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Original Article

Are synchronous and metachronous bilateral breast cancers different? An immunohistochemical analysis aimed at intrinsic tumor phenotype

Elżbieta Senkus[1], Jolanta Szade[2], Beata Pieczyńska[2], Anna Żaczek[3], Joanna Pikiel[4], Katarzyna Sosińska-Mielcarek[4], Agnieszka Karpińska[5], Jacek Jassem[1]

[1]Department of Oncology and Radiotherapy, Medical University of Gdańsk, Gdańsk, Poland; [2]Department of Pathomorphology, Medical University of Gdańsk, Gdańsk, Poland; [3]Laboratory of Cell Biology, Department of Medical Biotechnology, Intercollegiate Faculty of Biotechnology, University of Gdańsk and Medical University of Gdańsk, Gdańsk, Poland; [4]Regional Oncology Center, Gdańsk, Poland; [5]West-Pomeranian Oncology Center, Szczecin, Poland

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Abstract: The biology and pathomechanism of bilateral breast cancers is not fully understood. We compared the morphological and immunohistochemical characteristics of primary tumors in patients with synchronous bilateral breast cancers and metachronous bilateral breast cancers, with special focus on intrinsic tumor phenotype. Methods: Tumor morphology and expression of 8 immunohistochemical markers were assessed in tissue microarrays containing primary breast tumor cores from 113 metachronous bilateral breast cancers and 61 synchronous bilateral breast cancers. Analyzed markers included hormone receptors (estrogen receptor, progesterone receptor), HER2, Ki67, cytokeratin 5/6, E-cadherin, vimentin and epidermal growth factor receptor. Cutoff levels are provided in the table. Results: Metachronous bilateral breast cancers tumors had lower estrogen receptor expression (p=0.047) and higher expression of cytokeratin 5/6 (p=0.017) and of vimentin (p=0.008); in multivariate analysis only vimentin retained the significance (p=0.01). Ten (13%) and 11 (26%) of metachronous bilateral breast cancers and synchronous bilateral breast cancers had luminal A phenotype, 39 (50%) and 15 (36%) were luminal B HER2-negative, 13 (17%) and 12 (28%) - luminal B HER2-positive, 3 (4%) and 2 (5%) - HER2-positive (not luminal), and 12 (16%) and 2 (5%) had triple negative phenotype (p=0.07). Conclusion: Metachronous bilateral breast cancers, compared to synchronous bilateral breast cancers, are characterized by more aggressive phenotype, expressed by lower expression of estrogen receptor and stronger expression of cytokeratin 5/6 and vimentin; this does not, however, translate into differences in the distribution of intrinsic tumor phenotypes.

Keywords: Bilateral breast cancer, synchronous, metachronous, intrinsic phenotype, immunohistochemistry

Introduction

The risk of contralateral breast primary in breast cancer patients ranges between 2 and 15% and is estimated to be 2 to 6 times higher than that of first breast cancer in general population [1-3]. Some 30% of bilateral breast cancers occur synchronously, with the incidence of approximately $1.6/10^5$ person-years, which constitutes less than 2% of all breast cancers [4-6]. Apart from its low incidence, however, the number of such cases is about 100 times higher than could be expected by chance alone [4]. The annual risk of metachronous contralateral breast cancer in unselected breast cancer patients is 0.4-0.8% [1, 4, 6, 7]. Taking into account that (at least in older series) most patients had only one breast "at risk", the relative "per breast" risk may actually be even twice as high [2, 4]. Interestingly, the risk of metachronous contralateral breast cancer is similar to that observed in monozygote twin sisters of breast cancer patients [8].

The age incidence pattern of synchronous bilateral breast cancer (sBBC) recalls that of unilateral breast cancer (with absolute values being 50-100 times lower) [4]. The relative risk of metachronous bilateral breast cancer (mBBC) is particularly high in youngest patients. While

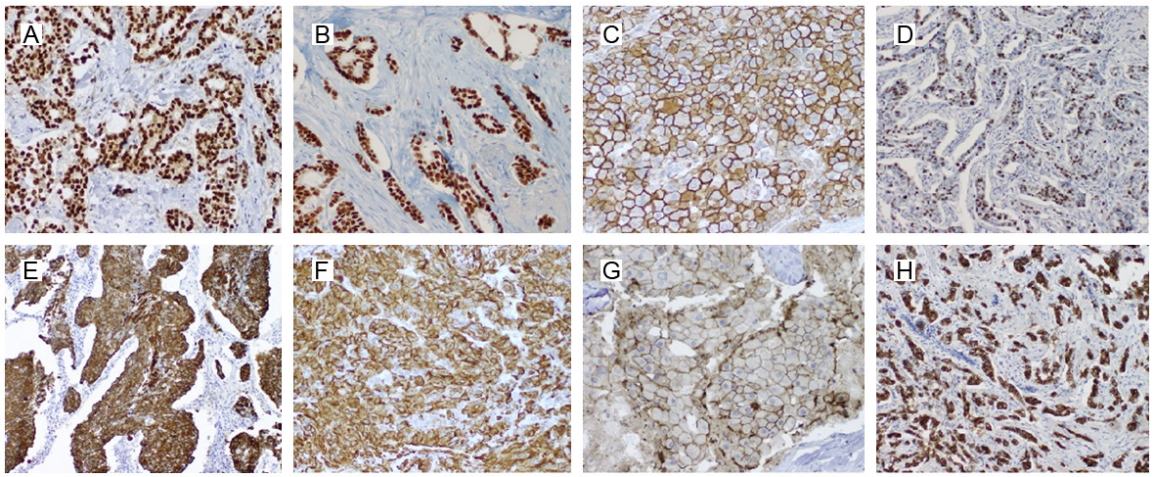

Figure 1. Immunohistochemistry staining for markers used in the study: A: estrogen receptor (x200), B: progesterone receptor (x200), C: HER2 (x200), D: Ki67 (x100), E: cytokeratin 5/6 (x100), F: vimentin (x200), G: EGFR (x200), H: E-cadherin (x100).

in unselected patients with a history of breast cancer the risk of developing a contralateral primary is approximately 5 times higher than that of breast cancer in an unaffected individual, this relative risk goes up to 23 in patients younger than 45, up to 50 - in those aged 30-34 and up to 100 - in 20-29-year-old population [2, 4, 6, 9]. Importantly, the incidence of second cancer in metachronous tumors may be modified by systemic therapy of the first cancer [6].

The appearance of two separate breast primaries may be a result of a genetic predisposition, exposure to common environmental risk factors or simply an accumulation of two unrelated events [3]. The incidence pattern of synchronous tumors, similar to unilateral breast cancer, suggests a relationship to accumulated exposure to environmental carcinogens [4, 5, 10]. In contrast, the high relative risk of metachronous contralateral breast cancer in young patients is suggestive of a genetic predisposition. Remarkably, *BRCA* mutations are more frequent among patients with metachronous cancers, although in one series *BRCA2* mutation was overrepresented in synchronous tumors [11, 12].

Different biology of sBBC and mBBC is also reflected in differences in histopathological features, stage and prognosis [10, 13-15]. Little is known, however, about the molecular characteristics of these two subtypes of bilateral breast cancer, in particular - their intrinsic phenotypes.

We undertook this study to analyze the distribution of immunohistochemically-defined surrogate intrinsic tumor phenotypes and related markers (estrogen receptor - ER, progesterone receptor - PgR, HER2, Ki67, cytokeratin 5/6 - CK5/6, vimentin, epidermal growth factor receptor - EGFR, E-cadherin - **Figure 1**) in tumor samples from patients with sBBC and mBBC. We believe that this study can add to the knowledge on pathomechanism of these tumors and possibly aid in guiding patient management.

Material and methods

The study was approved by the Ethics Committee of the Medical University of Gdańsk, Poland (NKEBN/280/2003 of 9-Jun-2003 and NKEBN/280-33/2007 of 6-Feb-2007). Cases were obtained from 4 institutions in Poland.

Available formalin-fixed paraffin-embedded tissue blocks from bilateral breast tumors were collected and centrally verified for diagnosis of invasive breast cancer and presence of sufficient invasive tumor to prepare tissue microarrays. Tumors were considered synchronous if diagnosed within 3 months. A total of 174 tumors diagnosed between 1985 and 2010 were available: 61 from patients with synchronous tumors (19 pairs of tumors from the same patient and 23 un-paired tumors) and 113 from patients with metachronous cancers (26 pairs of tumors from the same patient and 61 unpaired tumors, 44 first tumors and 69 second

Table 1. Antibodies used in the study

Antigen	Clone	Dilution	Incubation time	Epitope retrieval buffer pH	Positive control*	Supplier
ER[a]	6F11	1:800	overnight	9	endometrium	Novocastra[e]
PgR[b]	16	1:800	overnight	9	endometrium	Novocastra
HER-2	CB11	1:100	overnight	9	tonsil	Novocastra
Ki 67	MM1	1:1200	90 min.	6	tonsil, small bowel	Novocastra
CK5/6[c]	D5/16B4	1:800	overnight	9	tonsil, parotid gland, endometrium, urinary bladder	Millipore[f]
Vimentin	V9	1:100	overnight	9	tonsil, endometrium, testis	Novocastra
EGFR[d]	EGFR.113	1:20	90 min.	9	placenta, tonsil, testis, prostate	Novocastra
E-cadherin	36B5	1:32	overnight	9	tonsil, parotid gland, endometrium	Novocastra

*for negative control the same tissues and processing were used, apart from omitting the primary antibody. [a]estrogen receptor, [b]progesterone receptor, [c]cytokeratin 5/6, [d]epidermal growth factor receptor, [e]Leica Microsystems GmbH, Wetzlar, Germany, [f]Merck Millipore, Billerica, MA.

Table 2. Morphological features of sBBC and mBBC

	mBBC[a] (%)	sBBC[b] (%)	p (χ^2)
Number of patients (%)	113 (65)	61 (35)	
Histology (WHO 2003)			0.037
invasive ductal	98 (87)	47 (77)	
invasive lobular	5 (4)	8 (13)	
cribiform	2 (3)	3 (5)	
micropapillary	2 (2)	1 (2)	
tubular	-	2 (3)	
mucinous	2 (2)	-	
papillary	2 (2)	-	
medullary	2 (2)	-	
Grade			0.17
1	25 (22)	20 (33)	
2	42 (37)	24 (39)	
3	46 (41)	17 (28)	
DCIS[c]			0.63
absent	68 (60)	34 (56)	
non-extensive	19 (17)	9 (15)	
extensive	26 (23)	18 (29)	

[a]metachronous breast cancer, [b]synchronous breast cancer, [c]ductal carcinoma in situ.

tumors). All tumors were reviewed for histology in accordance with 2003 WHO classification [16], tumor grade and the presence and extent of intraductal component.

Tissue microarrays (TMA) were built using Manual Tissue Microarrayer 1 (Beecher Instr. Inc, Sun Prairie, WI), using 2 representative cores for each tumor. The blocks were cut into 4 μm thick sections and stained according to standard procedures, as described by manufacturers. Incubation with primary antibody was conducted overnight or for 90 min, depending on the antibody used (**Table 1**). The Novolink Polymer Detection System (Leica Microsystems GmbH, Wetzlar, Germany) was used for all the procedures, apart from the primary antibody and buffers used for antigen retrieval (DAKO, Glostrup, Denmark).

The histology review and immunohistochemistry scoring were carried out by a single experienced pathologist (JSz). ER and PgR were scored according to Allred criteria (with percentage and intensity scores noted separately) and HER2 - in accordance with ASCO/CAP guidelines [17]. For Ki67, the proportion of positive cells was divided into 3 categories: ≤14%, 15-30% and >30%. The proportion of cells stained for CK5/6, vimentin and EGFR was categorized into negative (<1%), 1-10% and >10%. E-cadherin score was considered positive when ≥1% of cells were stained.

Surrogate intrinsic phenotypes were determined using expression of steroid receptors, HER2 and Ki67 [18]. For HER2 (2+) cases, FISH analysis was not performed for the purpose of this study; for two patients the result was available in patients' records - these results were used only for determination of surrogate intrinsic tumor phenotype. Intrinsic phenotype was determined only for HER2 IHC 0, 1+ or 3+ patients and for those HER2 2+ for whom FISH result was available

Statistical methods

Correlation between synchronous/metachronous status and continuous variables (such as age) was tested by Mann-Whitney test, whereas correlations between the dichotomized variables were tested by χ^2 or Fisher's exact test. Odds ratios with 95% confidence intervals were calculated with logistic regression analysis.

Table 3. Antigen expression in sBBC and mBBC

	mBBC[a] (%)	sBBC[b] (%)	p (χ^2)
Estrogen receptor (Allred score)	*109 tumors*	*59 tumors*	0.26
negative (0-2/8)	22 (20)	6 (10)	
weak (3/8)	6 (6)	2 (3)	
moderate (4-5/8)	8 (7)	3 (5)	
strong (6-8/8)	73 (67)	48 (81)	
Progesterone receptor (Allred score)	*112 tumors*	*60 tumors*	0.15
negative (0-2/8)	38 (34)	11 (18)	
weak (3/8)	8 (7)	6 (10)	
moderate (4-5/8)	11 (10)	5 (8)	
strong (6-8/8)	55 (49)	38 (63)	
HER2	*111 tumors*	*58 tumors*	0.36
negative (0-1+)	60 (54)	29 (50)	
equivocal (2+)	34 (31)	15 (26)	
positive (3+)	17 (15)	14 (24)	
Ki67	*110 tumors*	*60 tumors*	0.15
≤14%	24 (22)	21 (35)	
15-30%	37 (34)	19 (32)	
>30%	49 (44)	20 (33)	
cytokeratin 5/6	*106 tumors*	*57 tumors*	0.05
<1%	67 (63)	44 (77)	
1-10%	11 (10)	7 (12)	
>10%	28 (26)	6 (11)	
vimentin	*108 tumors*	*58 tumors*	0.029
<1%	68 (63)	43 (74)	
1-10%	15 (14)	11 (19)	
>10%	25 (23)	4 (7)	
EGFR[c]	*108 tumors*	*58 tumors*	0.14
<1%	73 (68)	47 (81)	
1-10%	21 (19)	5 (9)	
>10%	14 (13)	6 (10)	
E-cadherin	*106 tumors*	*59 tumors*	0.99
negative	18 (17)	10 (17)	
positive	88 (83)	49 (83)	
Intrinsic phenotype	*77 tumors*	*42 tumors*	0.07
luminal A	10 (13)	11 (26)	
luminal B HER2-	39 (50)	15 (36)	
luminal B HER2+	13 (17)	12 (28)	
triple negative	12 (16)	2 (5)	
HER2+ (non luminal)	3 (4)	2 (5)	

[a]metachronous breast cancer, [b]synchronous breast cancer, [c]epidermal growth factor receptor.

Similarly, in multivariate analysis, logistic regression was used (stepwise backwards logistic regression, 95%). Statistical significance was assumed when $p<0.05$. Calculations were performed using Statistica software (Statsoft Co, USA, version 10), licensed to the Medical University of Gdańsk.

Results

Median age at diagnosis of mBBC and sBBC was 55 (49.5 and 56 years for first and second tumor, respectively) and 52 years, respectively (p=0.52) - **Table 2**. The median latency between first and second tumors was 75 months (range: 12-184 months) for patients with available pairs of tumors and 87 months (range 9-384 months) for all patients. The majority of tumors in all groups were invasive ductal carcinomas, but in sBBC lobular histology was more common (13% vs 4%, p=0.037). Grade 3 tumors were marginally more common in metachronous tumors (p= 0.09).

For TMA, the number of missing cores for particular assays ranged from 1 to 6% - most missing cores were from the oldest tumor blocks, for which the problems with fixation techniques were most pronounced.

Strong ER and PgR expression was present in 81% and 63% of sBBC, and 67% and 49% of mBBC, respectively (p=0.047 and 0.07, respectively, **Table 3**). Strong expression of CK5/6 (>10% of cells) was observed in 26% of mBBC and 11% of sBBC (p=0.017) and of vimentin (≥10% of cells) - in 23% of mBBC and 7% of sBBC. EGFR expression (≥1% of cells) was found in 32% of mBBC and 19% of sBBC (p=0.06). No differences in expression of HER2, Ki67 and E-cadherin were identified between subgroups.

Ten (13%) and 11 (26%) of mBBC and sBBC had luminal A phenotype, 39 (50%) and 15 (36%) were luminal B HER2-negative, 13 (17%) and 12 (28%) - luminal B HER2-positive, 3 (4%) and 2 (5%) - HER2-positive (not luminal), and 12 (16%) and 2 (5%) had triple negative phenotype (p=0.07).

Table 4. Uni- and multivariate analysis of factors associated with mBBC vs sBBC

	N (%)		Univariate analysis		Multivariate analysis	
	mBBC[a]	sBBC[b]	OR[c] (95% CI[d])	p (logistic regression)	OR (95% CI)	p (logistic regression)
Estrogen receptor (Allred score)						
negative, weak and moderate (0-6)	36 (33)	11 (9)	0.46 (0.21-1)	0.05	-	NS
strong (6-8)	73 (67)	48 (81)				
Progesterone receptor (Allred score)						
negative, weak and moderate (0-6)	57 (51)	22 (37)	0.56 (0.29-1.07)	0.07	-	NS
strong (6-8)	55 (49)	38 (63)				
HER2						
negative (0-1)	60 (54)	29 (50)	0.85 (0.45-1.6)	0.6	-	NS
positive (2+, 3+)	51 (46)	29 (50)				
Ki67						
≤14%	24 (22)	21 (35)	1.9 (0.95-3.9)	0.06	-	NS
>14%	86 (78)	39 (65)				
CK5/6[e]						
≤10%	78 (74)	51 (89)	3.05 (1.2-7.9)	0.02	-	NS
>10%	28 (26)	6 (11)				
vimentin						
≤10%	83 (77)	54 (93)	4.01 (1.3-12.4)	0.01	4.01 (1.3-12.4)	0.01
>10%	25 (23)	4 (7)				
E-cadherin						
negative	18 (17)	10 (17)	0.99 (0.33-3.01)	0.99	-	NS
positive	88 (83)	49 (83)				
EGFR[f]						
≤1%	73 (68)	47 (81)	2.01 (0.94-4.45)	0.07	-	NS
>1%	35 (32)	11 (19)				
grade						
1+2	67 (59)	44 (72)	1.3 (0.95-1.9)	0.09	-	NS
3	46 (41)	17 (28)				
DCIS[g]						
absent	68 (60)	34 (56)				
non-extensive	19 (17)	9 (15)	0.86 (0.6-1.24)	0.42	-	NS
extensive	26 (23)	18 (29)				

[a]metachronous breast cancer, [b]synchronous breast cancer, [c]odds ratio, [d]confidence interval, [e]cytokeratin 5/6, [f]epidermal growth factor receptor, [g]ductal carcinoma in situ.

As expected, there was a strong correlation between high tumor grade and lower ER and PgR scores (p=0.00009 and 0.00002, respectively) and between tumor grade and expression of Ki-67 (p<0.00001), CK5/6 (p=0.00001) and vimentin (p=0.00001). Not surprisingly, lack of E-cadherin expression was strongly correlated with lobular histology (p<0.00001). We also observed strong correlation of ER, PgR and HER2 with lower expression of CK5/6 (p<0.00001, 0.00001 and 0.046, respectively) and vimentin (p<0.00001, 0.00001 and 0.0025, respectively), and of ER and PgR with lack of EGFR expression (p=0.00001 and 0.0008, respectively). Additionally, high HER2 expression (2-3+) was more frequent in ductal histology (p=0.004) and in tumors with extensive DCIS (p=0.003), high Ki67 was seen more often in ER-low tumors (p=0.03), whereas expression of E-cadherin was lower in ER-high and HER2-low tumors (p=0.003 and 0.01, respectively).

In multivariate analysis, mBBC was related only to higher vimentin expression (OR=4.01, CI 1.3-12.4, p=0.01) - **Table 4**.

Additionally, in an exploratory analysis, sBBC and mBBC differed in terms of relationship between analyzed histopathological factors - **Table 5**. Overall, these relationships can be divided into three groups: (1) co-existence of

Table 5. Exploratory analysis of differences between expression of selected histopathological factors in subgroups of sBBC and mBBC (higher values in bold)

Subgroup	analyzed factor	frequency		p
		sBBC	mBBC	
G3	ER-low[a]	3/16 (19%)	**25/45 (56%)**	0.01
G3	PgR-low[b]	7/16 (44%)	**35/46 (76%)**	0.017
G3	HER2-low[c]	6/16 (38%)	**30/46 (65%)**	0.053
G3	CK5/6-high[d]	2/16 (12%)	**22/45 (49%)**	0.009
ER-low	G3	3/11 (27%)	**25/36 (69%)**	0.01
ER-low	Ki67-high[e]	5/10 (50%)	**34/36 (94%)**	0.0005
ER-low	CK5/6-high	2/10 (20%)	**30/35 (57%)**	0.04
ER-low	vimentin-high[f]	1/10 (10%)	**21/35 (60%)**	0.005
PgR-low	G3	7/22 (32%)	**35/57 (61%)**	0.02
PgR-low	vimentin-high	2/22 (9%)	**22/54 (41%)**	0.007
HER2-low	G3	6/29 (21%)	**30/60 (50%)**	0.008
HER2-low	vimentin-high	2/28 (7%)	**21/60 (35%)**	0.006
CK5/6-high	G3	2/6 (33%)	**22/28 (79%)**	0.03
CK5/6-high	ER-low	2/6 (33%)	**20/27 (74%)**	0.06
vimentin-high	ER-low	1/4 (25%)	**21/23 (91%)**	0.002
Ki67-low[g]	extensive DCIS	**12/21 (57%)**	5/24 (21%)	0.01
extensive DCIS	Ki67-low	**12/18 (67%)**	5/26 (19%)	0.0015
E-cadherin (-)	ductal	3/10 (30%)	**11/15 (73%)**	0.03

[a]Allred 0-5, [b]Allred 0-5, [c]IHC 0-1, [d]>10%, [e]>14%, [f]>10%, [g]≤14%.

factors related to tumor aggressiveness (G3, low expression of ER, PgR, high expression of CK5/6 and vimentin) - observed more frequently in mBBC; (2) co-existence of low Ki67 expression and extensive DCIS - more frequent in sBBC; (3) ductal histology in E-cadherin (-) tumors - more common in mBBC (in contrast to lobular histology found in majority of E-cadherin-negative sBBC tumors).

Discussion

Our study demonstrated that mBBC are characterized by lower ER and PgR expression and higher expression of CK5/6, vimentin and EGFR, whereas lobular histology was more common in sBBC. The morphology of mBBC tumors fits into a pattern more typical for tumors related to genetic predispositions, mostly *BRCA1* mutations [19]. Indeed, mBBC are believed to be related more to inherited predispositions, rather than environmental factors [4, 5]. In spite of being more common than in sBBC, *BRCA1/2* mutations in this population are, however, present only in a minority of patients [11, 12, 20]. Interestingly, it has been demonstrated that breast cancers in patients with strong family history and no mutation in *BRCA1* or *BRCA2* are different not only from *BRCA*-related tumors, but also from sporadic breast cancers [21], suggesting the existence of specific inherited factor.

As bilateral breast cancer by itself is not a routine indication for mutation testing, we have no knowledge on the *BRCA1/2* status in all our patients. Among the 53 *BRCA1*-tested patients, 10 carried a germline mutation: 8 with mBBC and 2 with sBBC (compared to 30 mBBC and 13 sBBC patients without *BRCA1* mutation). Owing to possible selection bias related to patterns of referral to genetic testing; it is, however, difficult to make any conclusions about mutation frequency in both populations.

The age of our sBBC and mBBC patients did not differ significantly, although many series report earlier occurrence of mBBC compared to sBBC, with median age in the latter group being as high as 65-71 years [22-28]. There is, however, a lack of consistency in the way the age of mBBC patients is reported. In most series, age at first and second presentation are reported separately, whereas we believe there is no biological difference between first and subsequent tumors (apart from a possible impact of treatment of the first tumor) and analyzed each tumor as a separate entity (to validate such an approach we compared the clinicopathological characteristics and biomarker expression in first vs second tumors: no significant differences were identified - data not shown). Actually, due to better tumor block availability for more recent tumors, majority of analyzed samples in the mBBC population in our study came from the second tumors- which obviously resulted in the increase of the median age in the entire group.

Our patients were, in general, younger than those described in British, American and Australian series [7, 10, 29-34], resembling more the age distribution in the German and Turkish population [20, 35]. On the other hand,

median age at first breast cancer presentation in a recent series from China is as low as 43 and 49 years for mBBC and sBBC, respectively [28].

Like in most series, the prevalent histology was invasive ductal [14, 15, 36-40]. Similarly to some other studies, the lobular histology was more common in synchronous tumors [6, 22, 26], although a single series from Nottingham reported higher incidence of lobular tumors among mBBC [34]. In some series, mBBC tend to demonstrate higher grade [35]; the data on long-term outcome are, however, contradictory (which may be related to different method of calculation of event-free survival in particular series: from first or second tumor) [22, 24, 27-29].

Lower steroid receptor expression in metachronous tumors in our series is consistent with other observations [22, 24, 26, 27, 35]. A few, in majority small, series reported on HER2 status in sBBC and mBBC, with no unique repetitive pattern [27, 28, 35, 38, 41, 42].

The expression of vimentin is upregulated in tumors undergoing epithelial-mesenchymal transition and is associated with the basal-like phenotype, but reported expression frequency in this population varies between 25 and 100%, as compared to 4-23% in tumors unselected for intrinsic phenotype [43-47]. No studies have specifically analyzed vimentin expression in sBBC and mBBC patients. In a single study, expression of this protein was among the parameters used to differentiate basal and non-basal triple-negative cancers: the frequency of the basal subtype in sBBC and mBBC cohorts was similar [48]. In contrast, in our study, vimentin was found to be the only biomarker independently related to mBBC, as opposed to sBBC. Its higher expression in the former group (37% vs 26%) may again suggest more aggressive phenotype than that seen in sBBC.

We are aware of only one small study analyzing E-cadherin expression in BBC patients [49] - with no data applicable to synchronous/metachronous subtypes. We have not found any difference in expression of this biomarker between analyzed groups, in spite of differences in frequency of lobular histology. This may partially be explained by the observation of higher incidence of ductal E-cadherin-negative tumors among mBBC (**Table 5**).

EGFR is typically expressed in triple negative and basal-like cancers [19]. No study has specifically assessed EGRF expression in BBC and, in unselected sporadic cases, its expression ranged widely from 1.5% to 44% [50-53], which encompasses both the values of 19% and 32% seen in our study. Again, marginally higher value seen in mBBC fits it into generally more aggressive phenotype.

We are not aware of any study assessing Ki67 expression specifically in synchronous/metachronous breast cancers. In general, expression above the 14% cut-off value in BBC seemed to be more common (65-78%) than in most unilateral cancer series [54]. There is no obvious explanation for this phenomenon, in particular in view of quite typical grade distribution.

Finally, the 5% and 16% incidence of triple-negative tumors (in sBBC and mBBC, respectively) in this series is remarkably lower than that of 30% observed in the only other study assessing intrinsic phenotypes specifically in BBC [48].

Our study is unique in analyzing the pattern of expression of extended panel of antigens related to the intrinsic subtypes of breast cancer in BBC - such a study has not been conducted before.

Another strength of our study is that patients were not selected for survival status (as is often the case in patients selected from genetic clinics), apart from surviving from the first malignancy long enough to develop the second one. However, as for metachronous tumors, the more recently diagnosed cases were more frequently available; this bias seems to be of minor impact.

Patients in this series were unselected for family history - this was primarily caused by the retrospective character of our study and the unavailability of family history in many cases. Family history, however, tends to be quite unreliable, particularly in small families [55]. Owing to large migrations and human losses in Poland during and after the Second World War, relatively few Polish patients are able to provide medical history for more than two preceding

generations. Remarkably, among proven *BRCA1* and *BRCA2* mutation carriers, 5-40% have no known family history of breast and ovarian cancers [56, 57].

Another limitation is the lack of available tissue for a proportion of paired tumors - this restricts the availability to assess the concordance of antigen expression between both tumors in the same individual. However, this was not the aim of our study, as this subject has been addressed extensively earlier. To increase the informative value and power of our study, we included also cases in which only a single tumor tissue was available. This was in line with our belief of each tumor being a result of a separate carcinogenic event.

Even lower power applies to comparison of intrinsic phenotypes, as we were not able to assess the HER2 amplification. However, since, on average among the IHC HER2 (2+) patients, some 15-20% are HER2 amplified, it is unlikely that this would change the main conclusions of our study.

Additionally, as tumors were collected over a long period of time and acquired from many institutions, differences in fixation techniques might have affected the IHC analysis. However, as this limitation applies in similar degree to both sBBC and mBBC, it probably had only a minor impact on the final results and conclusions.

The results of comparisons of sBBC and mBBC are obviously dependent on adapted threshold differentiating both BBC categories. As in most other series, we have adapted the 3-month cut-off, however values as long as 12-24 months have also been used in some studies and no strong data exist to support the choice of any particular period [10].

Our study is mainly descriptive and provides new data on the characteristics of bilateral breast cancers. However, it may also aid in management of multiple tumors and counseling (also regarding prophylactic contralateral mastectomy) as well as surveillance of patients with “single” breast cancer, with regard to the risks of developing second breast tumor.

In conclusion, mBBC are characterized by more aggressive phenotype, expressed by lower expression of ER and stronger expression of CK5/6 and vimentin, compared to sBBC; this does not, however, translate into differences in the distribution of intrinsic tumor phenotypes.

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Disclosure of conflict of interest

None.

Address correspondence to: Elżbieta Senkus, Department of Oncology and Radiotherapy, Medical University of Gdańsk, Dębinki 7, 80-211 Gdańsk, Poland. Tel: +48 58 349 2221; Fax: +48 58 349 2210; E-mail: elsenkus@gumed.edu.pl

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