagnosed at a younger age and had a higher tumour stage, histological grade and necrosis. No obvious differences were seen in lymphovascular invasion or lymph node metastasis. Lower ER and PR expression and higher Ki-67 expression were observed in BM+ BCs. Overall, 62.8% BM+ BCs had p53 mutations. Shorter DFS and OS were related to BM+ BCs (p=0.021 and 0.018, respectively). In multivariate analysis, CK5/6 and clinical stage were independent prognostic factors for DFS and OS. BM+ BCs had a higher proportion of high sTILs with increased CD3, CD8, FOXP3, CXCL13 and CD68 expression. PD-1 and PD-L1 expression was positively related to BM+ BCs.

Conclusion Our study demonstrated that more aggressive morphologic features and worse prognosis were associated with BM+ BCs, and its immune-activated status may support new therapeutic strategies, such as immunotherapy, as a potential treatment.

Introduction

Breast cancer has been defined as a heterogeneous disease that can be subdivided into at least four molecular intrinsic subtypes by gene expression profiling analysis, including luminal A, luminal B, HER2-overexpressing and basal-like breast cancer[1]. In clinical practice, immunohistochemistry (IHC) biomarkers of hormone receptor (HR) including estrogen receptor (ER) and progesterone receptor (PR), human epidermal growth factor receptor-2 (HER2) and Ki-67 proliferation index have been widely used for surrogate taxonomy [2], and great differences in prognostic features and therapies response have been distinguished from these subtypes [3] [4]. HR+/HER2- breast cancer, as the major subtype in breast cancer [5] [6], has shown greatly improved prognosis in standard endocrine therapy, but unfortunately, up to 27.0% of these tumours show de novo or acquired resistance [7] [8], resulting in recurrence and death events. Studies have illustrated mechanisms of resistance, such as ESR1 mutation[9], hormone-

insensitive cellular clones [10] and tumour heterogeneity [11]. In addition, discordance has been observed between immunohistochemistry IHC and genomic assay (e.g., Prediction Analysis of Microarray 50, PAM50) classification in 18%-30% HR+/HER2- breast cancer [12] [13] [14]. Approximately 16% of HR+/HER2- cases were basal-like triple-negative breast cancer (TNBC) according to molecular subtypes[12], potentially leading to more invasive biological behaviour and poor response to endocrine therapy. Thus, it is important to identify the basal-like phenotype of HR+/HER2- breast cancer in pathological practice.

Basal-like markers (BMs) have drawn attention for their prognostic value. BM is usually expressed in normal basal/myoepithelial cells of the breast, including basal cytokeratin (CK5/6, CK14 and CK17) [15] [16] and epidermal growth factor receptor (EGFR) [17]. BM expression is also found in more than 50% of triple-negative breast cancers (TNBCs), depending on the BM assay panel [5] [18] [19] [20], which are associated with high histological grade and shorter overall survival (OS) [21]. Similarly, a study showed that 9% of HER2-overexpressing breast tumours were BM-positive, with shorter 5-year overall survival [22]. For luminal tumours, several reports have suggested a subtype with BM coexpression (BM+ BCs). Blows et al. analysed subtypes of breast cancer from 12 studies based on IHC assays and found that BM (CK5/6 or EGFR) expression in luminal breast cancer (8%) was related to a higher mortality rate than BM-negative BCs [5]. Another study by Jeong et al. showed EGFR expression in 7% of HR+ breast tumours, which had a higher nuclear grade and shorter 5-year overall survival [23]. However, conclusions in other studies seem to be different. According to a meta-analysis (n=5040) that focused on luminal A subtypes, the association between survival and BM expression (CK5, CK5/6 or EGFR) was not significant, and BM+ BCs were more likely to have less aggressive clinicopathologic characteristics [24]. Later, Engstrom et al. retrospectively detected BM expression (CK5, CK14 and EGFR) in luminal breast cancers on a tissue microarray and found a lower risk of death in BM+ luminal HER2-negative tumours [25]. As most of these studies used meta-analysis and only one study evaluated BM expression on whole tissue sections, further research with single-centre data and comprehensive observation for morphology is necessary. Insufficient attention has been given to the clinicopathological characteristics of this subtype, especially its tumour immune microenvironment (TIME). Therefore, the clinical significance of BM expression in HR+/HER2- breast tumours remains controversial and unclear.

Our study retrospectively assessed BM expression in HR+/HER2- breast cancer and analysed the association between BM and clinicopathologic features, clinical outcome, benefit from adjuvant therapy (e.g., endocrine therapy and chemotherapy) and the immune microenvironment status to explore the clinical value of identifying the basal phenotype in HR+/HER2- breast cancer.

Materials And Methods

Case selection

The procedure of case-selection is shown in Fig. 1. Clinicopathologic data of patients diagnosed with HR+/HER2- invasive breast carcinomas of no special type (NST) (n=2756) at the Sun Yat-sen University Cancer Center (SYSUCC) during the years 2015–2019 were retrieved. On the basis of the previous studies mentioned above, CK5/6 and EGFR were selected as basal markers for this study and were more frequently used in clinical practice. Basal-like markers, including CK5/6 and EGFR, were detected by IHC staining. A total of 169 (6.1%) patients showed positive expression of at least one of these basal-like markers in the BM-positive group. Nine were excluded for their stage IV status at diagnosis, and ten were excluded for missing follow-up data. Overall, 180 cases with no expression of either CK5/6 or EGFR matched for histological types and clinical stage were randomly selected as the BM-negative group. All patients were operated at SYSUCC. Formalin-fixed, paraffin-embedded (FFPE) tissue specimens, including the tumour, sentinel lymph nodes, and axillary lymph nodes, were stained with haematoxylin and eosin (H&E). The archived H&E slides were retrospectively reviewed by two pathologists (JHH, PS) to reconfirm the diagnosis.

Clinicopathologic features analysis

Clinicopathological data, including age at diagnosis, tumour size, histological grade, lymph node metastasis (LNM) and lymphovascular invasion (LVI), local and systemic treatment, tumour recurrence status, distant metastasis, survival, were collected and analysed in the BM-positive (n=150) and BM-negative groups (n=180). The tumour stage based on the TNM stage was assessed according to the criteria established by the 8th edition American Joint Committee on Cancer (AJCC 8th) staging manual. ER, PR, and HER2 status were determined by immunohistochemical staining. ER and PR status were classified as negative using a cut-off of 1% according to the American Society of Clinical Oncology/College of American Pathologists guidelines[26]. HER2 status was defined as negative with 0, 1+ and 2+ on IHC without HER2 gene amplification on fluorescence in situ hybridization (FISH) [27]. The percentage of Ki-67-stained cells was assessed in tumour areas on average and recorded as continuous variables. A Ki-67 cut-off value of 20% was used to distinguish the surrogate intrinsic subtypes of luminal A (HR+, HER2- and Ki-67<20%) and luminal B1 (HR+, HER2- and Ki-67≥20%)[28] [2]. Representative tumour sections were also immunostained for CK5/6 (clone D5/16B4, Abcam, Cambridge, UK), EGFR (clone EP38Y, Abcam, Cambridge, UK), and p53 (polyclone, Abcam, Cambridge, UK). Survival data including overall survival (OS) and disease-free survival (DFS), were maintained.

Tumour immune microenvironment evaluation

Stromal tumour infiltrating lymphocytes (sTILs) were evaluated according to a five-step standardized scoring system developed by the International Immuno-oncology Biomarker Working Group [29] [30]. The percentage of stromal TILs (sTILs%) was considered a semiquantitative continuous parameter indicating how much of the demarcated stromal area exhibits dense mononuclear infiltrates.

IHC staining for different subsets of immune cells (IC), including CD3 (clone LN10, ZSJQ-BIO, Beijing, CHINA), CD8 (clone OTI3H6, ZSJQ-BIO, Beijing, CHINA), CD68 (clone PG-M1, ZSJQ-BIO, Beijing, CHINA), CXCL13 (polyclonal, ZSJQ-BIO, Beijing, CHINA), and FOXP3 (clone 236A/E7, Abcam, Cambridge, UK), was performed on 210 representative tumour sections (78 BM+ BCs; 132 BM- BCs). Details about the antibodies and experimental conditions are shown in Supplementary Table 1. Assessment of IHC was accomplished by two pathologists (JHZ, XC) blinded to the clinical and tumour information. The densities of CD3, CD8, FOXP3 and CD68 were recorded as the percentage of positive ICs over the total stromal cells. A semiquantitative H-score was used to assess CXCL13 expression on IC. First, intensity was scored as 0, 1, 2 and 3 for negative, weak, moderate and strong staining, respectively, and the percentage of IC over stromal cells for each intensity was assessed. Then, the H-score was calculated as follows: 3 × percentage of strongly stained IC + 2 × percentage of moderately stained IC + percentage of weakly stained IC (range, 0–300) [31].The median sTILs% and immune-associated biomarkers above were calculated to determine the low and high expression levels for the cohort.

IHC staining of PD-1 (clone D4 W2J, Cell Signalling Tech, Boston, USA) and PD-L1 (clone SP142, Abcam, Cambridge, UK) was detected in 61 cases, which had high sTILs% (>10%) (39 BM+ BCs; 22 BM- BCs). Any staining of PD-1 in the IC was used as the cut-off for positivity [32]. The percentage of PD-L1 labelling ≥1% of the area occupied by IC of any intensity was considered positive [33].

Statistical Analysis

The clinicopathological features were analysed using SPSS software (version 25.0, SPSS Inc, Chicago, USA). Categorical variables were compared between groups using the chi-square test. Correlations between BM expression and sTILs% as well as other immune-associated IHC markers were measured by the Pearson test. OS and DFS curves were drawn using the Kaplan–Meier method and were compared using log-rank tests. The hazard ratio (HR) and 95% confidence interval (CI) were measured using the Cox proportional hazards regression model. All tests were two-sided, and a p value <0.05 was considered statistically significant.

Results

Patient population and clinicopathologic features

The clinicopathological features are summarized in **Table 1 and Table 2,** and the typical morphological features are shown in **Fig. 2.** Of the 330 patients, 329 (99.7%) were female. The median age at diagnosis was 48 years (range, 26-79 years). Tumour sizes were most frequent between 0.5 and 5.0 cm (T1–T2), with a median of 2.2 cm. Compared to BM- BCs, BM+ BCs occurred at a younger age (median, 47 vs. 50 years, p=0.031), with larger tumour sizes (median, 2.5 cm vs. 2.0 cm, p=0.024). No significant differences were observed between the groups in tumour stage (pT), lymph node metastasis (pN) or TNM stage. Histologically, of the 210 BCs with available tissue sections, higher histological grade (grade I, 0 vs. 2.3%;

grade II, 30.8% vs. 75.8%; grade III, 69.2% vs.22.0%; p=0.008), and necrosis (26.9% vs. 10.6%; p=0.002) were more frequently found in BM+ BCs, while other features, such as LVI, ductal carcinoma in situ (DCIS), calcification, fibrotic focus (FF) and tertiary lymphatic structures (TLS), did not differ between the groups. Regarding clinical management, fewer BM+ BCs received endocrine therapy than BM- BCs (87.2% vs. 74.0%, p=0.002). Other regimens, such as surgical methods, neoadjuvant therapy and adjuvant therapy, did not show significant differences between the groups.

Table 1

Comparison of clinical features of HR+/HER2- breast cancer by BM IHC expression status (n=330).

Characteristics	Total (n=330)	BM+ (*n*=150)	BM- (*n*=180)	*p*
Age at diagnosis, *n* (%)				
Median age, years	48	47	50	**0.031**
<40	49 (14.8)	25 (16.7)	24 (13.3)	0.067
40-55	194 (58.8)	93 (62.0)	101 (56.1)	
>55	87 (26.4)	32 (21.3)	55 (30.6)	
pT, *n* (%)				0.061
pT1	152 (46.1)	58 (38.7)	94 (52.2)	
pT2	156 (47.3)	85 (56.7)	71 (39.4)	
pT3	17 (5.2)	5 (3.3)	12 (6.7)	
pT4	5 (1.5)	2 (1.3)	3 (1.7)	
pN, *n* (%)				0.412
pN0	174 (52.7)	83 (55.3)	91 (50.6)	
pN1	94 (28.5)	42 (28.0)	52 (28.9)	
pN2	35 (10.6)	10 (6.7)	25 (13.9)	
pN3	27 (8.2)	15 (10.0)	12 (6.7)	
TNM staging				0.536
	88 (26.7)	34 (22.7)	54 (30.0)	
a	131 (39.7)	67 (44.7)	64 (35.6)	
b	47 (14.2)	23 (15.3)	24 (13.3)	
a	35 (10.6)	9 (6.0)	26 (14.4)	
b	2 (0.6)	2 (1.3)	0	
c	27 (8.2)	15 (10.0)	12 (6.7)	
Surgery, n (%)				0.218
Breast conservation	53 (16.1)	20 (13.3)	33 (18.3)	
Mastectomy	277 (83.9)	130 (86.7)	147 (81.7)	
Chemotherapy, n (%)				**0.018**
Yes	265 (80.3)	129 (86.0)	136 (75.6)	
No	65 (19.7)	21 (14.0)	44 (24.4)	

Endocrine therapy, n (%)				**0.002**
Yes	268 (81.2)	111 (74.0)	157 (87.2)	
No	62 (18.8)	39 (26.0)	23 (12.8)	
Radiotherapy, *n* (%)				0.883
Yes	118 (35.8)	53 (35.3)	65 (36.1)	
No	212 (64.2)	97 (64.7)	115 (63.9)	
Local relapse *n* (%)				1.000
Yes	2 (0.6)	1 (0.7)	1 (0.6)	
No	328 (99.4)	149 (99.3)	179 (99.4)	
Distant Metastasis, *n* (%)				**0.047**
Yes	38 (11.5)	23 (15.3)	15 (8.3)	
No	292 (88.5)	127 (84.7)	165 (91.7)	
Death, *n* (%)				0.055
Yes	16 (4.8)	11 (7.3)	5 (2.8)	
No	314 (95.2)	139 (92.7)	175 (97.2)	

Table 2

Association of BM expression with pathological features in HR+/HER2- breast cancer (n=210).

Characteristics	Total(n=210)	BM+ (n=78)	BM- (n=132)	*p*
Histological grade, *n* (%)				**0.008**
I	3 (1.4)	0	3 (2.3)	
II	124 (59.0)	24 (30.8)	100 (75.8)	
III	83 (39.5)	54 (69.2)	29 (22.0)	
Ductal carcinoma in situ, *n* (%)				0.050
Yes	91 (43.3)	27 (34.6)	64 (48.5)	
No	119 (56.7)	51 (65.4)	68 (51.5)	
lymphovascular invasion (LVI), *n* (%)				0.384
Yes	73 (34.9)	24 (31.2)	49 (37.1)	
No	136 (65.1)	53 (68.9)	83 (62.9)	
Uncertain	1	1	-	
Necrosis				**0.002**
Yes	35 (16.7)	21 (26.9)	14 (10.6)	
No	175 (83.3)	57 (73.1)	118 (89.4)	
Calcification				0.069
Yes	31 (14.8)	7 (9.0)	24 (18.2)	
No	179 (85.2)	71 (91.0)	108 (81.8)	
Fibrotic focus (FF)				
Yes	37 (17.6)	14 (17.9)	23 (17.4)	0.923
No	173 (82.4)	64 (82.1)	109 (82.6)	
Tertiary lymphatic structure (TLS)				0.750
Yes	7 (3.3)	3 (3.8)	4 (3.0)	
No	203 (96.7)	75 (96.2)	128 (97.0)	

Biomarkers

As shown in **Table 3 and Fig. 3**, compared to BM- BCs, BM+ BCs were associated with lower expression of ER (0-50% positive, 1.7% vs. 61.3%, $p<0.001$) and PR (0-50% positive, 33.9% vs. 82.7%, $p<0.001$). A higher Ki-67 index was also observed in BM+ BCs (Ki-67>50% positive, 40.7% vs. 7.8%, $p<0.001$). The majority of

BM+ BCs was luminal B HER2- subtypes (86.7% vs. 66.1%, p<0.001). BM+ BCs harboured mutated p53 more frequently than BM- BCs (66.7% vs. 40.0%, p<0.001). Among the BM+ BCs, all cases showed EGFR expression while CK5/6 expression was less common (n=78, 52.0%).

Table 3

Comparison of biomarkers and intrinsic subtypes between BM+ BCs and BM- BCs.

Characteristics, *n*	Total (n=330)	BM+ (n=150)	BM- (n=180)	*p*
ER, *n* (%)				**<0.001**
0-20%+	69 (20.9)	69 (46.0)	0	
21-50%+	26 (7.89)	23 (15.3)	3 (1.7)	
>50%+	235 (71.2)	58 (38.7)	177 (98.3)	
PR, *n* (%)				**<0.001**
0-20%+	148 (44.8)	105 (70.0)	43 (23.9)	
21-50%+	37 (11.2)	19 (12.7)	18 (10.0)	
>50%+	145 (43.9)	26 (17.3)	119 (66.1)	
HER2				0.359
0	209 (63.3)	99 (66.0)	110 (61.1)	
1+or 2+	121 (36.7)	51 (34.0)	70 (38.9)	
Ki-67, *n* (%)				**<0.001**
0-15%+	81 (24.5)	20 (13.3)	61 (33.9)	
16%-50%	174 (52.7)	69 (46.0)	105 (58.3)	
>50%+	75 (22.7)	61 (40.7)	14 (7.8)	
Intrinsic subtypes, *n* (%)				**<0.001**
Luminal A	81 (24.5)	20 (13.3)	61 (33.9)	
Luminal B HER2-	249 (75.5)	130 (86.7)	119 (66.1)	
BM, *n* (%)				-
EGFR positive	150 (45.5)	150 (100)	-	
CK5/6 positive	0	0	-	
Both positive	78 (23.6)	78 (52)	-	
p53 status, *n* (%)				**<0.001**
Mutant-tpye	164 (51.6)	92 (66.7)	72 (40.0)	
Wild-type	154 (48.4)	46 (33.3)	108 (60.0)	
Not known	12	12	0	

Prognosis of BM-positive HR+/HER2- breast cancer

The follow-up time of our cohort ranged from 7.10-72.17 months, with a median of 34.42 months. Survival curves for DFS and OS are shown in **Fig. 4**. Both DFS and OS were shorter in BM+ BCs (DFS: *p*=0.034, HR=1.96, 95%CI: 1.04-3.69; OS: *p*=0.037, HR=2.93, 95%CI: 1.02-8.45). When CK5/6 status was taken into consideration, significantly poorer DFS and OS were also observed (DFS: *p*=0.033, HR=2.03, 95%CI: 1.06-3.90; OS: *p*=0.007, HR=3.88, 95%CI: 1.45-1.39) in BM+ BCs. Univariate and multivariate analyses demonstrated that CK5/6 was an independent prognostic factor for both DFS (*p*=0.004, HR=4.32, 95% CI: 1.66-11.61) and OS (*p*=0.003, HR=2.82, 95% CI: 1.44-5.55) in HR+/HER2- BCs (**Table 4**). Meanwhile, clinical stage also played an independent prognostic role in DFS (*p*<0.001, HR=1.67, 95% CI: 1.27-2.20) and OS (*p*<0.001, HR=1.86, 95% CI: 1.55-2.24)

Table 4

Univariate analysis and multivariate analysis of HR+/HER2- breast cancer

	Univariate analysis			**Multivariate analysis**		
	p	**HR**	**95% CI**	***p***	**HR**	**95% CI**
DFS						
Clinical stage	0.001	1.62	1.24-2.13	**<0.001**	**1.67**	**1.27-2.20**
CK5/6 (pos. vs. neg.)	0.007	3.88	1.45-1.39	**0.004**	**4.32**	**1.60-11.61**
EGFR (pos. vs. neg.)	0.047	2.93	1.02-8.45	0.580	-	-
ER	0.026	0.97	0.97-1.00	0.990	-	-
PR	0.010	0.98	0.96-0.99	0.122	-	-
Ki-67	0.016	1.02	1.00-1.04	0.585	-	-
OS						
Clinical stage	<0.001	1.81	1.51-2.15	**<0.001**	**1.86**	**1.55-2.24**
CK5/6 (pos. vs. neg.)	0.033	2.03	1.06-3.90	**0.003**	**2.82**	**1.44-5.55**
Chemotherapy (yes vs. no)	0.030	4.85	1.17-20.11	0.110	-	-
Radiation (yes vs. no)	0.001	2.86	1.51-5.43	0.055	-	-
EGFR (pos. vs. neg.)	0.038	1.96	1.04-3.69	0.314	-	-
PR	0.025	0.99	0.98-1.00	0.175	-	-
Ki-67	0.006	1.02	1.01-1.03	0.218	-	-

Among the BM+ patients who received endocrine therapy (n=111, 74.0%), combining adjuvant chemotherapy did not affect their prognosis in terms of either DFS or OS (*p*=0.457 and 0.670,

respectively). On the other hand, it was noteworthy that the OS of BM+ BCs who had been treated with neoadjuvant therapy (n=15, 10.0%) was not improved but rather decreased, compared with those who did not receive the treatment (p=0.037). As the cases were limited, the response to neoadjuvant therapy in BM+ BCs needs further attention.

Immune profiles of BM-positive HR+/HER2- breast cancer

The expression of immunue biomarkers is summarized in **Table 5**. Of the 210 cases with available tissue blocks for IHC staining, the median sTILs% was 10%. A total of 149 (71.0%) had low sTIL% and 61 (29.0%) had high sTIL%. The sTIL% was notably higher in BM+ BCs than in BM- BCs (50.0% vs.16.7%; p<0.001), with medians of 15% and 5%, respectively. Significantly positive correlations were observed between CD3, CD8, FOXP3, CXCL13, CD68 and BM expression. Examples of immune marker expression and heatmap are displayed in **Fig. 5**. Among the 61 HR+/HER2- breast cancers, more BM+ BCs showed positive expression of PD-1 (76.9% vs. 50.0%, p=0.031) and PD-L1 (61.5% vs. 32.1%, p=0.010) than BM- BCs.

Table 5

Correlation between BM status and sTILs%, CD3, CD8, FOXP3, CXCL13, PD-1, PD-L1.

N=210	Total (n=210)	BM+ (%) (n=78)	BM- (%) (n=132)	Correlation coefficient	*p*
TILs				0.36	**<0.001**
Low	149 (71.0)	39 (50.0)	110 (83.3)		
High	61 (29.0)	39 (50.0)	22 (16.7)		
CD3				0.25	**<0.001**
Low	132 (62.9)	37 (47.4)	95 (72.0)		
High	78 (37.1)	41 (52.6)	87 (28.0)		
CD8				0.21	**0.002**
Low	130 (61.9)	38 (48.7)	92 (69.7)		
High	80 (38.1)	40 (51.3)	40 (30.3)		
FOXP3				0.26	**<0.001**
Low	139 (66.2)	39 (50.0)	100 (75.8)		
High	71 (33.8)	39 (50.0)	32 (24.2)		
CXCL13				0.26	**0.001**
Low	143 (68.1)	41 (52.6)	102 (77.3)		
High	67 (31.9)	37 (47.4)	30 (22.7)		
CD68				0.15	**0.036**
Low	127 (60.5)	40 (51.3)	87 (65.9)		
High	83 (39.5)	38 (48.7)	45 (34.1)		
N=61					
PD-1				0.28	**0.031**
Positive	41 (67.2)	30 (76.9)	11 (50.0)		
Negative	20 (32.8)	9 (23.1)	11 (50.0)		
PD-L1				0.33	**0.010**
Positive	30 (49.2)	24 (61.5)	6 (27.3)		
Negative	31 (50.8)	15 (38.5)	16 (72.7)		

Discussion

Basal marker expression and their poor prognostic impact in TNBC have been extensively studied [34] [18] [35], but only a few studies have focused on their expression and prognostic value in luminal breast cancer, and the conclusions have been controversial. According to a collaborative study that included 10159 breast cancer cases from multiple centres, approximately 8% of luminal HER2-negative patients had basal marker expression (defined as CK5/6 and/or EGFR positive), and they showed a poor prognosis compared to luminal HER2-negative basal-negative patients [5]. In another meta-analysis (n=5040) that focused on luminal A subtypes (HR+, HER2-), the association between survival and basal marker expression (defined as CK5, CK5/6 or EGFR positive) was not significant, while BM+ tumours were more likely to have less aggressive clinicopathologic characteristics [24]. In contrast, a retrospective study that detected basal marker (CK5, CK14 and EGFR) expression in luminal breast cancers on a tissue microarray showed a lower risk of death in BM+ luminal HER2-negative tumours (n=324) than in BM- luminal HER2- tumours (n=870) [25]. It should be noted that, to date, there has been no accepted consensus on the definition of basal markers, and detecting basal markers was not recommended for breast cancer diagnosis, potentially leading to different criteria, incomplete data and even diverging results of the basal-like phenotype. Previous studies have shown that the combined detection of CK5/6 and EGFR has convenient and predictive value to define basal breast cancer [18] [36]. CK5/6 is regarded as one of the most common cytokeratins in basal breast cancer, and its expression in TNBC is associated with poor prognosis [37]. EGFR, as a member of the HER family of casein kinase receptor proteins, also showed a relationship with worse survival in breast cancer [38]. Furthermore, EGFR is also a treatment target with several available drugs, such as cetuximab and gefitinib [39], which can help to identify a basal-like subset that might benefit from those agents.

As one of the largest single-centre studies, we compared the differences between clinicopathologic and immune-associated features, clinical outcomes and BM status. We found that patients with BM+ HR+/HER2- tumours had some clinicopathologic features in common with basal-like breast cancer. For example, patients with BM+ tumours were younger. More aggressive tumour features occurred in BM+ tumours, including higher tumour stage and histological grade. They were more likely to have lower ER and PR expression, a higher Ki-67 index and more p53 mutations, which served as an indicator of aggressive biological behaviour and poor outcomes. Clearly shorter DFS and OS were found in BM+ breast cancer in the K-M survival analysis. In further multivariate analysis, CK5/6 was a risk factor for both DFS and OS. These results support the notion that BM+ BCs can be regarded as a subtype of HR+/HER2- breast cancer with a poor prognosis.

According to this study, traditional adjuvant treatment, including chemotherapy and endocrine therapy, did not help prolong the survival of BM+ BCs; thus, further exploration of new regimens should be a future area of focus. The immune system plays an important role in both the elimination and progression of breast cancer. The TIME, after a complex transformation initially from an inflammatory reaction, is associated with immune escape and tumour progression [40]. A higher number of sTILs was observed in HER2+ breast cancers and TNBCs than in luminal breast cancers[41]. Moreover, PD-L1 positive

expression was more frequently seen in TNBCs[42], and this has been a focus of clinical trials for the prospect of immunotherapy[43]. PD-L1-targeted drugs have also been approved for patients with unresectable locally advanced or metastatic TNBC[44]. To our knowledge, there has been little prior research on BM+ BC TIME characteristics. By assessment of whole slide sections, we observed relatively higher sTILs% in the BM+ cohort, which reflected a relatively activated immune status in these tumours. According to a previous study by Denkert et al. [45], increased TILs may be an adverse predictive factor of response to neoadjuvant therapy in ER+/HER2- tumours, although they predicted a better response in TNBC and HER2-overexpressing breast cancer. In our study, the survival of BM+ patients who had received neoadjuvant therapy did not improve. Since few cases that received neoadjuvant therapy were included in our research, the explicit impact of the treatment in these patients should be prospectively analysed. Higher levels of TILs and PD-L1-positive immune cells in this subtype might be a hint for new therapy options, such as immune checkpoint inhibitors.

Our study has several limitations. The clinical and pathological information was mostly collected retrospectively, leading to the possibility of missing data. In addition, limited tissue blocks for TIME assays and short follow-up time in our cohort restricted the analysis, and these patients should be further observed. Furthermore, patients included in this study received different adjuvant regimens, which may have influenced their survival outcomes to some extent. Despite these limitations, this study highlighted the biological behaviour of BM+ HR+/HER2- breast cancer and provided a preliminary exploration of its TIME characteristics.

Conclusion

Our study indicated that more aggressive morphologic features and worse prognosis were associated with the basal-like phenotype of HR+/HER2- breast cancer, and its immune-activated status may support new therapeutic strategies, such as immunotherapy, as a potential treatment for patients of this subset.

Abbreviations

BC: breast cancer

BM: basal-like marker

CD: cluster of differentiation

CI: confidence interval

CK5/6: Cytokeratin 5/6

CXCL13: (C-X-C motif) ligand 13

DFS: disease-free survival

EGFR: epidermal growth factor receptor

ER: estrogen receptor

FOXP3: Forkhead box P3

HER2: human epidermal growth factor receptor-2

HR+: hormone receptor positive

HR: hazard ratio

IC: immune cell

IHC: immunohistochemistry

LVI: lymphovascular invasion

OS: overall survival

PAM50: Prediction Analysis of Microarray 50

PD-1: Programmed cell death protein 1

PD-L1: Programmed cell death ligand 1

PR: progesterone receptor

sTIL: stromal tumor-infiltrating lymphocyte

TIL: tumor-infiltrating lymphocyte

TIME: tumor immune microenvironment

TNP: triple-negative phenotype

Declarations

Acknowledgements

Not applicable.

Authors' contributions

JHZ and LP participated in the generation, interpretation and analysis of data and drafted the manuscript. ZSZ, KMC and JBL carried out the experiment. ML, XC, RZL and PS participated in the

acquisition and interpretation of pathologic data. JHH and PS provided the idea and organized the study, directed data generation and analysis, and edited the manuscript. All authors read and approved the final manuscript.

Funding

This work was funded by the National Natural Science Foundation of China (81902679), the Guangdong Natural Science Foundation (2021A1515010760), and the Science and Technology Planning Project of Guangdong Province (032515).

Ethics approval and consent to participate

This study was conducted in accordance with the ethical standards of the research committee of the Sun Yat-sen University Cancer Center (BL-B2022-036-01). All patients were systemically asked for consent to use their data anonymously for analysis and publication.

Availability of data and materials

The authenticity of this article has been validated by uploading the key raw data onto the Research Data Deposit public platform (www.researchdata.org.cn).

Consent for publication

Not applicable.

Competing interests

There are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

References

1. Perou, C.M., et al., *Molecular portraits of human breast tumours.* Nature, 2000. **406**(6797): p. 747-52.
2. Curigliano, G., et al., *De-escalating and escalating treatments for early-stage breast cancer: the St. Gallen International Expert Consensus Conference on the Primary Therapy of Early Breast Cancer 2017.* Ann Oncol, 2017. **28**(8): p. 1700-1712.
3. Kennecke, H., et al., *Metastatic behavior of breast cancer subtypes.* J Clin Oncol, 2010. **28**(20): p. 3271-7.

4. Ali, S., et al., *Current Management Strategies in Breast Cancer by Targeting Key Altered Molecular Players.* Front Oncol, 2016. **6**: p. 45.
5. Blows, F.M., et al., *Subtyping of breast cancer by immunohistochemistry to investigate a relationship between subtype and short and long term survival: a collaborative analysis of data for 10,159 cases from 12 studies.* PLoS Med, 2010. **7**(5): p. e1000279.
6. Voduc, K.D., et al., *Breast cancer subtypes and the risk of local and regional relapse.* J Clin Oncol, 2010. **28**(10): p. 1684-91.
7. Cuzick, J., et al., *Effect of anastrozole and tamoxifen as adjuvant treatment for early-stage breast cancer: 10-year analysis of the ATAC trial.* Lancet Oncol, 2010. **11**(12): p. 1135-41.
8. Early Breast Cancer Trialists' Collaborative, G., *Aromatase inhibitors versus tamoxifen in early breast cancer: patient-level meta-analysis of the randomised trials.* Lancet, 2015. **386**(10001): p. 1341-1352.
9. Takeshita, T., et al., *Analysis of ESR1 and PIK3CA mutations in plasma cell-free DNA from ER-positive breast cancer patients.* Oncotarget, 2017. **8**(32): p. 52142-52155.
10. Johnston, S.R. and M. Dowsett, *Aromatase inhibitors for breast cancer: lessons from the laboratory.* Nat Rev Cancer, 2003. **3**(11): p. 821-31.
11. Curtis, C., et al., *The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups.* Nature, 2012. **486**(7403): p. 346-52.
12. Whitworth, P., et al., *Chemosensitivity predicted by BluePrint 80-gene functional subtype and MammaPrint in the Prospective Neoadjuvant Breast Registry Symphony Trial (NBRST).* Ann Surg Oncol, 2014. **21**(10): p. 3261-7.
13. Guiu, S., et al., *Molecular subclasses of breast cancer: how do we define them? The IMPAKT 2012 Working Group Statement.* Ann Oncol, 2012. **23**(12): p. 2997-3006.
14. Prat, A., et al., *Clinical implications of the intrinsic molecular subtypes of breast cancer.* Breast, 2015. **24 Suppl 2**: p. S26-35.
15. Gusterson, B. and C.J. Eaves, *Basal-like Breast Cancers: From Pathology to Biology and Back Again.* Stem Cell Reports, 2018. **10**(6): p. 1676-1686.
16. Gusterson, B.A., et al., *Basal cytokeratins and their relationship to the cellular origin and functional classification of breast cancer.* Breast Cancer Res, 2005. **7**(4): p. 143-8.
17. van Agthoven, T., et al., *Differential expression of estrogen, progesterone, and epidermal growth factor receptors in normal, benign, and malignant human breast tissues using dual staining immunohistochemistry.* Am J Pathol, 1994. **144**(6): p. 1238-46.
18. Cheang, M.C., et al., *Basal-like breast cancer defined by five biomarkers has superior prognostic value than triple-negative phenotype.* Clin Cancer Res, 2008. **14**(5): p. 1368-76.
19. Bidard, F.C., et al., *Does triple-negative phenotype accurately identify basal-like tumour? An immunohistochemical analysis based on 143 'triple-negative' breast cancers.* Ann Oncol, 2007. **18**(7): p. 1285-6.

20. Tan, D.S., et al., *Triple negative breast cancer: molecular profiling and prognostic impact in adjuvant anthracycline-treated patients.* Breast Cancer Res Treat, 2008. **111**(1): p. 27-44.
21. Alshareeda, A.T., et al., *Characteristics of basal cytokeratin expression in breast cancer.* Breast Cancer Res Treat, 2013. **139**(1): p. 23-37.
22. Bagaria, S.P., et al., *Prognostic value of basal phenotype in HER2-overexpressing breast cancer.* Ann Surg Oncol, 2012. **19**(3): p. 935-40.
23. Jeong, Y., et al., *EGFR is a Therapeutic Target in Hormone Receptor-Positive Breast Cancer.* Cell Physiol Biochem, 2019. **53**(5): p. 805-819.
24. Sung, H., et al., *Heterogeneity of luminal breast cancer characterised by immunohistochemical expression of basal markers.* Br J Cancer, 2016. **114**(3): p. 298-304.
25. Engstrom, M.J., M. Valla, and A.M. Bofin, *Basal markers and prognosis in luminal breast cancer.* Breast Cancer Res Treat, 2017. **163**(2): p. 207-217.
26. Hammond, M.E., et al., *American Society of Clinical Oncology/College Of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer.* J Clin Oncol, 2010. **28**(16): p. 2784-95.
27. Wolff, A.C., et al., *Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer: American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Focused Update.* J Clin Oncol, 2018. **36**(20): p. 2105-2122.
28. Untch, M., et al., *Primary Therapy of Patients with Early Breast Cancer: Evidence, Controversies, Consensus: Opinions of German Specialists to the 14th St. Gallen International Breast Cancer Conference 2015 (Vienna 2015).* Geburtshilfe Frauenheilkd, 2015. **75**(6): p. 556-565.
29. Salgado, R., et al., *The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014.* Ann Oncol, 2015. **26**(2): p. 259-71.
30. Dieci, M.V., et al., *Update on tumor-infiltrating lymphocytes (TILs) in breast cancer, including recommendations to assess TILs in residual disease after neoadjuvant therapy and in carcinoma in situ: A report of the International Immuno-Oncology Biomarker Working Group on Breast Cancer.* Semin Cancer Biol, 2018. **52**(Pt 2): p. 16-25.
31. Wei, Y., et al., *CXCL13 expression is prognostic and predictive for postoperative adjuvant chemotherapy benefit in patients with gastric cancer.* Cancer Immunol Immunother, 2018. **67**(2): p. 261-269.
32. Matikas, A., et al., *PD-1 protein and gene expression as prognostic factors in early breast cancer.* ESMO Open, 2020. **5**(6): p. e001032.
33. Schmid, P., et al., *Atezolizumab plus nab-paclitaxel as first-line treatment for unresectable, locally advanced or metastatic triple-negative breast cancer (IMpassion130): updated efficacy results from a randomised, double-blind, placebo-controlled, phase 3 trial.* Lancet Oncol, 2020. **21**(1): p. 44-59.
34. Rakha, E.A., et al., *Breast carcinoma with basal differentiation: a proposal for pathology definition based on basal cytokeratin expression.* Histopathology, 2007. **50**(4): p. 434-8.

35. Prat, A., et al., *Molecular characterization of basal-like and non-basal-like triple-negative breast cancer.* Oncologist, 2013. **18**(2): p. 123-33.
36. Abdelrahman, A.E., et al., *Prognostic impact of EGFR and cytokeratin 5/6 immunohistochemical expression in triple-negative breast cancer.* Ann Diagn Pathol, 2017. **28**: p. 43-53.
37. Nielsen, T.O., et al., *Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma.* Clin Cancer Res, 2004. **10**(16): p. 5367-74.
38. Viale, G., et al., *Invasive ductal carcinoma of the breast with the "triple-negative" phenotype: prognostic implications of EGFR immunoreactivity.* Breast Cancer Res Treat, 2009. **116**(2): p. 317-28.
39. El Guerrab, A., et al., *Anti-EGFR monoclonal antibodies and EGFR tyrosine kinase inhibitors as combination therapy for triple-negative breast cancer.* Oncotarget, 2016. **7**(45): p. 73618-73637.
40. Emens, L.A., *Breast Cancer Immunotherapy: Facts and Hopes.* Clin Cancer Res, 2018. **24**(3): p. 511-520.
41. Stanton, S.E., S. Adams, and M.L. Disis, *Variation in the Incidence and Magnitude of Tumor-Infiltrating Lymphocytes in Breast Cancer Subtypes: A Systematic Review.* JAMA Oncol, 2016. **2**(10): p. 1354-1360.
42. Dill, E.A., et al., *PD-L1 Expression and Intratumoral Heterogeneity Across Breast Cancer Subtypes and Stages: An Assessment of 245 Primary and 40 Metastatic Tumors.* Am J Surg Pathol, 2017. **41**(3): p. 334-342.
43. *ClinicalTrials.gov.* 2020.
44. *FDA approves atezolizumab for PD-L1 positive unresectable locally advanced or metastatic triple-negative breast cancer. FDA U.S. Food and Drug.* 2020.
45. Denkert, C., et al., *Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: a pooled analysis of 3771 patients treated with neoadjuvant therapy.* Lancet Oncol, 2018. **19**(1): p. 40-50.

Figures

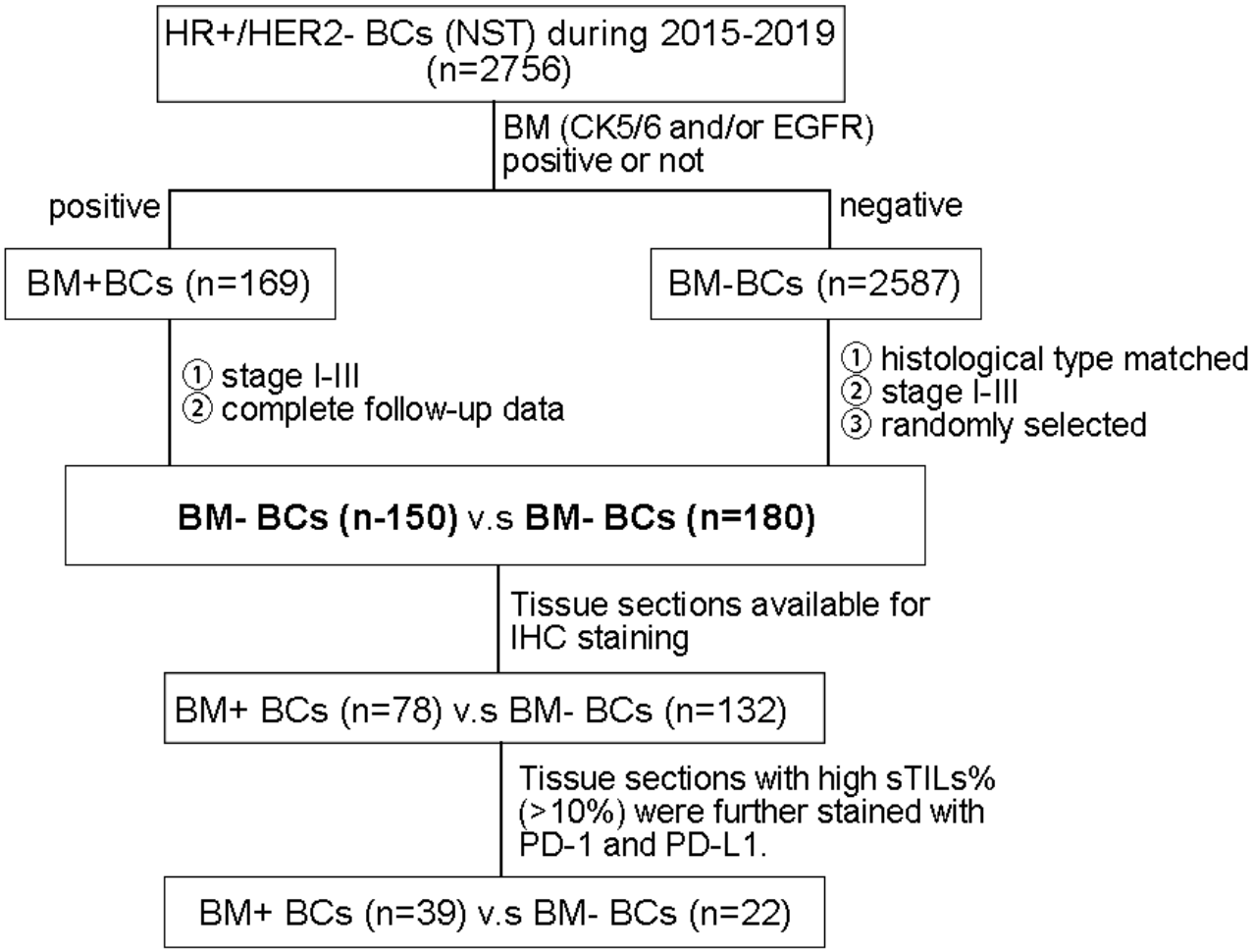

Figure 1

The flowchart of cases selection.

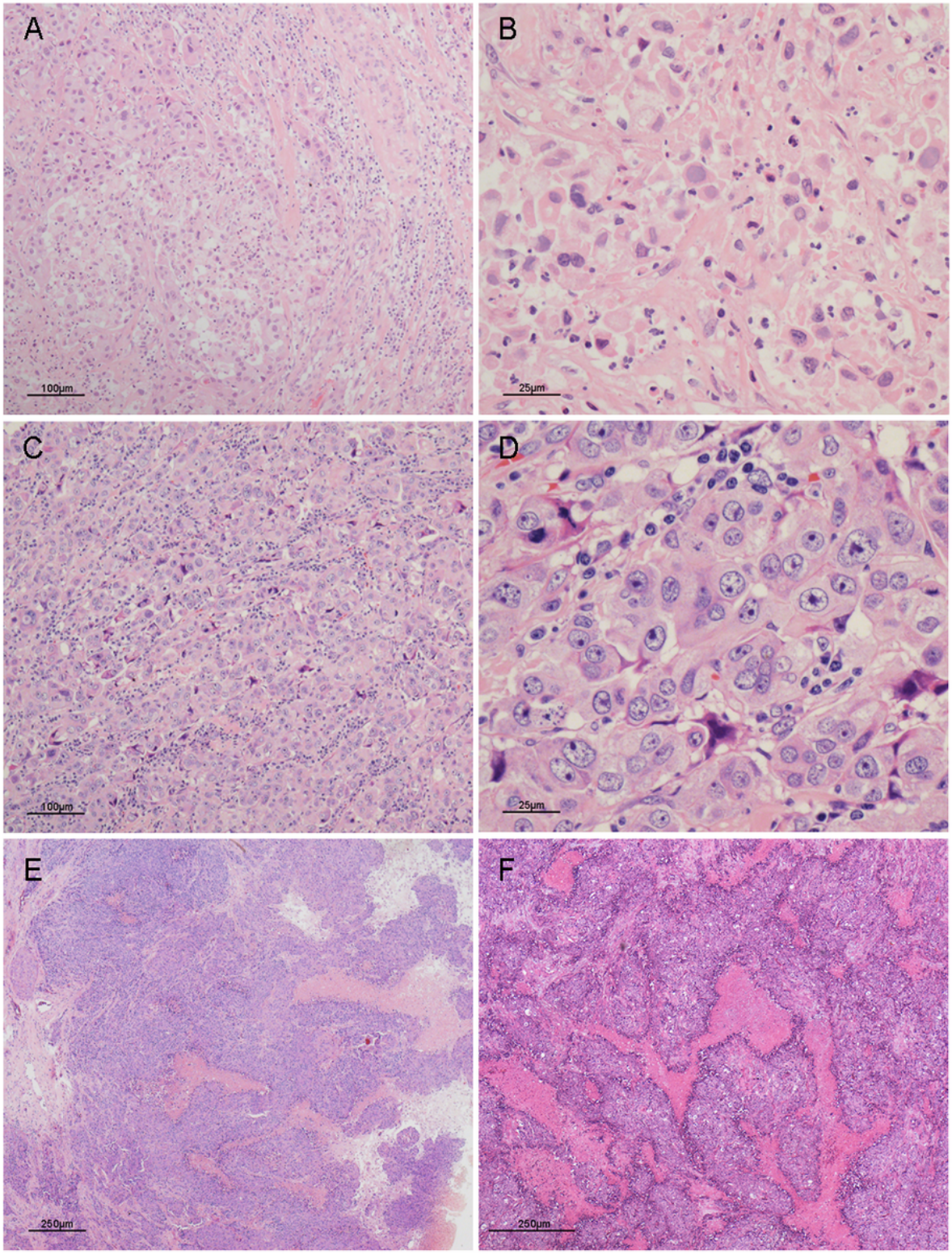

Figure 2

Examples of morphological features in BM+ BCs. BM BCs consisted of tumour cells with high histological grade, including less or little tubule formation (A, C) and obvious nuclear pleomorphism (B, D). In addition, necrosis was more easily observed in BM+ BCs (E, F).

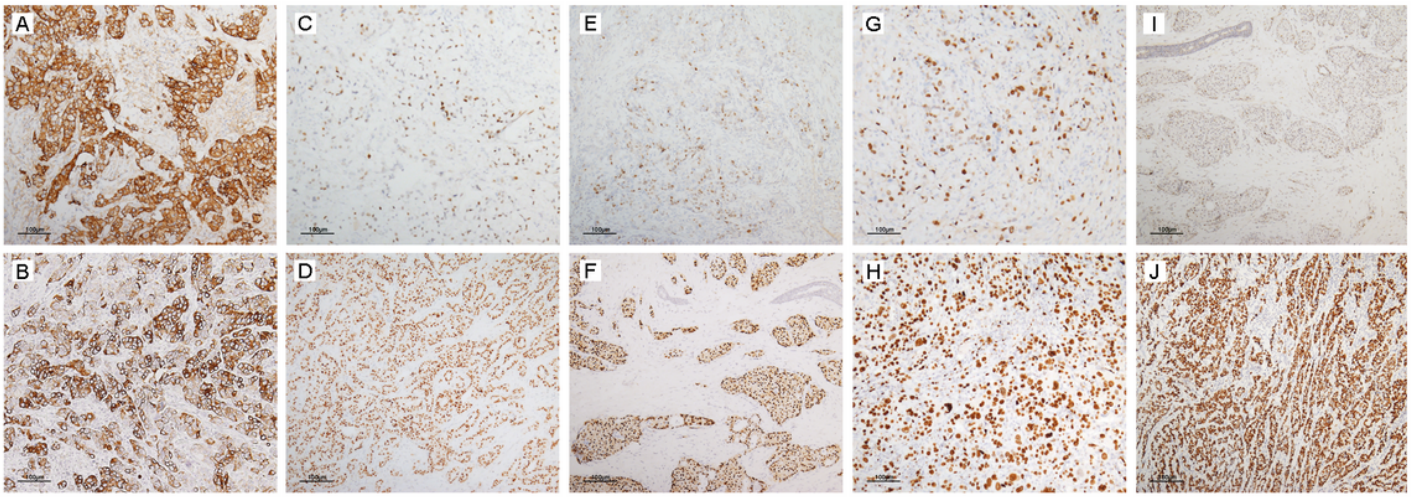

Figure 3

Biomarker features in BM+ BCs. EGFR and CK5/6 showed strongly positive expression (A, B). Low expression of ER, PR and Ki-67 was displayed in C, E and G, and high expression was displayed in D, F and H. p53 expression are shown in I (wild-type) and J (mutant-type).

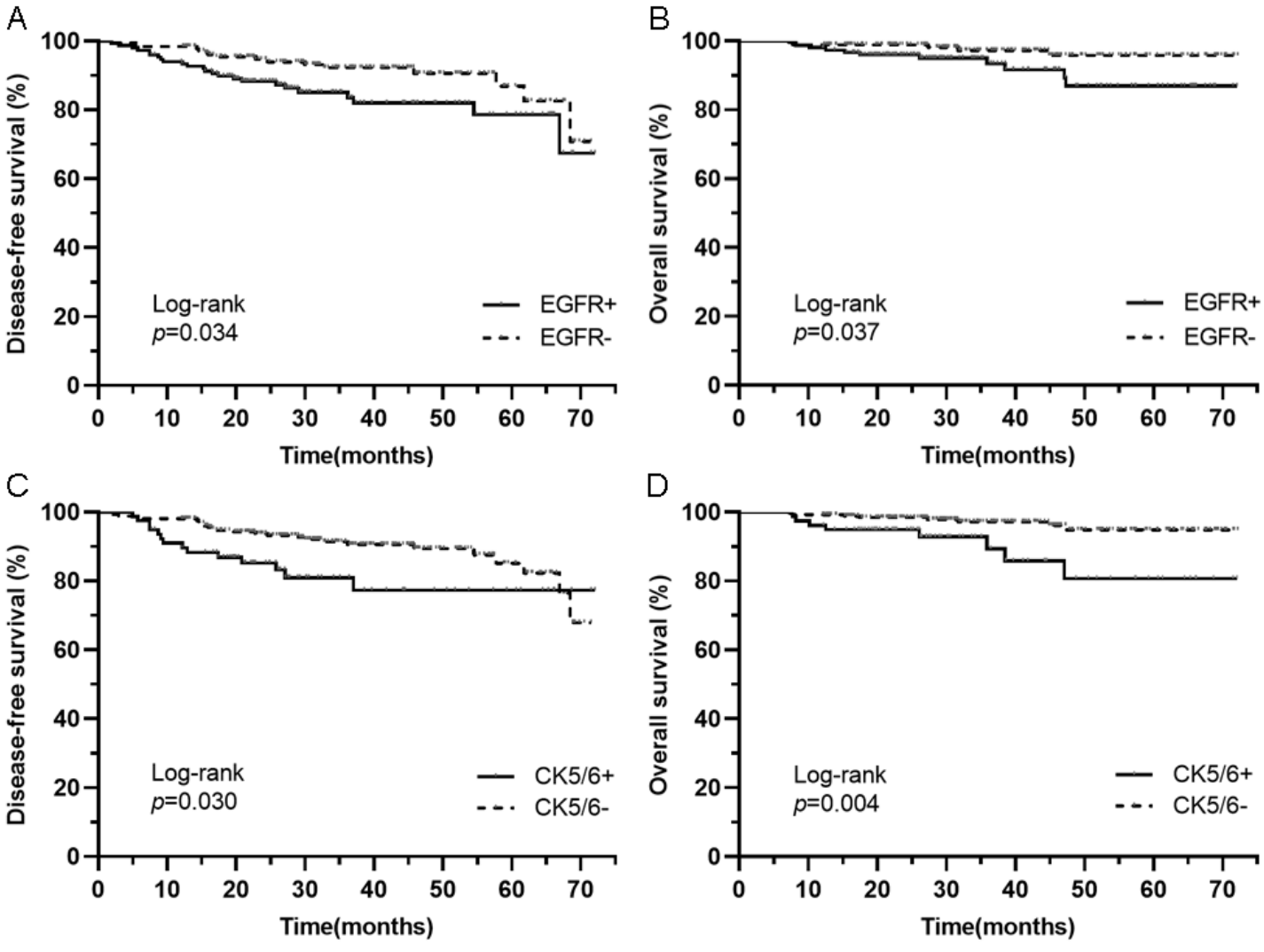

Figure 4

K-M survival curves in BM+ BCs and BM- BCs. Both DFS and OS were significantly shorter in BM+ BCs, including EGFR- (A, B) and CK5/6-positive BCs (C, D).

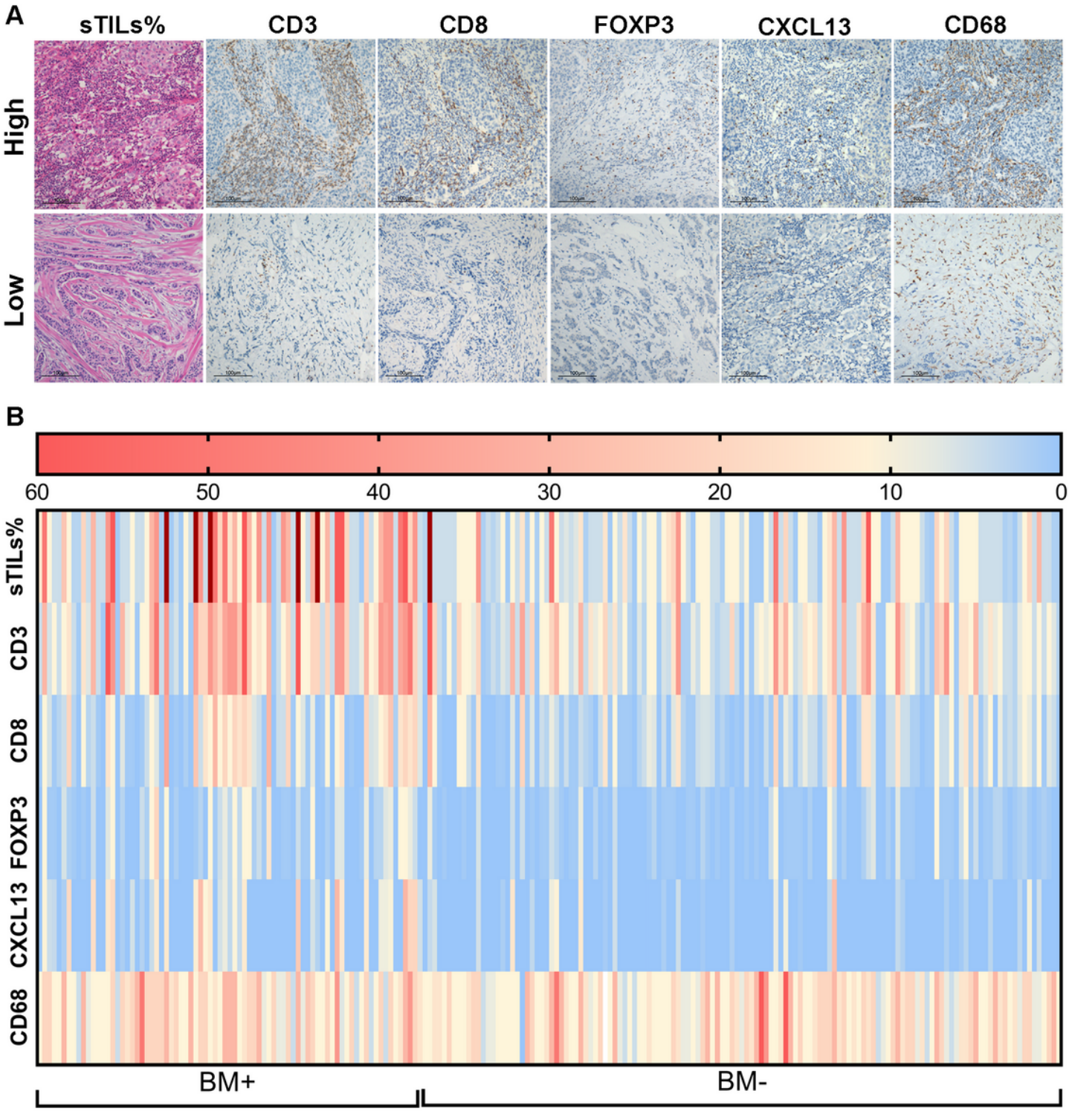

Figure 5

Examples of high expression of immune biomarker in BM+ BCs and low expression in BM- BCs are shown in A. The level of sTILs% and expressions of immune biomarker of 210 samples are shown in the heatmap (B), in which each column represents a sample, and colours change from blue to red with increasing proportions (%).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementarytable1.docx