doi:10.4149/neo_2014_043

The biological markers and results of treatment in male breast cancer patients. The Cracow experience.

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Received August 7, 2013 / Accepted October 9, 2013

Male breast cancer is a rare form of carcinoma with an incidence rate of approximately 0.5-1% compared with cases of breast carcinoma as a whole. Male breast cancer reacts effectively to endocrine therapy because of a high frequency of hormone receptor expression.

The aim of the present study was the assessment of correlations between stage, grade, expression of steroid receptors, basal/mesenchymal markers and proliferation index, as well as analysis of the impact of the above-mentioned parameters on overall (OS) and disease-free survival (DFS) in the group of 32 male breast cancer patients, treated at the Centre of Oncology in Cracow.

We showed the significant positive correlation between MIB-1 LI and tumor stage, and hormone receptors (ER or PgR) immunonegativity, and expression of EGFR, vimentin ($p<0.05$) and P-cadherin (the last at statistical border). The presence of any of basal or masenchymal markers correlated with a more advanced tumor stage. Moreover tumors without vimentin expression were characterised by lower MIB-1 LI and were more frequently EGFR immunonegative.

We found that hormone receptor negativity, vimentin immunopositivity and high MIB-1 LI are significant independent indicators of poor OS and DFS for male breast cancer patients ($p<0.05$).

Key words: male breast cancer, basal markers, mesenchymal markers, nodal status, hormonal receptors

Male breast cancer is a rare form of carcinoma with an incidence rate of approximately 0.5-1% compared with cases of breast carcinoma as a whole [1, 2, 3, 4]. Its peak incidence is noted in the seventh decade of life [5, 6, 7]. The most significant risk factors are an increased concentration of estrogen and obesity, that are a result of estrogen overproduction, impaired metabolism, and disturbances in the concentration of estrogen and androgen (i.e. in Klinefelter's syndrome) as well as the use of estrogenes in prostate cancer treatment [6, 8, 9, 10, 11]. Other factors include alcoholism, liver cirrhosis, the presence of hereditary BRCA1 and BRCA2 gene mutations and exposure to ionizing radiation, the electromagnetic field and increased temperature [6, 8, 9, 12, 13, 14, 15, 16, 17].

According to Anderson et al., male breast cancer is characterised by similar biological factors to those present in the female variant of the cancer [18]. However, these tumors are diagnosed in more advanced stages of the disease, are characterised by higher degree of differentiation (more frequently G1, G2 grade) and a lower mitotic index in comparison to female breast cancer [5, 19]. There are also differences in the frequency of the expression of hormone receptors and growth factor receptors [19, 20]. Besides, the prognosis for male breast cancer is worse [5, 18, 20, 21]. A study by Baojiang et al. of 5-year survival rates of patients suffering from male breast carcinoma and female breast carcinoma produced the following results: 61.2% and 68.7% (DFS), and 75.% and 82.9% (OS), respectively [20].

Male breast cancer has a higher (over 90%) hormone receptor expression and, as a consequence, reacts more effectively to endocrine therapy [6, 16, 17, 19, 22, 23, 24, 25, 26, 27].

The treatment of male breast carcinoma is based on standard methods, more specifically surgical procedures [6, 21, 25,

Table 1. The clinical, pathological and treatment characteristic of 32 male patients with breast cancer.

Clinical feature		No of patients	%
positive family history		2	6,3
stage of disease			
	T1	4	12.5
	T2	4	12.5
	T3	1	3.1
	T4	12	37.5
	Tx	11	34.4
	N0	8	25.0
	N1	9	28.1
	N2	3	9.4
	N3	1	3.1
	Nx	11	34.4
pathological stage of nodes	pN+	17	53.1
tumour grade			
	G1	8	25.0
	G2	17	53.1
	G3	5	15.6
	Gx	2	6.3
surgery	mastectomy	31	96.8
	breast-conserving surgery	1	3.2
adjuvant therapies	radiotherapy	12	37.5
	chemotherapy	10	31.3
	endocrine therapy	18	56.3
localization of distant metastases	lungs	6	54.5
	bones	5	45.5
	skin	2	18.2
	brain	1	9.1
	lymph nodes (distant)	1	9.1

26]. Breast amputations are more frequent in male patients than in female [6, 15, 17, 26]. According to Nilsson et al., mastectomy is performed in 92% of male patients and 44% of female patients [28]. The adjuvant treatment employed in such cases (radiotherapy, chemotherapy, hormone therapy) depends on the presence of prognostic and predictive features, which are indications for this type of treatment [5, 6, 12, 15, 16, 23, 25, 26, 28, 29, 30, 31].

The aim of the present study was to: (i) describe the clinical and biological characteristics of breast cancers of male patients treated at the Centre of Oncology in Cracow, (ii) assess correlations between stage, grade, expression of steroid receptors, basal/mesenchymal markers and proliferation index, (iii) and analyse impact of the above-mentioned parameters on overall and disease-free survival.

Patients and methods

Patients. Between 1950 and 2010, 81 male patients with breast cancer were treated at the Centre of Oncology in Cracow. These cases represented 0.5% of all (17.320) patients treated for breast cancer in this period. The biological markers were tested in 32 cases only and this group is the subject of the following publication. The size of the group was determined by the fact that the assessment of biological parameters were carried out on archival formalin-fixed, paraffin-embedded sections, which in some cases were not adequate for immunohistochemistry (small amount of tissue in paraffin block, poor quality of material). The patients (with marker assessment) were treated between 1976 and 2010 and represented 0.2% of all the patents suffering from breast cancer during this period.

The consent to perform the above-mentioned tests was given by the Bioethic Committee of the Regional Medical Chamber in Cracow.

The age of the 32 patients ranged from 34 up to 84 years and the mean value was equal to 62.72 ± 12.49 on average (median: 63 years). A positive family history was noted in 2 patients (6.3%). In table 1 the clinical and pathological characteristics and treatment methods of the 32 analysed patients are presented. The majority of the patients (40%) were diagnosed with stage T3-T4 cancer. In 25% of the patients no clinical features of regional lymph node metastases were noted, while in 17 patients (53.1%) axillary lymph node metastases were confirmed in pathological examination.

All patients received surgical treatment. A total of 31 patients (96.8%) underwent a mastectomy, whereas 1 patient (3.2%) underwent a tumorectomy with axillary lymphadenectomy. After the surgery, 26 patients (81.2%) received adjuvant therapy: radiotherapy, chemotherapy and endocrine therapy.

A total of 12 patients (37.5%) received radiotherapy, whereas 8 of the patients underwent additional chemotherapy and/or endocrine therapy. Chemotherapy was administered to 10 patients, whereas 18 patents (56.3%) were treated with endocrine therapy combined with tamoxifen. Adjuvant therapy was administered as the only treatment in 9 patients, whereas in the remaining patients adjuvant therapy was applied simultaneously with radiotherapy (3 patients) and/or chemotherapy (6 patients).

Pathological material. Archival tumor specimens were obtained from the Department of Tumor Pathology and were reviewed by pathologist to confirm histological diagnosis and tumor grade (according to Elston-Ellis modified version of the Bloom-Richardson scale).

Immunohistochemistry. Four μm sections, prepared from tissues fixed in 10% neutral buffered formalin and embedded in paraffin, were mounted on SuperFrost® Plus slides (Menzel-Gläser, Braunschweig, Germany), deparaffinised and hydrated through a series of xylenes and alcohols.

Following antigen retrieval (techniques summarized in table 2), slides were incubated in 3% H_2O_2 diluted in methanol for 30 min. to block the activity of endogenous peroxidases. Twenty min. incubation with 2.5% horse normal serum was applied to block non-specific binding of antibodies. Next, slides were incubated with primary antibodies: for P-cadherin – 1h incubation at 37°C, for other antibodies – overnight

Table 2. Immunohistochemical procedures used for visualization of markers.

Marker /antibody	Clone	Dilution	Manufacturer	Antigen retrieval technique	No of immunopositive / no of assessed cases (%)
ER	6F11	1/100	Leica Biosystems [1]	TRS, pH=6.1 DAKO[2], 50 min., 96°C	25/27 (78.1)
PgR	PGR/2	1/200			
HER2	polyclonal	1:250	DAKO [2]		1/25 (3.1)
Ki-67	MIB-1	1:75	DAKO [2]		24/24 (100.0)
Cytokeratin 5/6	D5/16 B4	1:50	DAKO [2]		4/29 (12.5)
Cytokeratin 5	XM26	1:80	Thermo [3]		
P-cadherin	56	1:200	BD [4]		11/23 (34.4)
SMA	αsm-1	1:50	Leica Biosystems[1]		1/25 (3.1)
Vimentin	V9	1:200	BioGenex[5]	Not applied	2/28 (6.3)
EGFR	H11	1:200	DAKO[2]	Proteinase K, 10 min., 37°C	3/28 (9.4)

[1] Leica Biosystems Newcastle Ltd, Newcastle, UK
[2] DakoCytomation Denmark A/S, Glostrup, Denmark
[3] Thermo, Fisher Scientific, Fremont, CA, USA
[4] BD Biosciences Pharmingen, BD Transduction Laboratories™, Franklin Lakes, NJ, USA
[5] BioGenex Laboratories Inc., San Ramin, USA

incubation at 4°C (clones, dilutions and manufacturers are provided in table 2). Proteins labeled with primary antibodies were visualized with BrightVision detection system (Immunologic, Duiven, The Netherlands) and 3,3'-diaminobenzidine (DAB) (Vector Laboratories, Inc., Burlingame, USA). Hematoxylin was used for nuclear counterstaining. The details of the immunohistochemical (IHC) stainings, together with the number of stained cases and number of slides with positive staining, are shown in Table 2. Staining pattern for ER, Ki-67, CK5/6, P-cadherin, SMA, vimentin and EGFR are presented in figure 1 (a, b, c, d, e, f, g, h, i respectively).

IHC evaluation. IHC stainings were evaluated in the invasive component of the tumors, only. ERα (figure 1a)/PgR, CK5/6 / CK5 (figure 1c), SMA (figure 1f), vimentin (figure 1h), and EGFR expression (figure 1i) was considered positive if >1% of tumor cells showed immunopositivity (nuclear for ER/PgR (figure 1a), and cytoplasmic/membranous for other markers (figure 1c, d, f, h, i). According to ASCO recommendation only tumors with complete intensive (3+) membranous HER2 staining of >30% of cells were considered positive [32]. P-cadherin immunopositivity was defined as complete strong membranous staining observed in >10% of cells or strong cytoplasmic staining in >50% of cells (figure 1d). In case of SMA and vimentin immunopositivity of stromal cells (figure 1 e, g star) or of myoepithelial cells (figure 1e arrow) was not taken into account.

MIB-1 labelling index (MIB-1 LI) was calculated as the percentage of Ki-67 immunopositive cells (figure 1b). Between 500 and 1000 cells (at ×400 magnification) were counted in 5 – 10 fields for each slide.

Statistical methods. The Mann-Whitney (for continuous variables) and Kruskal-Wallis, ANOVA and Chi^2 (for categorised variables) tests were employed to determine the relationship between the results of the biological analysed tests.

The probability of overall survival (OS) and disease-free survival (DFS) was calculated using the Kaplan-Meier method. The log rank test was applied to assess the influence of different factors on the results.

A Cox model multivariate analysis was also carried out. The significance level for all the tests was set at $\alpha=0.05$.

Results

Frequency and pattern of markers expression. In table 2 the frequency of markers expression was presented. The lack of results for some markers is the effect of a small amount of tissue in paraffin blocks or small fragments of tumor tissue that hindered obtaining reliable data.

ER expression was noted in 22 carcinomas (68.8%), PgR in 20 (62.5%), while steroid receptor immunopositivity (ER and/or PgR) was present in 25 tumors (78.1%).

The proliferation index (MIB-1 LI) ranged from 11.1% to 71.1% (average 35.3% ±15.37, median: 31.6%). In 53.1% the MIB-1 LI was less than or equal to the cut-off value, which was 35%. The above-mentioned cut-off value was established at mean value.

Correlations between studied markers and clinicopathological parameters. The relationships between the results of the immunohistochemical analysis are presented in table 3.

Significantly higher MIB-1LI was noted for more advanced tumor stage, hormone receptors (ER or PgR) immunonegativity, and expression of EGFR, vimentin ($p<0.05$) and P-cadherin (the last at statistical border). On the other hand, the presence of any of basal or masenchymal markers correlated with a more advanced tumor stage. Moreover, tumors with lack of vimentin expression were characterised by lower MIB-1LI and more frequently EGFR immunonegativily.

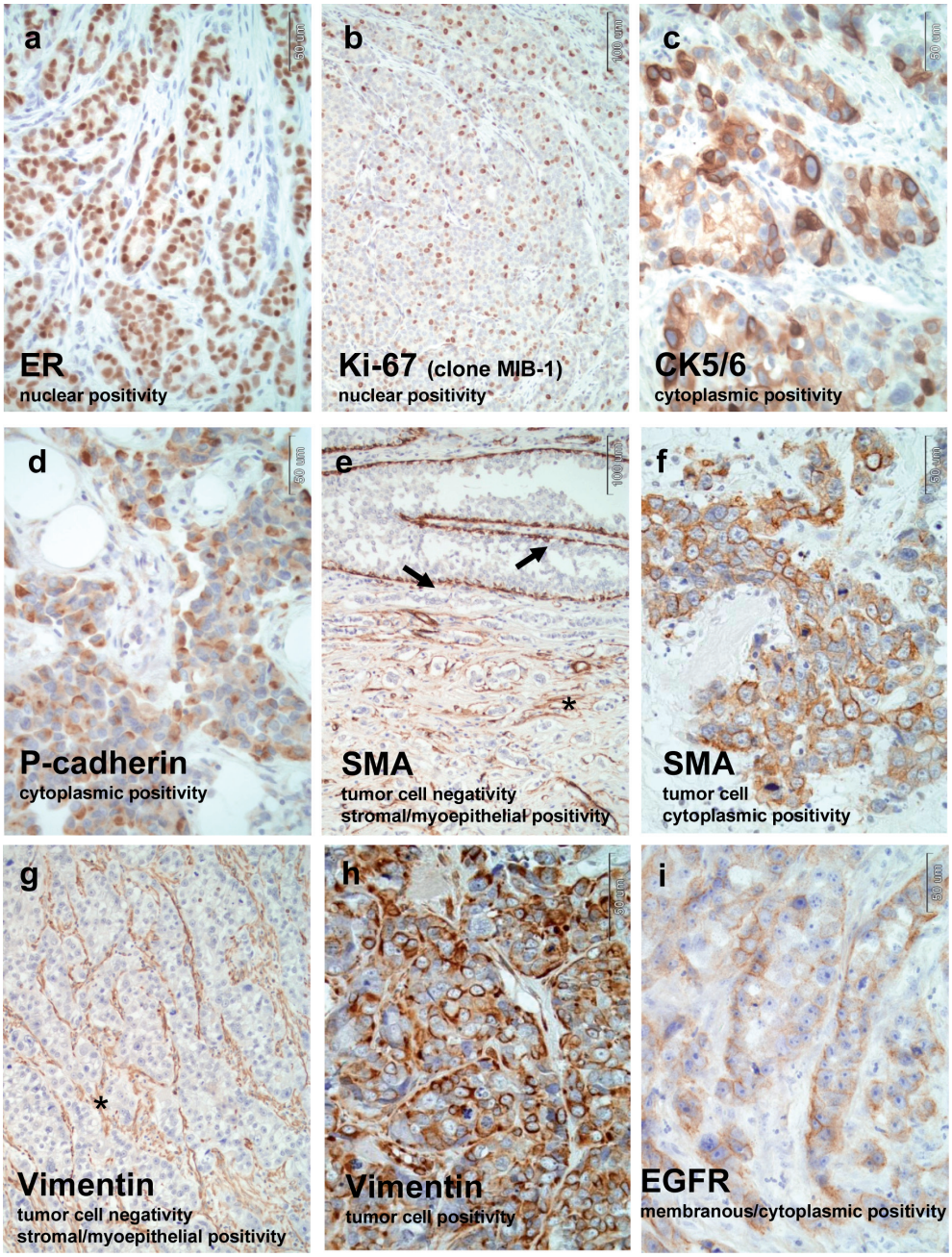

Figure 1. Results of immunohistochemical analysis of male breast cancer. Estrogen receptor immunopositivity in almost 100% of tumor cells (a), expression of Ki-67 (visualized using clone MIB-1) (b), cytoplasmic localization of CK5/6 (c) and P-cadherin (d), SMA expression in myoepithelial cells (e, arrow), stromal cells (e, star), and tumor cells (f), stromal vimentin immunopositivity (g, star), and positive reaction in tumor cells (h), weak membranous EGFR expression (i). Microphotographs a, c, d, f, h, i were taken at 40×, while b, e, g at 20× objective magnification.

Survival analysis. In the studied group of 32 male patients, the follow-up period was between 1 and 302 months (average 73 months, median: 46 months). In this period 4 patients (12.5%) suffered from the onset of local recurrences. These recurrences occurred between 7 and 70 months (average: 28.3 months) following the treatment. In 11 patients (34.4%) the cancer spread within a period of 1 and 251 months was observed. Table 1 presents the locations of distant metastases. The most common locations for metastases were the lungs and bones.

A second cancer was noted in 3 patients (9.4%) (non-small cell lung cancer in 2 patients, prostate cancer in 1 patient)

Table 3. The relationships between the results of the immunohistochemical analysis.

Parameters	Expression						MIB-1LI	
	P-cadherin			vimentin			mean value ±SD	p
	positive		p	positive		p		
tumour grade:								
G1	1/ 8	12.5%		0/8	-		30.7 ± 7.5	
G2	4/17	23.5%	0.0081	0/17	-	0.0251	29.5 ± 8.3	0,0036
G3	5/5	100.0%		2/5	40.0%		59.4 ± 10.7	
MIB-1LI			0.1645					
≤35%	6/15	40.0%		0/15	-	0.0224	-	-
>35%	5/7	71.4%		2/7	28.6%			
hormonal receptors (ER/PgR)			0.0755			0.0825		0.0290
positive	9/25	36.0%		1/25	4.0%		32.6 ± 13.0	
negative	2/2	100.0%		1 /2	50.0%		64.0 ± 10.0	
EGFR								
positive	2/3	66.7%	0.0654	1/3	33.3%	0.0309	55.9 ± 15.7	0.0181
negative	8/25	32.0%		0/25	-		30.5 ± 10.7	
vimentin								
positive	2/2	100.0%	0.0847	-	-	-	62.2 ± 7.4	0.0474
negative	9/20	45.0%					33.3 ± 13.6	
P-cadherin								
positive	-	-	-	2/11	1.2%	0.0847	43.3 ± 17.1	0.0652
negative				0/11	0		27.0 ± 10.2	

between 12 and 251 months after they had received treatment for breast carcinoma.

Twenty patients died in the follow-up period. Ten of them died from breast cancer (through the recurrence and/or spread of the cancer), two patients died as a result of a second cancer, whereas 8 patients died from non-oncological diseases.

The estimated 5-year survival rates were 57.3% (OS), and 63.1% (DFS) (figure 2a, e respectively).

None of therapeutic features (methods of adjuvant treatment: radiotherapy, chemotherapy and endocrine therapy) had significant influence on both overall and disease-free survival rates. The 5-year OS and DFS according to presence or absence method of treatment were as following: 53.5% vs 59.2% , p=0.8931 and 42.3% vs 74.1%, p=0.1149 (adjuvant radiotherapy), 51.4% vs 57.8%, p=0.5160 and 60.0% vs 66.5%, p=0.6811 (adjuvant chemotherapy), 58.0% vs 48.2%, p=0.7117 and 53.1% vs 78.6%, p=0.8150 (endocrine therapy).

It was shown that patient's OS strongly depends on the following factors: (i) tumor grade (5-year survival rates: G1 – 62.5%, G2 – 66.5%, G3 – 20%, p=0.0186) (Figure 2b), (ii) the hormone receptor status (5-year survival results: ER/PgR negativity – 22.2%, and positivity – 62%, p=0.0123) (Figure 2c) and (iii) vimentin (5-year survival for vimentin immunonegativity – 60%, and immunopositivity – 0%, p=0.0118) (Figure 2d). Furthermore, the disease-free survival rates depend on: (i) status of lymph nodes (5-year survival rate results: pN0 in 80%, pN+ in 47.5%, p=0.0329) (Figure 2f), (ii) hormone receptor expression (5-year survival results: receptor immunonegativity – 22.2%, receptor immunopositivity – 68.9%, p=0.0053) (figure 2g) and (iii) MIB-1LI (5-year survival results: MIB-1IL ≤ 35% in 74.3%, MIB-1 IL < 35% in 35.7%, p=0.0543) (Figure 2h).

The final results of the multivariate analysis were presented in table 4. We found that

hormone receptor negativity is a significant indicator of poor overall and disease-free survival (table 4). In addition, vimentin immunopositivity and high MIB-1LI are significant independent prognostic factors of poor overall and disease-free survival respectively (table 4).

Discussion

Male breast cancer is a rare form of carcinoma. In the literature the size of the analysed groups ranges from 31 to 118 patients, studied over a periods from 14 to 47 years [15, 16, 20, 21, 27, 28, 29, 31, 33]. The number of male patients treated for breast carcinoma at the Institute of Oncology in

Table 4. The results of Cox proportional hazards for 32 male breast cancer patients.

Variable and value	RR	95% CI	p
Overall survival			
ER/PgR immunonegativity	4.12	0.090 – 0.659	0.0054
vimentin immunopositivity	21.8	2.602 – 182.699	0.0045
Disease-free survival			
MIB-1LI > 35%	5.2	1.335 – 20.252	0.0175
ER/PgR immunonegativity	6.94	0.041 – 0.498	0.0022

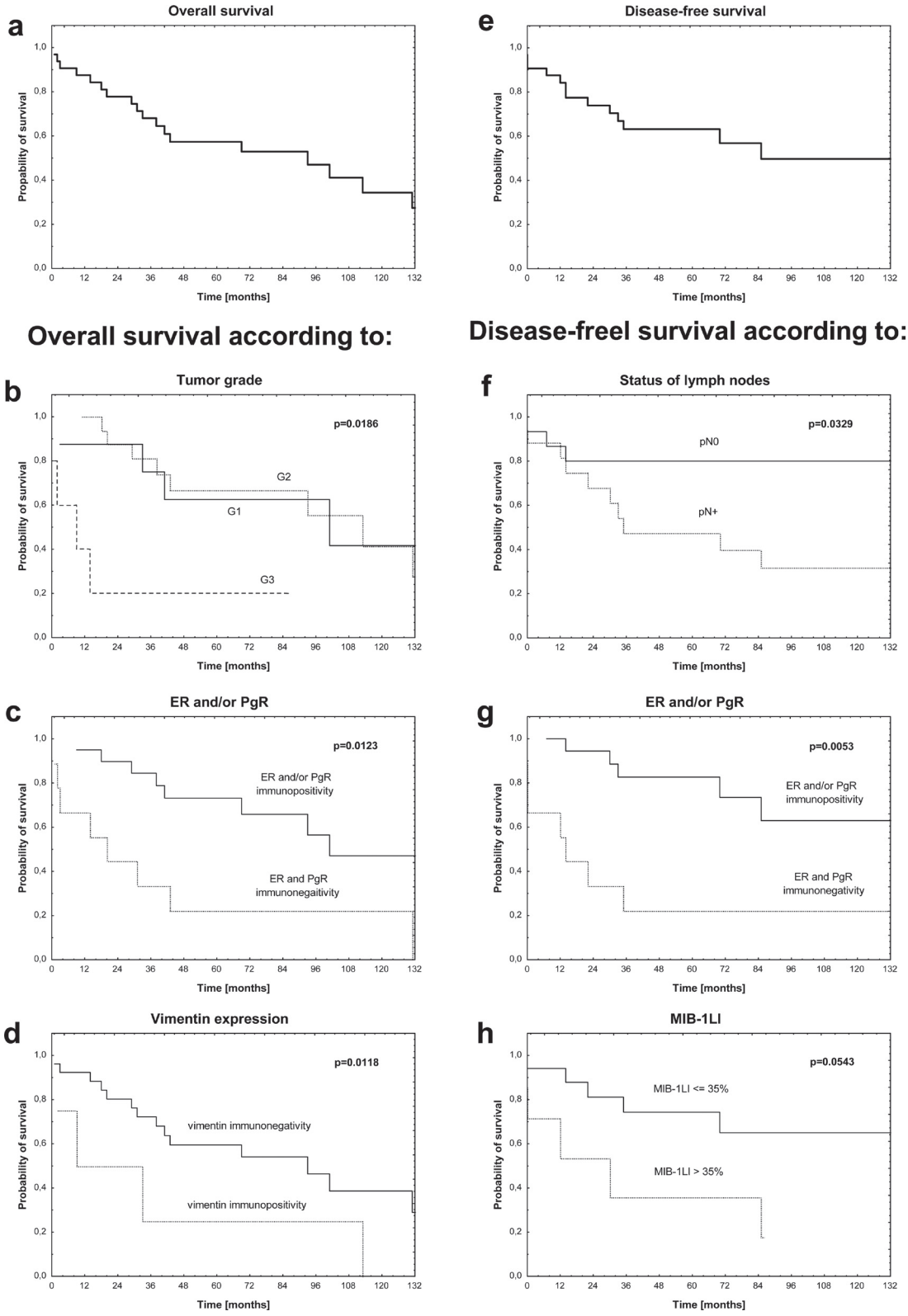

Figure 2. Overall survival in male patients with breast cancer: whole group (a), and according to: tumor grade (b), ER and/or PgR status (c) and vimentin expression (d).
Disease-free survival in male patients with breast cancer: whole group (e), and according to: status of lymph nodes (f), ER and/or PgR status (g) and MIB-1 LI (h).

Cracow is similar. Between 1960 and 2010, a total 81 patients were treated, while biological markers were carried out on the 32 patients treated between 1976 and 2010 year. The size of this group was limited as the biological markers assessments were carried out on archival paraffin sections, which in some cases were unreliable.

In our study the 5-year survival rates was 57.3% (OS) and 63.1% (DFS). These rates are comparable to the results presented in the literature, which are as follows: 41-75% for 5-year (OS) and 60-73% (DFS) [20, 21, 25, 28, 31, 34].

It was indicated that the most important prognostic factor for male breast cancer patients is the presence of metastases in the lymph nodes [21, 27, 31, 34]. Our observations are similar. We observed lymph nodes involvement in 17 out of the 32 patients (53.1%). Other authors reported lymph nodes metastases in 46-50% of the male patients with breast cancer [25, 27, 34]. In our study the presence of lymph node metastases significantly decreases cancer-free survival rates. The 5-year OS rate depends on the status of the lymph nodes. For pN0 is 80.0% and for pN+ is 47% .

The other, frequently reported, significant factor determining a patient's prognosis was hormone receptor status. We found the correlation between the hormone receptor negativity and a poor prognosis (relative risk is 4.12 and 6.94 for OS and DFS, respectively). It should be pointed out that in our study 20 out of the 32 patients (62.9%) showed expression of ER and/or PgR. Due to this fact, male patients suffering from breast cancer responded better to hormonal therapy. These statement is in agreement with other authors indicating a higher survival rate (over 90%) for patients with such tumors [6, 17, 19, 23, 25, 26, 27].

We observed significantly higher MIB-1LI for ER/PgR negative carcinomas (64%) than for positive ones (32.6%) (p=0.0290). The cut-off point for MIB-1LI found in our study for male breast carcinomas is relatively high (35%) but comparable to that reported by other authors (13.25% – 30%) [35, 36, 37, 38, 39]. This might be the result of application of a very sensitive visualization system (utilizing polymerised peroxidases as DAB substrate) and inclusion of patients with more advanced clinical stage.

Relation between MIB-1LI or steroid receptor status and patients survival in male breast cancer is not surprising as these markers are known prognostic indicators [35, 36, 37, 38, 39].

The multivariate analysis indicated that the high MIB-1LI was the negative prognostic factor which determined shorter disease-free survival rates. The relative risk related to the above-mentioned factor was 6.94. Moreover, it was shown that P-cadherin and vimentin expression was observed more frequently in high grade tumors. This result is in agreement with other authors results who noted relation between expression of basal and/or mesenchymal markers and basal breast cancer immunophenotype or high grade [39, 40]. It was also observed that the vimentin expression was independing prognostic factor for OS related to relative risk of 21.8.

Poor survival found for patients with carcinomas presenting vimentin expression (mesenchymal marker) might be explained by higher aggressiveness of tumors showing features of epithelial-mesenchymal transition (process related to increased invasiveness and metastatic potential) [41].

According to the literature, the hormone receptor negativity as well as vimentin expression was related to increased tumor proliferative activity, while vimentin immunopositivity was the main factors leading to a negative prognosis for patients with breast cancer [40, 42, 43]. Moreover vimentin, CK5/6 and EGFR expression is noted more frequently in cancers with hormone receptor immunonegativity[44].

Prognostic significance of the biological markers found in our series of male breast cancer need to be confirmed on a larger group of patients.

Conclusions

1. In male breast cancer the steroid receptor immunopositivity (ER and/or PgR) was present in 25 (78.1%) cases.
2. The estimated 5-year OS rate was 57.3%. For the same time point DFS rate was 63.1%
3. The hormone receptor negativity, vimentin immunopositivity and high MIB-1 LI are significant independent prognostic factor of poor male breast cancer patients survival.

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