Histol Histopathol (2018) 33: 55-64

http://www.hh.um.es

Histology and Histopathology

From Cell Biology to Tissue Engineering

Are synchronous and metachronous bilateral breast cancers different? An immunohistochemical analysis focused on cell cycle regulation

Elżbieta Senkus[1], Jolanta Szade[2], Beata Pieczyńska[2], Michał Kunc[3], Agnieszka Pliszka[1] and Jacek Jassem[1]

[1]Department of Oncology and Radiotherapy, [2]Department of Pathomorphology, Medical University of Gdańsk and [3]University Clinical Centre, Gdańsk, Poland

Summary. Introduction. The biology and pathomechanisms of bilateral breast cancers is not fully understood. We compared the morphological and immunohistochemical characteristics of primary tumors in patients with synchronous (sBBC) and metachronous bilateral breast cancers (mBBC), with special focus on cell cycle regulation and its correlation with markers determining intrinsic phenotype.

Methods. Immunohistochemical expression of $p16^{Ink4A}$, $p21^{(WAF1/CIP1)}$, $p27^{Kip1}$, p53, cyclin A, cyclin B, cyclin D1, cyclin D3 and cyclin E was assessed in tissue microarrays containing primary breast tumor cores from 113 mBBC and 61 sBBC. Expression of these markers was correlated with tumor grade and expression of estrogen receptor (ER), human epidermal growth factor receptor 2 (HER2) and Ki-67.

Results. In univariate analysis, mBBC demonstrated higher expression of $p16^{Ink4A}$ (both cytoplasmic: p=0.002 and nuclear: p=0.014), cyclin A (p=0.024) and B (cytoplasmic; p=0.015). In multivariate analysis mBBC were associated with lower expression of p21: p=0.038 and higher cytoplasmic expression of cyclin B: p=0.019. Lower ER expression for all BBCs and mBBC, respectively, was associated with stronger p16 expression (cytoplasmic: both p<0.000001 and nuclear: p<0.000001, p=0.00002), p53: p<0.000001, p=0.000001, cyclin A: p=0.00002, p=0.00045, cyclin B (cytoplasmic: p=0.00037, 0.00015 and nuclear: both p=0.0004) and cyclin E: p=000003, p<0.000001, and weaker expression of p27: p=0.00003, p=0.0001 and cyclin D1: both p<0.000001; for sBBC some of these correlations were absent. Higher p27 score correlated with lower HER2 expression in sBBC: p=0.018, whereas higher HER2 expression was associated with higher p53: 0.024 and cyclin E: p=0.048 expression in all BBC and higher nuclear expression of cyclin B in sBBC: p=0.027. Higher Ki-67 expression was correlated with higher expression of p16 (cytoplasmic: p=0.000015, p=0.086, p=0.0002 and nuclear: p=0.000009, p=0.016, p=0.00003) in all subsets [all BBC, sBBC (non-significant for cytoplasmic score), mBBC, respectively], p21 (all BBC: p=0.05) and sBBC: p=0.017), p53 (all BBC: p=0.0003 and mBBC: p=0.0002), cyclin A: all p<0.000001, cyclin B (cytoplasmic: p<0.000001, p=0.004, p<0.000001, respectively and nuclear: p=0.0002, p=0.047, p=0.0026, respectively), cyclin D3 (all BBC: p=0.005 and mBBC: p=0.02) and cyclin E (all BBC: p<0.000001 and mBBC: p=0.000002), and lower expression of cyclin D1 (all BBC: p=0.046 and mBBC: p=0.035) and p27 (sBBC: p=0.048).

Conclusion. Compared to sBBC, mBBC are characterized by lower expression of p21 and higher cytoplasmic expression of cyclin B, suggesting its more aggressive behavior. Correlations between cell-cycle regulation proteins and markers of breast cancer phenotype parallel those reported for unilateral breast cancer.

Key words: Bilateral breast cancer, Synchronous, Metachronous, Cell cycle, Intrinsic phenotype, Immunohistochemistry

Offprint requests to: Elżbieta Senkus, Department of Oncology and Radiotherapy, Medical University of Gdańsk, Dębinki 7, 80-211 Gdańsk, Poland. e-mail: elsenkus@gumed.edu.pl

DOI: 10.14670/HH-11-887

Introduction

Bilateral breast cancers (BBC) are a heterogenous group of tumors with specific risk factors, prognosis and treatment. Tumor in the contralateral breast may either grow synchronically (sBBC) or metachronically (mBBC), displaying different phenotypes.

The risk of contralateral primary 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 (Dawson et al., 1991; Chen et al., 1999; Vaittinen and Hemminki, 2000). In a recent series of over 4000 breast cancer patients treated at one institution over more than 30 years, the incidence of sBBC among all breast cancers was 1% and mBBC - 7% (Jobsen et al., 2015). Approximately 30% of BBC occur synchronously, with the incidence of approxi-mately $1.6x10^{-5}$ person-years, which constitutes less than 2% of all breast cancers (Kollias et al., 2004; Hartman et al., 2005; Schaapveld et al., 2008). The annual risk of mBBC cancer in unselected breast cancer patients ranges from 0.4 to 0.8% (Chen et al., 1999, 2001; Hartman et al., 2007; Schaapveld et al., 2008). Considering that (at least in older series) most patients had only one breast "at risk", the relative "per breast" risk may actually be even doubled (Vaittinen and Hemminki, 2000; Hartman et al., 2005). Interestingly, the risk of mBBC is similar to that observed in monozygotic twin sisters with breast cancer, suggesting a genetic background (Peto and Mack, 2000).

The development of two separate breast primaries may result from a genetic predisposition, exposure to common environmental risk factors, or simply an accumulation of two unrelated and incidental events (Dawson et al., 1991). The incidence pattern of sBBC is similar to that of unilateral breast cancer, suggesting a relationship to accumulated exposure to environmental carcinogens (Kollias et al., 2004; Hartman et al., 2005; Howe et al., 2005). In contrast, the high relative risk of contralateral mBBC in young patients is suggestive of a genetic predisposition. Remarkably, *BRCA* mutations are more frequent among patients with mBBC, although in one series *BRCA2* mutations were overrepresented in synchronous tumors (Bergthorsson et al., 2001; Rogozińska-Szczepka et al., 2004).

Different biology of sBBC and mBBC is also reflected by differences in histopathological features, stage and prognosis (Safal et al., 2002; Hartman et al., 2005; Howe et al., 2005; Takahashi et al., 2005). Little is known, however, about the molecular characteristics of these two subtypes of BBC.

The cell-cycle is a complicated machinery depending on various regulators. Interactions between cyclins, cyclin-dependent kinases (CDKs) and their inhibitors (CDKIs) are essential for the successful completion of the entire cell cycle (Casimiro et al., 2012). Cyclins are a group of proteins activating cyclin-dependent kinases. Several events may lead to cyclin overexpression in cells, e.g. gene amplification, defected proteasome degradation or abnormal ubiquitination (Hwang and Clurman, 2005). Overexpression of cyclins is a known factor involved in carcinogenesis in many tissues, including breast. For example, cyclin D/CDK4 and cyclin E/CDK2 complexes inactivate retinoblastoma (Rb) protein, leading to progression from G1 to S phase (Connell-Crowley et al., 1997). Cell cycle progression may be regulated by CDKIs, which belong to two families: the INK4 inhibitors (p16, p15, p19, and p18) and the Cip/Kip inhibitors (p21, p27, and p53). The first ones are G1-phase specific and block the function of CDK4 and CDK6, whereas the latter act regardless of the cycle phase (Deshpande et al., 2005).

The role of the cell-cycle regulators in breast cancer pathogenesis and their clinical relevance is well established. Cyclin D1 amplification is an early event in breast carcinogenesis and serves as a marker of malignant transformation (Velasco-Velázquez et al., 2011). As cyclin D1 expression is regulated by estrogen receptor, its changes may impact endocrine responsiveness in tamoxifen-resistant tumors (Kilker et al., 2004). Similarly, cyclin E overexpression contributes to the pathogenesis of breast cancer, whereas cyclin A overexpression is associated with poor prognosis (Geng et al., 2001; Husdal et al., 2006). In turn, some of the CDKIs showed predictive and prognostic value in breast cancer patients. The relocation of p27 from the nucleus to the cytoplasm may drive anti-HER2 therapy resistance, whereas p21 loss confers tamoxifen-stimulated growth of breast cancer (Abukhdeir et al., 2008). While many studies have shed some light on the

Table 1. Patient and tumor characteristics.

Variable	mBBC[a] (%)	sBBC[b] (%)	p
Number of patients (%)	113 (65)	61 (35)	
median age (years)	55	52	0.52
median latency between tumors (months)	75	0	N/A
Histology			0.037
non-special type	98 (87)	47 (77)	
invasive lobular	5 (4)	8 (13)	
other	10 (9)	6 (10)	
Grade			0.17
1	25 (22)	20 (33)	
2	42 (37)	24 (39)	
3	46 (41)	17 (28)	
Estrogen receptor (Allred score)	109 tumors	59 tumors	0.047
0-6	36 (33)	11 (9)	
6-8	73 (67)	48 (81)	
HER2	111 tumors	58 tumors	0.6
0, 1+	60 (54)	29 (50)	
2+, 3+	51 (46)	29 (50)	
Ki-67	110 tumors	60 tumors	0.15
≤30%	61 (56)	40 (67)	
>30%	49 (44)	20 (33)	

[a]: metachronous bilateral breast cancer, [b]: synchronous bilateral breast cancer, N/A: not applicable.

Table 2. 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, breast cancer	Novocastra[b]
HER-2	CB11	1:100	overnight	9	breast, tonsil, breast cancer	Novocastra
Ki 67	MM1	1:1200	90 min.	6	breast, tonsil	Novocastra
$p16^{Ink4A}$	JC8	1/200	90 min.	6	CIN3	Santa Cruz[c]
$p21^{(WAF1/CIP1)}$	DCS-60.2	**	**	**	colon, non-small cell lung carcinoma	Ventana[d]
$p27^{Kip1}$	SX53G8	**	**	**	colon, non-small cell lung carcinoma	Ventana
p53	DO-7	1/200	overnight	9	breast, tonsil, placenta	Novocastra
cyclin A	6E6	1/100	90 min.	6	tonsil, small intestine	Novocastra
cyclin B	7A9	1/40	90 min.	6	tonsil	Novocastra
cyclin D1	P2D11F11	1/50	90 min.	6	liver	Novocastra
cyclin D3	DCS-22	1/30	60 min.	9	tonsil	Novocastra
cyclin E	13A3	1/60	60 min.	9	placenta	Novocastra

*: for negative control the same tissues and processing were used, apart from omitting the primary antibody. **: stainings were performed on autostainer according to manufacturers' instructions. [a]: estrogen receptor, [b]: Leica Microsystems GmbH, Wetzlar, Germany, [c]: Santa Cruz Biotechnology, Inc., Dallas, TX, [d]: Ventana Medical Systems, Inc., Tucson, AZ.

role and clinical relevance of the cell-cycle proteins in breast cancer, such knowledge in relation to BBC is limited.

We have previously demonstrated that compared to sBBC, mBBC are characterized by lower expression of ER and stronger expression of CK5/6 and vimentin (Senkus et al., 2014a). We also showed that compared to unilateral breast cancers, sBBC present more often with low grade, high ER expression and low expression of cytokeratin 5/6 (CK5/6), epidermal growth factor receptor (EGFR) and E-cadherin, and are less often of triple-negative phenotype. In turn, compared to sporadic cancers, mBBC demonstrate lower ER, progesterone receptor (PgR) and HER2 expression, higher Ki6 and vimentin expression, are more often of triple-negative and less often of HER2-positive phenotype. Overall BBC, compared to sporadic tumors present with lower HER2 expression, higher Ki-67 expression and are less often of HER2-positive phenotype (Senkus et al., 2014b).

The aim of the current study was to analyze the distribution of markers related to cell cycle regulation ($p16^{Ink4A}$, $p21^{(WAF1/CIP1)}$, $p27^{Kip1}$, p53, cyclin A, cyclin B, cyclin D1, cyclin D3, cyclin E) in primary tumor samples of sBBC and mBBC. We have also compared expression profiles of sBBC and mBBC, and analyzed correlations between the cell-cycle regulators and expression of markers determining surrogate (IHC based) intrinsic phenotype (estrogen receptor - ER, progesterone receptor - PgR, HER2, Ki67) for all BBC subpopulations.

Materials 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

Table 3. Antigen expression in sBBC and mBBC tumors.

Score	sBBC[a] (%)	mBBC[b] (%)	p (χ^2)
p16 (cytoplasmic) score	57 tumors	108 tumors	**0.002**
0-6	51 (89)	77 (71)	
7-8	6 (11)	31 (29)*	
p16 (nuclear) score	57 tumors	108 tumors	**0.014**
0-6	48 (84)	76 (70)	
7-8	9 (16)	32 (30)*	
p21 score	54 tumors	104 tumors	0.051
0-6	38 (70)	87 (84)	
7-8	16 (30)**	17 (16)	
p27 score	56 tumors	105 tumors	0.72
0-6	31 (55)	55 (52)	
7-8	25 (45)	50 (48)	
p53 score	58 tumors	109 tumors	0.15
3-5	46 (79)	75 (69)	
6	12 (21)	34 (31)	
Cyclin A score	55 tumors	103 tumors	**0.024**
2-4	30 (55)	37 (36)	
5-7	25 (45)	66 (64)*	
Cyclin B (cytoplasmic) score	58 tumors	106 tumors	**0.015**
2-4	54 (93)	83 (78)	
5-6	4 (7)	23 (22)*	
Cyclin B (nuclear) %	58 tumors	106 tumors	0.061
0	56 (97)	93 (88)	
1-2	2 (3)	13 (12)**	
Cyclin D1 score	57 tumors	104 tumors	0.25
2-6	27 (47)	59 (57)	
7-8	30 (53)	45 (43)	
Cyclin D3 score	56 tumors	106 tumors	0.67
0-2	4 (7)	6 (6)	
3-8	52 (93)	100 (94)	
Cyclin E score	54 tumors	106 tumors	0.43
0-3	26 (48)	58 (55)	
4-8	28 (52)	48 (45)	

[a]: synchronous bilateral breast cancer, [b]: metachronous bilateral breast cancer, *: subgroups with significantly higher expression of analyzed markers; **: subgroups with a trend of higher expression of analyzed markers.

Polish institutions. Clinical data were encoded, thus no individual written consent of patients was required.

Archival formalin-fixed paraffin-embedded tissue blocks from bilateral breast tumors were collected and centrally verified for diagnosis of invasive breast cancer and for 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 un-paired tumors; 44 first tumors and 69 second tumors) (Table 1).

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 2). 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). Tumor samples were characterized for the expression of the following markers: $p16I^{nk4A}$, $p21^{(WAF1/CIP1)}$, $p27^{Kip1}$, p53, cyclin A, cyclin B, cyclin D1, cyclin D3, cyclin E.

The immunohistochemistry scoring was carried out by an experienced pathologist (JSz). ER was scored according to Allred criteria (with percentage and intensity scores noted separately) and HER2 - in accordance with ASCO/CAP guidelines [ASCO/CAP]. For Ki-67, the proportion of positive cells was divided into 3 categories: ≤14%, 15-30% and >30%.

Expression of $p16^{Ink4A}$, $p21^{(WAF1/CIP1)}$, $p27^{Kip1}$, p53, cyclin A, cyclin B, cyclin D1, cyclin D3 and cyclin E was assessed in the nucleus, whereas for $p16^{Ink4A}$ and cyclin B cytoplasmic expression was also assessed.

Expression of $p16^{Ink4A}$, $p21^{(WAF1/CIP1)}$, $p27^{Kip1}$, cyclin A, cyclin B, cyclin D1, cyclin D3 and cyclin E was scored as a percentage of stained cells on a 0-5 scale (0: 0%, 1: 1-5%, 2: 6-25%, 3: 26-50%, 4: 51-75%, 5: >75%). Expression of cells stained for p53 was scored on a 0-3 scale (0: 0%, 1: 1-10%, 2: 11-50%, 3: >50%). If some staining percentages could not be counted, scales were simplified to encompass only existing scores. Intensity of staining for all cell-cycle markers was scored as 1 - weak, 2 - moderate and 3 - strong. Overall expression score was obtained by adding scores of

Table 4. Uni- and multivariate analysis of immunohistochemical markers in sBBC vs mBBC.

Marker		N (%)		Univariate analysis		Multivariate analysis	
		sBBC[a]	mBBC[b]	OR[c] (95% CI[d])	p (logistic regression)	OR (95% CI)	p (logistic regression)
p16 (cytoplasmic) score	0-6	51 (89)	77 (71)	0.29 (0.11-0.75)	**0.011**	-	NS
	7-8	6 (11)	31 (29)				
p16 (nuclear) score	0-6	48 (84)	76 (70)	0.45 (0.2-1.01)	0.054	-	NS
	7-8	9 (16)	32 (30)				
p21 score	0-6	38 (70)	87 (84)	2.15 (0.99-4.71)	0.054	2.38 (1.05-5.37)	0.038
	7-8	16 (30)	17 (16)				
p27 score	0-6	31 (55)	55 (52)	0.89 (0.46-1.7)	0.718	-	NS
	7-8	25 (45)	50 (48)				
p53 score	3-5	46 (79)	75 (69)	0.58 (0.27-1.22)	0.151	-	NS
	6	12 (21)	34 (31)				
Cyclin A score	2-4	30 (55)	37 (36)	0.47 (0.24-0.91)	**0.025**	-	NS
	5-7	25 (45)	66 (64)				
Cyclin B (cytoplasmic) score	2-4	54 (93)	83 (78)	0.27 (0.09-0.82)	**0.02**	0.26 (0.08-0.8)	0.019
	5-6	4 (7)	23 (22)				
Cyclin B (nuclear) %	0	56 (97)	93 (88)	0.26 (0.06-1.17)	0.08	-	NS
	1-2	2 (3)	13 (12)				
Cyclin D1 score	2-6	27 (47)	59 (57)	1.46 (0.76-2.79)	0.256	-	NS
	7-8	30 (53)	45 (43)				
Cyclin D3 score	0-2	4 (7)	6 (6)	0.78 (0.21-2.89)	0.71	-	NS
	3-8	52 (93)	100 (94)				
Cyclin E score	0-3	26 (48)	58 (55)	1.3 (0.67-2.51)	0.432	-	NS
	4-8	28 (52)	48 (45)				

[a]: synchronous bilateral breast cancer, [b]: metachronous bilateral breast cancer, [c]: odds ratio, [d]: confidence interval. The results that are statistically significant are typed in bold.

stained cell percentage and staining intensity (for cyclin B nuclear expression no separate score assessment was conducted due to limited number of positive staining). Cut-off points were optimized to allow best discrimination between groups.

Statistical methods

Correlation between synchronous and metachronous status and dichotomized variables were tested by χ^2 or Fisher's exact test. Odds ratios with 95% confidence intervals were calculated with logistic regression analysis. Similarly, in a multivariate analysis, logistic regression was used (stepwise backwards logistic regression, 95%). Statistical significance was assumed for $p < 0.05$. Calculations were performed using STATA 11 (StataCorp LP, College Station, TX) - license No. 30110532736.

Results

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

Compared to sBBC, mBBC demonstrated higher expression of $p16^{Ink4A}$ (both cytoplasmic: p=0.002 and nuclear: p=0.014), cyclins A (p=0.024) and B (cytoplasmic: p=0.015) (Table 3).

The multivariate analysis used multiparameter logistic regression model including tumor grade and expression of ER, HER2, Ki-67, p16 (cytoplasmic and nuclear), p21, p27, p53 and cyclins A, B (cytoplasmic and nuclear), D1, D3 and E. The step-wise analysis identified p21 and cyclin B (cytoplasmic) as independently correlated with the occurrence of sBBC vs mBBC (Table 4).

Table 5. Correlation between ER expression and cell-cycle regulation markers in all BBC, sBBC and mBBC.

Marker	BBC			sBBC[a]			mBBC[b]		
	ER low (%)	ER high (%)	p (χ^2)	ER low (%)	ER high (%)	p (χ^2)	ER low (%)	ER high (%)	p (χ^2)
p16 (cytoplasmic) score	46 tumors	117 tumors	**<0.000001**	10 tumors	47 tumors	**0.027**	36 tumors	70 tumors	**<0.000001**
0-6	21 (46)	106 (91)		7 (70)	44 (94)		14 (39)	62 (89)	
7-8	25 (54) *	11 (9)		3 (30)*	3 (6)		22 (61)*	8 (11)	
p16 (nuclear) score	46 tumors	117 tumors	**<0.000001**	10 tumors	47 tumors	**0.02**	36 tumors	70 tumors	**0.00002**
0-6	22 (48)	101 (86)		6 (60)	42 (89)		16 (44)	59 (84)	
7-8	24 (52)*	16 (14)		4 (40)*	5 (11)		20 (56)*	11 (16)	
p21 score	44 tumors	113 tumors	0.38	9 tumors	45 tumors	0.18	35 tumors	68 tumors	0.74
0-6	37 (84)	88 (78)		8 (89)	30 (67)		29 (83)	58 (86)	
7-8	7 (16)	25 (22)		1 (11)	15 (33)		6 (17)	10 (14)	
p27 score	45 tumors	113 tumors	**0.00003**	10 tumors	45 tumors	0.074	35 tumors	68 tumors	**0.0001**
0-6	36 (80)	49 (43)		8 (80)	22 (49)		28 (80)	27 (40)	
7-8	9 (20)	64 (57)*		2 (20)	23 (51)**		7 (20)	41 (60)*	
p53 score	44 tumors	119 tumors	**<0.000001**	9 tumors	47 tumors	**0.006**	35 tumors	72 tumors	**0.000001**
3-5	17 (39)	101 (85)		4 (44)	40 (85)		13 (37)	61 (85)	
6	27 (61)*	18 (15)		5 (56)*	7 (15)		22 (63)*	11 (15)	
Cyclin A score	41 tumors	115 tumors	**0.00002**	8 tumors	46 tumors	0.059	33 tumors	69 tumors	**0.00045**
2-4	6 (15)	61 (53)		2 (25)	28 (61)		4 (12)	33 (48)	
5-7	35 (85)*	54 (47)		6 (75)**	18 (49)		29 (88)*	36 (52)	
Cyclin B (cytoplasmic) score	45 tumors	118 tumors	**0.00037**	10 tumors	48 tumors	0.67	35 tumors	70 tumors	**0.0015**
2-4	30 (67)	106 (90)		9 (90)	45 (94)		21 (60)	61 (87)	
5-6	15 (33)*	12 (10)		1 (10)	3 (6)		14 (40)*	9 (13)	
Cyclin B (nuclear) %	45 tumors	118 tumors	**0.0004**	10 tumors	48 tumors	0.25	35 tumors	70 tumors	**0.0004**
0	35 (78)	113 (96)		10 (100)	46 (96)		25 (71)	67 (96)	
1-2	10 (22)*	5 (4)		0 (0)	2 (4)		10 (29)*	3 (4)	
Cyclin D1 score	44 tumors	115 tumors	**<0.000001**	10 tumors	47 tumors	**0.00024**	34 tumors	68 tumors	**<0.000001**
2-6	41 (93)	43 (37)		10 (100)	17 (36)		31 (91)	26 (38)	
7-8	3 (7)	72 (63)*		0 (0)	30 (64)*		3 (9)	42 (62)*	
Cyclin D3 score	45 tumors	114 tumors	0.9	10 tumors	46 tumors	0.08	35 tumors	68 tumors	0.36
0-2	3 (7)	7 (6)		2 (20)	2 (4)		1 (3)	5 (7)	
3-8	42 (93)	107 (94)		8 (80)	44 (96)**		34 (97)	63 (93)	
Cyclin E score	44 tumors	114 tumors	**0.000003**	9 tumors	45 tumors	0.32	35 tumors	69 tumors	**<0.000001**
0-3	10 (23)	73 (64)		3 (33)	23 (51)		7 (20)	50 (72)	
4-8	34 (77)*	41 (36)		6 (67)	22 (49)		28 (80)*	19 (28)	

[a]: synchronous bilateral breast cancer, [b]: metachronous bilateral breast cancer, ER low - ER Allred score 0-5, ER high - ER Allred score 6-8. *: subgroups with significantly higher expression of analyzed markers; **: subgroups with a trend of higher expression of analyzed marker.

In the next step, we correlated expression of cell-cycle regulation proteins with markers determining breast cancer phenotype (ER, HER2, Ki-67) for both the whole BBC population, and mBBC and sBBC subgroups. Lower ER expression was associated with stronger expression of p16 (both cytoplasmic and nuclear) p53, cyclin A, cyclin B (both cytoplasmic and nuclear), and cyclin E, for both all BBC and mBBC (Table 5). Some of these relationships were not found in the sBBC subgroup, possibly due to smaller sample size. In this subset, the positive correlation with higher ER levels was observed only for p27 expression (NS for sBBC) and cyclin D1.

Higher p27 score correlated with lower HER2 expression in sBBC, whereas higher HER2 expression was associated with higher p53 and cyclin E expression in all BBC, and with higher nuclear expression of cyclin B in sBBC (Table 6).

Like for ER, there was a consistent pattern of positive correlation between Ki-67 levels and higher expression of most cycle-regulation markers, including p16 (both cytoplasmic and nuclear), p21, p53, cyclin A, cyclin B (both cytoplasmic and nuclear), cyclin D3 and cyclin E (Table 7.). Some of these relationships were not significant in the sBBC and mBBC subgroups (although the pattern of correlations was similar), possibly due to smaller sample sizes. The only exception was cyclin D1, which negatively correlated with Ki-67 expression both for all BBC and mBBC. A similar trend (borderline significant only for sBBC) was also observed for p27.

In the step-wise multivariate analysis, only p21, cyclin A and cyclin B (cytoplasmic) expression was independently associated with occurrence of sBBC vs mBBC (Table 8).

Table 6. Correlation between HER2 expression and cell-cycle regulation markers in all BBC, mBBC and sBBC.

	BBC			sBBC[a]			mBBC[b]		
Marker	HER2 low (%)	HER2 high (%)	p (χ^2)	HER2 low (%)	HER2 high (%)	p (χ^2)	HER2 low (%)	HER2 high (%)	p (χ^2)
p16 (cytoplasmic) score	123 tumors	40 tumors	0.64	39 tumors	17 tumors	0.87	84 tumors	23 tumors	0.73
0-6	94 (76)	32 (80)		35 (90)	15 (88)		59 (70)	17 (74)	
7-8	29 (24)	8 (20)		4 (10)	2 (12)		25 (30)	6 (26)	
p16 (nuclear) score	123 tumors	40 tumors	0.97	39 tumors	17 tumors	0.31	84 tumors	23 tumors	0.65
0-6	92 (75)	30 (75)		34 (87)	13 (76)		58 (69)	17 (74)	
7-8	31 (25)	10 (25)		5 (13)	4 (24)		26 (31)	6 (26)	
p21 score	117 tumors	39 tumors	0.22	36 tumors	17 tumors	0.9	81 tumors	22 tumors	0.2
0-6	39 (33)	9 (23)		9 (25)	4 (24)		30 (37)	5 (23)	
7-8	78 (67)	30 (77)		27 (75)	13 (76)		51 (63)	17 (77)	
p27 score	119 tumors	39 tumors	0.3	38 tumors	17 tumors	**0.018**	81 tumors	22 tumors	0.71
0-6	41 (34)	17 (44)		8 (21)	9 (53)		33 (41)	8 (36)	
7-8	78 (66)	22 (56)		30 (79)*	8 (47)		48 (59)	14 (64)	
p53 score	125 tumors	40 tumors	**0.024**	40 tumors	17 tumors	0.086	85 tumors	23 tumors	0.07
3-5	95 (76)	23 (58)		34 (85)	11 (65)		61 (72)	12 (52)	
6	30 (24)	17 (42)*		6 (15)	6 (35)**		24 (28)	11 (48)**	
Cyclin A score	116 tumors	40 tumors	0.48	36 tumors	17 tumors	0.17	80 tumors	23 tumors	0.9
2-4	51 (44)	15 (37)		22 (61)	7 (41)		29 (36)	8 (35)	
5-7	65 (56)	25 (63)		14 (39)	10 (59)		51 (64)	15 (65)	
Cyclin B (cytoplasmic) score	123 tumors	40 tumors	0.85	40 tumors	17 tumors	0.83	83 tumors	23 tumors	0.56
2-4	103 (84)	33 (83)		37 (93)	16 (94)		66 (80)	17 (74)	
5-6	20 (16)	7 (17)		3 (7)	1 (6)		17 (20)	6 (26)	
Cyclin B (nuclear) %	123 tumors	40 tumors	0.84	40 tumors	17 tumors	**0.027**	83 tumors	23 tumors	0.55
0	112 (91)	36 (90)		40 (100)	15 (88)		72 (87)	21 (91)	
1-2	11 (9)	4 (10)		0 (0)	2 (12)*		11 (13)	2 (9)	
Cyclin D1 score	120 tumors	39 tumors	0.2	39 tumors	17 tumors	0.95	81 tumors	22 tumors	0.08
2-6	60 (50)	24 (62)		18 (46)	8 (47)		42 (52)	16 (73)	
7-8	60 (50)	15 (38)		21 (54)	9 (53)		39 (48)**	6 (27)	
Cyclin D3 score	121 tumors	38 tumors	0.067	38 tumors	17 tumors	0.16	83 tumors	21 tumors	0.2
0-2	10 (8)	0 (0)		4 (17)	0 (0)		6 (7)	0 (0)	
3-8	111 (92)	38 (100)**		34 (83)	17 (100)		77 (93)	21 (100)	
Cyclin E score	120 tumors	39 tumors	**0.048**	37 tumors	17 tumors	0.2	83 tumors	22 tumors	0.16
0-3	68 (57)	15 (38)		20 (54)	6 (35)		48 (58)	9 (41)	
4-8	52 (43)	24 (62)*		17 (46)	11 (65)		35 (42)	13 (59)	

[a]: synchronous bilateral breast cancer, [b]: metachronous bilateral breast cancer, HER2 low - IHC 0-1, HER2 high - IHC score 2-3. *: subgroups with significantly higher expression of analyzed markers; **: subgroups with a trend of higher expression of analyzed marker.

Discussion

This study presents a pathological analysis of a relatively large sample of BBC, with a focus on markers related to cell proliferation. To our knowledge this is the first study addressing this issue in BBC. We have also compared expression profiles of sBBC and mBBC and analyzed correlations between the cell cycle regulators and ER, HER2 and Ki-67 status.

Abnormalities of CDKIs have been reported for a variety of human malignancies, including breast cancer, and some of them showed prognostic and predictive importance. In breast cancer, high p16 immunoreactivity (both nuclear and cytoplasmic) have been shown to be associated with a more aggressive phenotype (high grade, high Ki-67 and negative ER status) (Milde-Langosch et al., 2001). Similar associations, and generally higher p16 scores in mBBC were also found in our study. These relationships may be partly explained by a very long p16 half-life, allowing its accumulation in rapidly dividing cells, such as those with high Ki-67 index (Milde-Langosch et al., 1998, 2001). In turn, several studies indicated that p21 expression may be related to better prognosis and more indolent phenotype (Elledge and Allred, 1998; Mathoulin-Portier et al., 2000; Göhring et al., 2001), whereas its loss may be predictive for tamoxifen resistance (Abukhdeir et al., 2008). In our study lower p21 immunoreactivity was observed in mBBC, which may correspond to its overall more aggressive behavior.

Mutated TP53 is present in around one fourth of breast cancer samples (The Cancer Genome Atlas Network, 2012). This abnormality is involved in three main carcinogenesis events: early tumorigenesis, tumor growth and metastasis. Thus, defective p53 is considered the driving oncogene in breast cancer (Walerych et al.,

Table 7. Correlation between Ki-67 expression and cell-cycle regulation markers in all BBC, mBBC and sBBC.

	BBC			sBBC[a]			mBBC[b]		
	Ki-67 low (%)	Ki-67 high (%)	p (χ^2)	Ki-67 low (%)	Ki-67 high (%)	p (χ^2)	Ki-67 low (%)	Ki-67 high (%)	p (χ^2)
p16 (cytoplasmic) score	95 tumors	69 tumors	**0.000015**	37 tumors	20 tumors	0.086	58 tumors	49 tumors	**0.0002**
0-6	85 (98)	42 (61)		35 (95)	16 (80)		50 (86)	26 (53)	
7-8	10 (11)	27 (39)*		2 (5)	4 (20)**		8 (14)	23 (47)*	
p16 (nuclear) score	95 tumors	69 tumors	**0.000009**	37 tumors	20 tumors	**0.016**	58 tumors	49 tumors	**0.00003**
0-6	91 (96)	49 (71)		37 (100)	17 (85)		54 (93)	32 (65)	
7-8	4 (4)	20 (29)*		0 (0)	3 (15)*		4 (7)	17 (35)*	
p21 score	91 tumors	67 tumors	0.05	34 tumors	20 tumors	**0.017**	57 tumors	47 tumors	0.36
0-5	51 (56)	27 (40)		18 (53)	4 (20)		33 (58)	23 (49)	
6-8	40 (44)	40 (60)*		16 (47)	16 (80)*		24 (42)	24 (51)	
p27 score	94 tumors	66 tumors	0.075	37 tumors	19 tumors	**0.048**	57 tumors	47 tumors	0.4
0-6	45 (48)	41 (62)		17 (46)	14 (74)		28 (49)	27 (57)	
7-8	49 (52)	25 (38)		20 (54)*	5 (26)		29 (51)	20 (43)	
p53 score	98 tumors	68 tumors	**0.0003**	38 tumors	20 tumors	0.56	60 tumors	48 tumors	**0.0002**
3-5	81 (83)	39 (57)		31 (82)	15 (75)		50 (83)	24 (50)	
6	17 (17)	29 (43)*		7 (18)	5 (25)		10 (17)	24 (50)*	
Cyclin A score	91 tumors	66 tumors	**<0.000001**	35 tumors	20 tumors	**<0.000001**	56 tumors	46 tumors	**<0.000001**
2-4	63 (69)	4 (6)		30 (86)	0 (0)		33 (59)	4 (9)	
5-7	28 (31)	62 (94)*		5 (14)	20 (100)*		23 (41)	42 (91)*	
Cyclin B (cytoplasmic) score	95 tumors	68 tumors	**<0.000001**	38 tumors	20 tumors	**0.004**	57 tumors	48 tumors	**<0.000001**
2-4	94 (99)	42 (62)		38 (100)	16 (80)		56 (98)	26 (54)	
5-6	1 (1)	26 (38)*		0 (0)	4 (20)*		1 (2)	22 (46)*	
Cyclin B (nuclear) %	95 tumors	68 tumors	**0.0002**	38 tumors	20 tumors	**0.047**	57 tumors	48 tumors	**0.0026**
0	93 (98)	55 (81)		38 (100)	18 (90)		55 (96)	37 (77)	
1-2	2 (2)	13 (19)*		0 (0)	2 (10)*		2 (4)	11 (23)*	
Cyclin D1 score	94 tumors	67 tumors	**0.046**	37 tumors	20 tumors	0.41	57 tumors	47 tumors	**0.0035**
2-6	44 (47)	42 (63)		19 (51)	8 (40)		25 (44)	34 (72)	
7-8	50 (53)*	25 (37)		18 (49)	12 (60)		32 (56)*	13 (28)	
Cyclin D3 score	93 tumors	68 tumors	**0.005**	36 tumors	20 tumors	0.12	57 tumors	48 tumors	**0.02**
0-2	10 (11)	0 (0)		4 (11)	0 (0)		6 (11)	0 (0)	
3-8	83 (89)	68 (100)*		32 (89)	20 (100)		51 (89)	48 (100)*	
Cyclin E score	92 tumors	68 tumors	**<0.000001**	34 tumors	20 tumors	0.4	58 tumors	48 tumors	**0.000002**
0-3	64 (70)	20 (29)		20 (59)	6 (30)		44 (76)	14 (29)	
4-8	28 (30)	48 (71)*		14 (41)	14 (70)		14 (24)	34 (71)*	

[a]: synchronous breast cancer, [b]: metachronous breast cancer, Ki-67 low - ≤30%, Ki-67 high - >30%. *: subgroups with significantly higher expression of analyzed markers; **: subgroups with a trend of higher expression of analyzed marker.

2012). In this series p53 expression correlated with low ER and higher HER2 for both sBBC and mBBC. Higher p53 score was also associated with higher Ki-67 in mBBC. Similarly, Ackerman et al. (1995) did not demonstrate significant differences in p53 expression between sBBC and mBBC, whereas other studies confirmed the correlation between p53 mutations and Ki-67 expression in BBC (Özer et al., 1998). Interestingly, the prevalence of p53 alterations in BBC does not seem to be higher compared to unilateral breast cancers (Lidereau and Soussi, 1992). Nevertheless, in one study TP53 mutations were detected in 50% BBC and in 26% unilateral cases respectively (p<0,01), and within BBC were more frequent in mBBC (Kinoshita et al., 1995). Other breast cancer studies showed that HER2 driven proliferation of breast cancer cells is dependent on mutated p53 (Casalini et al., 2001) and the coexistence of p53 accumulation and HER2 overexpression carries poor prognosis (Yamashita et al., 2004). Interestingly, in the present BBC series, high p53 score correlated with high HER2.

Homeostasis aberrations of the cyclin-CDK system lead to dysregulation of the cell cycle and eventually to malignant transformation. In breast cancer, overexpression of cyclin A, B1 and E seems to correlate with high tumor grade, high Ki-67 index and HER2 overexpression, whereas cyclin D1 correlates positively with ER, PgR and non-basal histology (Husdal et al., 2006; Aaltonen et al., 2009a,b; Boström et al., 2009). Unsurprisingly, in our study mBBC tumors showed stronger cyclin A and B immunoreactivity, cyclin A and B expression correlated with high Ki-67 in both mBBC and sBBC, and cyclin E - in mBBC, and cyclin D1 had a significant positive correlation with ER.

A study by Lodén et al. (2002) implies that cyclin D1 mediates proliferation in ER-positive cancer cells. In turn, cyclin E may drive proliferation in ER negative breast cancer (Aaltonen et al., 2009a,b). Our results suggest that this phenomenon may also refer to BBC. Moreover, in our cohort high expression of cyclin E was found to positively correlate with HER2 overexpression. Interestingly, cyclin E overexpression and the ensuing increase in CDK2 activity is a mechanism of trastuzumab resistance in HER2 positive breast cancer (Scaltriti et al., 2011).

This study demonstrated a clear and consistent pattern of positive correlation of most cell-cycle regulation proteins (and a negative correlation for p27 and cyclin D1), with more aggressive tumor phenotype, as well as with lower ER and higher Ki-67 expression. No such clear pattern emerged for HER2, so the identified correlations might have been incidental.

In this series patients were not selected for survival status (as is often the case in patients selected from genetic clinics), apart from surviving long enough to develop second malignancy. However, as for the mBBC, the more recently diagnosed cases were generally more available, a possible bias seems to be of minor importance.

A major limitation of this study is the lack of both long term patient outcomes and the clinical data, such as disease stage, family history or exposure to other risk factors for breast cancer.

To increase the informative value and statistical power of our study, we included both matched tumors from the same patients and also cases in which only one of the two paired BBC was available. This was in line with our belief that each tumor represented a separate carcinogenic event.

However, the lack of available tissue for a proportion of paired tumors precluded a reliable comparative analysis of matched tumors from the same patients. This was not the aim of our study though, as this subject has been addressed earlier (Russnes et al., 2011).

Owing to the relative rarity of BBC, our material was collected over a long period of time and acquired from many institutions. In consequence, 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.

In conclusion, we demonstrated that compared to sBBC, mBBC are characterized by lower expression of p21 and higher cytoplasmic expression of cyclin B, suggesting its more aggressive behavior. Additionally, our study confirmed that a number of previously described correlations between cell-cycle regulation

Table 8. Multivariate analysis of factors associated with expression of cell cycle regulation proteins.

Cell cycle regulation protein	Related parameters	OR (95% CI)	p
p16 (cytoplasmic) score	ER	0.12 (0.05-0.30)	0.000
	Ki-67	2.78 (1.11-6.93)	0.028
p16 (nuclear) score	ER	0.18 (0.08-0.41)	0.000
p21 score	**mBBC[a] vs sBBC[b]**	**2.35 (1.06-5.19)**	**0.034**
p27 score	ER	5.14 (2.26-11.68)	0.000
p53 score	ER	0.11 (0.05-0.24)	0.000
	HER2	2.89 (1.21-6.88)	0.017
Cyclin A score	Ki-67	37.86 (12.13-118.20)	0.000
	mBBC vs sBBC	**0.38 (0.15-0.95)**	**0.039**
Cyclin B (cytoplasmic) score	Grade	8.19 (2.09-32.11)	0.003
	Ki-67	25.08 (3.07-204.81)	0.003
	mBBC vs sBBC	**0.27 (0.07-0.98)**	**0.046**
Cyclin B (nuclear) %	Ki-67	7.23 (1.48-35.37)	0.015
	ER	0.29 (0.09-0.96)	0.043
Cyclin D1 score	ER	23.43 (6.83-80.35)	0.000
Cyclin D3 score	Ki-67	8.19 (2.09-360.61)	0.005
Cyclin E score	ER	0.25 (0.10-0.61)	0.002
	Grade	2.57 (1.08-6.13)	0.034
	Ki-67	2.45 (1.04-5.75)	0.039

[a]: metachronous breast cancer, [b]: synchronous breast cancer, the results that are statistically significant are typed in bold.

proteins and breast cancer phenotype are also valid for BBC.

Acknowledgements. The authors thank Claudia Wiewiora for English editing of the manuscript. This work was financially supported by the Polish Ministry of Science and Higher Education (grant no. NN 407 206435).

Funding source. This work was financially supported by the Polish Ministry of Science and Higher Education (grant no. NN 407 206435)

Conflict of interest. None declared

References

Aaltonen K., Amini R.-M., Heikkilä P., Aittomäki K., Tamminen A., Nevanlinna H. and Blomqvist C. (2009a). High cyclin B1 expression is associated with poor survival in breast cancer. Br. J. Cancer 100, 1055-1060.

Aaltonen K., Amini R.-M., Landberg G., Eerola H., Aittomäki K., Heikkilä P., Nevanlinna H. and Blomqvist C. (2009b). Cyclin D1 expression is associated with poor prognostic features in estrogen receptor positive breast cancer. Breast Cancer Res. Treat. 113, 75-82.

Abukhdeir A.M., Vitolo M.I., Argani P., De Marzo A.M., Karakas B., Konishi H., Gustin J.P., Lauring J., Garay J.P., Pendleton C., Konishi Y., Blair B.G., Brenner K., Garrett-Mayer E., Carraway H., Bachman K.E. and Park B.H. (2008). Tamoxifen-stimulated growth of breast cancer due to p21 loss. Proc. Natl. Acad. Sci. USA 105, 288-293.

Ackerman J., Baunoch D.A., Gimotty P., George J., Lane M.A. and Dawson P.J. (1995). The role of p53 mutations in bilateral breast carcinoma. Mod. Pathol. 8, 244-248.

Bergthorsson J.T., Ejlertsen B., Olsen J.H., Borg A., Nielsen K.V, Barkardottir R.B., Klausen S., Mouridsen H.T., Winther K., Fenger K., Niebuhr A., Harboe T.L. and Niebuhr E. (2001). BRCA1 and BRCA2 mutation status and cancer family history of Danish women affected with multifocal or bilateral breast cancer at a young age. J. Med. Genet. 38, 361-368.

Boström P., Söderström M., Palokangas T., Vahlberg T., Collan Y., Carpen O. and Hirsimäki P., (2009). Analysis of cyclins A, B1, D1 and E in breast cancer in relation to tumour grade and other prognostic factors. BMC Res. Notes 2, 140.

Casalini P., Botta L. and Menard S. (2001). Role of p53 in HER2-induced Proliferation or Apoptosis. J. Biol. Chem. 276, 12449-12453.

Casimiro M.C., Crosariol M., Loro E., Li Z. and Pestell R.G. (2012). Cyclins and cell cycle control in cancer and disease. Genes Cancer 3, 649-657.

Chen Y., Thompson W., Semenciw R. and Mao Y. (1999). Epidemiology of contralateral breast cancer. Cancer Epidemiol. Biomarkers Prev. 8, 855-861.

Chen Y., Semenciw R., Kliewer E., Shi Y. and Mao Y. (2001). Incidence of second primary breast cancer among women with a first primary in Manitoba, Canada. Breast Cancer Res. Treat. 67, 35-40.

Connell-Crowley L., Harper J.W. and Goodrich D.W. (1997). Cyclin D1/Cdk4 regulates retinoblastoma protein-mediated cell cycle arrest by site-specific phosphorylation. Mol. Biol. Cell 8, 287-301.

Dawson P.J., Maloney T., Gimotty P., Juneau P., Ownby H. and Wolman S.R. (1991). Bilateral breast cancer: one disease or two? Breast Cancer Res. Treat. 19, 233-244.

Deshpande A., Sicinski P. and Hinds P.W. (2005). Cyclins and cdks in development and cancer: a perspective. Oncogene. 24, 2909-2915.

Elledge R.M. and Allred D.C. (1998). Prognostic and predictive value of p53 and p21 in breast cancer. Breast Cancer Res. Treat. 52, 79-98.

Geng Y., Yu Q., Whoriskey W., Dick F., Tsai K.Y., Ford H.L., Biswas D.K., Pardee A.B., Amati B., Jacks T., Richardson A., Dyson N. and Sicinski P. (2001). Expression of cyclins E1 and E2 during mouse development and in neoplasia. Proc. Natl. Acad. Sci. USA 98, 13138-13143.

Göhring U.J., Bersch A., Becker M., Neuhaus W. and Schöndorf T. (2001). p21(waf) correlates with DNA replication but not with prognosis in invasive breast cancer. J. Clin. Pathol. 54, 866-870.

Hartman M., Czene K., Reilly M., Adolfsson J., Bergh J., Adami H.O., Dickman P.W. and Hall P. (2007). Incidence and prognosis of synchronous and metachronous bilateral breast cancer. J. Clin. Oncol. 25, 4210-4216.

Hartman M., Czene K., Reilly M., Bergh J., Lagiou P., Trichopoulos D., Adami H.-O. and Hall P. (2005). Genetic implications of bilateral breast cancer: a population based cohort study. Lancet Oncol. 6, 377-382.

Howe H.L., Weinstein R., Alvi R., Kohler B. and Ellison J.H. (2005). Women with multiple primary breast cancers diagnosed within a five year period, 1994-1998. Breast Cancer Res. Treat. 90, 223-232.

Husdal A., Bukholm G. and Bukholm I.R.K. (2006). The prognostic value and overexpression of cyclin A is correlated with gene amplification of both cyclin A and cyclin E in breast cancer patient. Cell. Oncol. 28, 107-116.

Hwang H.C. and Clurman B.E. (2005). Cyclin E in normal and neoplastic cell cycles. Oncogene 24, 2776-2786.

Jobsen J.J., van der Palen J., Ong F., Riemersma S. and Struikmans H. (2015). Bilateral breast cancer, synchronous and metachronous; differences and outcome. Breast Cancer Res. Treat. 153, 277-283.

Kilker R.L., Hartl M.W., Rutherford T.M. and Planas-Silva M.D. (2004). Cyclin D1 expression is dependent on estrogen receptor function in tamoxifen-resistant breast cancer cells. J. Steroid Biochem. Mol. Biol. 92, 63-71.

Kinoshita T., Ueda M., Enomoto K., Ikeda T., Kikuchi K., Ishii S. and Kitajima M. (1995). Comparison of p53 gene abnormalities in bilateral and unilateral breast cancer. Cancer 76, 2504-2509.

Kollias J., Pinder S.E., Denley H.E., Ellis I.O., Wencyk P., Bell J.A., Elston C.W. and Blamey R.W. (2004). Phenotypic similarities in bilateral breast cancer. Breast Cancer Res. Treat. 85, 255-261.

Lidereau R. and Soussi T. (1992). Absence of p53 germ-line mutations in bilateral breast cancer patients. Hum. Genet. 89, 250-252.

Lodén M., Stighall M., Nielsen N.H., Roos G., Emdin S.O., Ostlund H. and Landberg G. (2002). The cyclin D1 high and cyclin E high subgroups of breast cancer: separate pathways in tumorogenesis based on pattern of genetic aberrations and inactivation of the pRb node. Oncogene 21, 4680-4690.

Mathoulin-Portier M.P., Viens P., Cowen D., Bertucci F., Houvenaeghel G., Geneix J., Puig B., Bardou V.J. and Jacquemier J. (2000). Prognostic value of simultaneous expression of p21 and mdm2 in breast carcinomas treated by adjuvant chemotherapy with antracyclin. Oncol. Rep. 7, 675-680.

Milde-Langosch K., Ocon E., Becker G. and Löning T. (1998). p16/MTS1 inactivation in ovarian carcinomas: high frequency of reduced protein expression associated with hyper-methylation or mutation in endometrioid and mucinous tumors. Int. J. Cancer 79, 61-65.

Milde-Langosch K., Bamberger A.M., Rieck G., Kelp B. and Löning T. (2001). Overexpression of the p16 cell cycle inhibitor in breast cancer is associated with a more malignant phenotype. Breast Cancer Res. Treat. 67, 61-70.

Özer E., Canda T. and Kuyucuoðlu F. (1998). p53 mutations in bilateral breast carcinoma. Correlation with Ki-67 expression and the mean nuclear volume. Cancer Lett. 122, 101-106.

Peto J. and Mack T.M. (2000). High constant incidence in twins and other relatives of women with breast cancer. Nat. Genet. 26, 411-414.

Rogozińska-Szczepka J., Utracka-Hutka B., Grzybowska E., Maka B., Nowicka E., Smok-Ragankiewicz A., Zientek H., Steffen J. and Wojciechowska-Łacka A. (2004). BRCA1 and BRCA2 mutations as prognostic factors in bilateral breast cancer patients. Ann. Oncol. 15, 1373-1376.

Russnes H.G., Kuligina E.S., Suspitsin E.N., Voskresenskiy D.A., Jordanova E.S., Cornelisse C.J., Borresen-Dale A.-L. and Imyanitov E.N. (2011). Paired distribution of molecular subtypes in bilateral breast carcinomas. Cancer Genet. 204, 96-102.

Safal M., Lower E.E., Hasselgren P.O., Hungness E.S., Gazder P., Aron B., Shaughnessy E.A. and Yassin R. (2002). Bilateral synchronous breast cancer and HER-2/neu overexpression. Breast Cancer Res. Treat. 72, 195-201.

Scaltriti M., Eichhorn P.J., Cortes J., Prudkin L., Aura C., Jimenez J., Chandarlapaty S., Serra V., Prat A., Ibrahim Y.H., Guzman M., Gili M., Rodriguez O., Rodriguez S., Perez J., Green S.R., Mai S., Rosen N., Hudis C. and Baselga J. (2011). Cyclin E amplification/overexpression is a mechanism of trastuzumab resistance in HER2+ breast cancer patients. Proc. Natl. Acad. Sci. USA 108, 3761-3766.

Schaapveld M., Visser O., Louwman W.J., Willemse P.H.B., De Vries E.G.E., Van Der Graaf W.T.A., Otter R., Coebergh J.W.W. and Van Leeuwen F.E. (2008). The impact of adjuvant therapy on contralateral breast cancer risk and the prognostic significance of contralateral breast cancer: A population based study in the Netherlands. Breast Cancer Res. Treat. 110, 189-197.

Senkus E., Szade J., Pieczyńska B., Zaczek A., Pikiel J., Sosińska-Mielcarek K., Karpińska A. and Jassem J., (2014a). Are synchronous and metachronous bilateral breast cancers different? An immunohistochemical analysis aimed at intrinsic tumor phenotype. Int. J. Clin. Exp. Pathol. 7, 353-363.

Senkus E., Szade J., Pieczyńska B., Żaczek A., Świerblewski M., Biernat W. and Jassem J. (2014b). Are bilateral breast cancers and breast cancers coexisting with ovarian cancer different from solitary tumors? A pair-matched immunohistochemical analysis aimed at intrinsic tumor phenotype. Pathol. Int. 64, 508-517.

Takahashi H., Watanabe K., Takahashi M., Taguchi K., Sasaki F. and Todo S. (2005). The impact of bilateral breast cancer on the prognosis of breast cancer: a comparative study with unilateral breast cancer. Breast Cancer 12, 196-202.

The Cancer Genome Atlas Network. (2012). Comprehensive molecular portraits of human breast tumours. Nature 490, 61-70.

Vaittinen P. and Hemminki K. (2000). Risk factors and age-incidence relationships for contralateral breast cancer. Int. J. Cancer 88, 998-1002.

Velasco-Velázquez M.A., Li Z., Casimiro M., Loro E., Homsi N. and Pestell R.G. (2011). Examining the role of cyclin D1 in breast cancer. Future Oncol. 7, 753-765

Walerych D., Napoli M., Collavin L. and Del Sal G. (2012). The rebel angel: mutant p53 as the driving oncogene in breast cancer. Carcinogenesis 33, 2007-2017.

Yamashita H., Nishio M., Toyama T., Sugiura H., Zhang Z., Kobayashi S. and Iwase H. (2004). Coexistence of HER2 over-expression and p53 protein accumulation is a strong prognostic molecular marker in breast cancer. Breast Cancer Res. 6, R24.

Accepted March 9, 2017