The routine immunohistochemical evaluation in Paget disease of the nipple

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Paget disease (PD) of the nipple with coexisting intraductal (DCIS) and invasive carcinoma of the breast comprises 0.6–1.8% of all malignant epithelial neoplasms of this organ. Unlike invasive ductal carcinoma, there are many controversies concerning histological features of PD and the significance of the immunohistochemical characteristics of this neoplasm, which limits the optimal treatment protocols. Therefore, we decided to verify the immunohistochemical markers of PD basing on the retrospective analysis of postoperative material from 69 patients treated surgically. Microscopic examination revealed partial (7 cases) or total (62 cases) replacement of the squamous epithelium of the nipple with nests of atypical glandular cells spreading in an area ranging from 0.2 to 2.5 cm. DCIS coexisting with the PD lesions was present in all examined patients, and infiltrating carcinoma occurred in 31 (44.9%) patients. Both intraepidermal and DCIS components presented c-erbB2 overexpression. Positive estrogen and progesterone receptor staining was observed only in 7 (10.1%) and 2 (2.7%) tumours, respectively. Ki-67 proliferation index of PD cells ranged from 10% to 30%, whereas in DCIS it varied from 4% to 20%. The value of Ki-67 index exceeding 25% in the intraepidermal component of PD was associated with worse overall survival rate.

Key words: Paget disease, breast carcinoma, DCIS, Ki-67 (MiB1) index.

Introduction

Paget disease (PD) of the nipple comprises only 0.6-1.8% of all malignant epithelial neoplasms of this organ [1-5]. According to the definition of the World Health Organisation, PD is characterised by the presence of malignant glandular epithelial cells within the squamous epithelium of the nipple [6]. The cancer cells are large with abundant clear cytoplasm, atypical nuclei and prominent nucleoli. The population of tumour cells varies from single cells dispersed among keratinocytes to numerous cells arranged in nests or tubular structures. In the latter case the cells infiltrate nearly whole epidermis causing its ulceration.

The intraepidermal component of PD is accompanied by ductal carcinoma in situ (DCIS), that usually involves more than one lactiferous duct and, frequently, distant ducts of the gland. Infiltrating carcinoma of the breast, as an additional component of PD [6], occurs in 30–79% of patients [1, 2, 4, 7-12].

There are considerable discrepancies in published data concerning the incidence of DCIS and invasive carcinoma accompanying PD [1, 2, 4, 7-9, 11, 13-16], as well as the rate of PD cells presenting estrogen and/or progesterone receptor expression [2, 3, 7, 14]. In contrast with ductal carcinoma, the importance of the abovementioned histological and immunohistochemical features of PD has not been definitely established.

Controversies in morphological and immunophenotypic parameters of the tumour hinder optimal treatment decisions, concerning surgical as well as adjuvant treatment methods. Therefore, the purpose of this study is to determine the distinctive immunophenotypic characteristics of PD on the basis of routine immunohistochemical staining in breast carcinoma patients considered as candidates to adjuvant treatment.

Material and methods

Clinical data

The study was based on the retrospective analysis of postoperative material from 69 female PD patients treated surgically in the Department of Surgical Oncology, Centre of Oncology Maria Skłodowska-Curie Memorial Institute, Cracow Branch from 1973 to 2002. The patients age ranged from 33 to 88 years, with mean value of 56.9 years (SD 12.4) and median value of 57 years. There were 11 (15.9%) premenopausal patients, 16 (23.2%) in the menopausal period and 42 (60.9%) after menopause.

In 56.6% of patients physical examination did not reveal any breast mass. In the group of patients with palpable tumour, 6 (8.7%) had a tumour of 2 cm in size or less, in 12 (17.4%) patients the tumour measured more than 2 cm but not more than 5 cm, and in 12 (17.4%) patients its size exceeded 5 cm. The vast majority of patients (n = 65) started oncological treatment with radical surgery; only in 4 (5.8%) cases surgical treatment followed the administration of neoadjuvant chemotherapy. Thirteen (18.8%) patients underwent Halsted radical mastectomy, 52 (75.4%) patients – modified radical mastectomy (Madden technique), and 4 (5.8%) patients – simple mastectomy. The method of surgical treatment depended on the stage of the disease.

In all cases, the postoperative tissue samples retrieved from the archives of the Department of Tumour Pathology, Centre of Oncology Maria Skłodowska-Curie Memorial Institute, Cracow Branch, were reassessed by two pathologists. During the microscopic examination the most representative samples of the nipple and breast tumour (both the intraductal and invasive component) were selected for additional stainings.

Immunohistochemical staining

The immunohistochemical stainings were performed on formalin-fixed paraffin-embedded tissue samples of the nipple and breast tumour. The paraffin sec-

Table I. Antibodies used for immunohistochemical studies and staining procedures

ANTIGEN	CLONE	MANUFACTURER	DILUTION	INCUBATION TIME	DETECTION SYSTEM	ANTIGEN RETRIEVAL TECHNIQUE
Estrogen receptor	GF11	Novocastra	1 : 1500	Overnight, 4°C	UltraVision*	Citrate buffer pH = 6.0 Microwave oven, 2 × 10 min
Progesterone receptor	SP2	LabVision	1 : 500	30 min, room temp.	UltraVision*	Citrate buffer pH = 6.0 Water bat, 20 min
c-erbB2	CB11	Novocastra	1 : 300	Overnight, 4°C	UltraVision*	Citrate buffer pH = 6.0 Microwave oven, 2 × 10 min
c-erbB2	CBE1	Novocastra	1 : 80	Overnight, 4°C	UltraVision*	Citrate buffer pH = 6.0 Microwave oven, 2 × 10 min
P53	BP53-12	Novocastra	1 : 150	Overnight, 4°C	UltraVision*	Citrate buffer pH = 6.0 Microwave oven, 2 × 10 min
P53	PAb1801	Novocastra	1 : 40	Overnight, 4°C	UltraVision*	Citrate buffer pH = 6.0 Microwave oven, 2 × 10 min
Ki-67	MiB1	DAKO	1 : 100	Overnight, 4°C	UltraVision*	Citrate buffer pH = 6.0 Microwave oven, 2 × 10 min

**Detection system – UltraVision Large Volume Detection System Thermo Scientific Cat. No. TP125HL*

tions, up to 5 μm thick, were mounted onto SuperFrost(+) slides and dried at 60°C for 24 hours, then deparaffinized in xylene (2 × 30 minutes), rehydrated in absolute alcohol followed by 96% alcohol (for 5 minutes in each concentration) and finally rinsed in distilled water. In the rehydrated sections endogenous peroxidase was blocked with 3% hydrogen peroxide for 15 minutes. Afterwards, the sections were immersed in sodium citrate buffer (pH 6.0), heated 3 times in a microwave oven (540 Watt) for 7 minutes. During c-erbB2 antigen staining, epitope retrieval in microwave oven was omitted, whereas during Ki-67 proliferative antigen staining, epitope retrieval followed additional trypsin digestion of the examined sections (Sigma Code-No. T7168) for 15 minutes in room temperature. After washing in TRIS, the sections were successively incubated with blocking serum, primary antibody and detection system components. The data concerning dilution and exposition time of antibodies and sera used are depicted in Table I. Finally, the slides were incubated in DAB solution (DAKO-S3000) with 3% hydrogen peroxide, counterstained in Harris hematoxyllin, dehydrated and coversliped in Canada Balsam.

The evaluation of Ki-67 (MiB1) proliferative index was based on the percentage of nuclei stained. In each case 500 cells were counted, 100 cells in 5 high-power fields (magnification 400×), and the score was expressed as arithmetic mean. The immunoreactivity for steroid receptors (estrogen and progesterone) and P53 protein (BP53-12 and P53-1801) was assessed according to Remmele score; the reaction intensity and percentage of nuclei stained were determined. In the evaluation of c-erbB2 antigen immunoreactivity, the intensity of cytoplasmic membrane staining was taken into consideration; both intracellular (CB11 antibody) and extracellular (CBE1 antibody) protein domain stainings were examined. In each case the reactions were assessed independently in the interaepidermal, *in situ* and invasive component.

Statistical analysis

The survival curves were estimated with Kaplan-Meier method and compared with the log-rank test. Considering low incidence of the studied disease and resulting limited case number, the tendency towards statistical significance was also accentuated (p value ranging from 0.05 to 0.10).

Results

Microscopic appearance of the nipple and breast parenchyma lesions

Microscopic examination revealed partial (7 cases) or total (62 cases) replacement of the multilayered squamous epithelium of the nipple with nests of atypical glandular cells spreading in an area ranging from 0.2 to 2.5 cm (median 1.0 cm, mean 1.2 cm). In all 69 examined cases the described glandular cells were consistent with adenocarcinoma cells, ie. contained abundant clear cytoplasm and centrally or pericentrally located nucleus with large central nucleolus (high nuclear grade) (Fig. 1.). DCIS coexisting with the lesions of the nipple was present in all 69 examined patients. Infiltrating carcinoma occurred in 31 (44.9%) patients; in all of them the invasive component was invasive ductal carcinoma, not otherwise specified (NOS). Histological grade of breast carcinoma, according to Elston-Ellis modification of Bloom-Richardson grading system, was assessed in 16 (51.6%) cases; 1 of the tumours was well differentiated carcinoma (Bloom-Richardson grade I), 9 were moderately differentiated carcinomas (grade II), and 6 – poorly differentiated carcinomas (grade III).

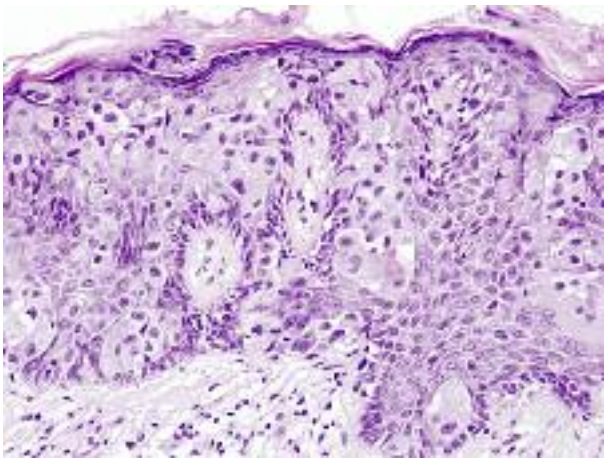


Fig. 1. Microscopic appearance of the intraepidermal component of Paget disease, HE

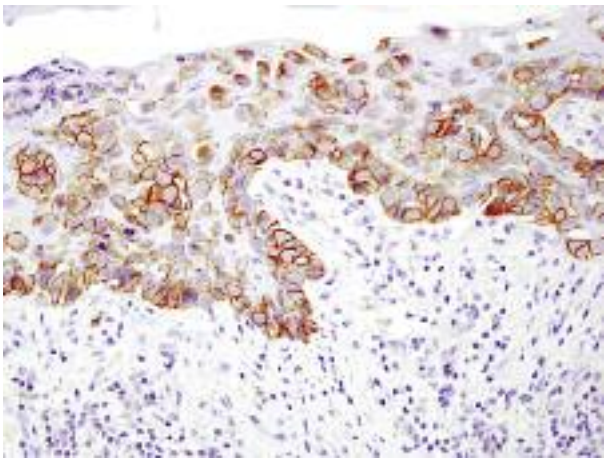


Fig. 2. Strong (+++) positive membranous reaction to c-erbB2 (HER2) antigen in Paget disease cells

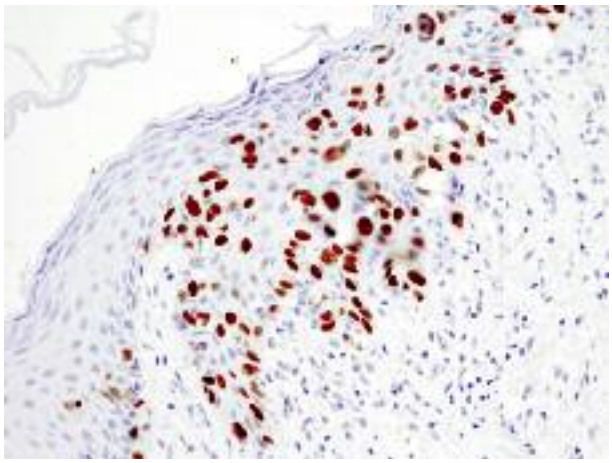


Fig. 3. Positive reaction with antibody against P53 antigen in Paget disease cells

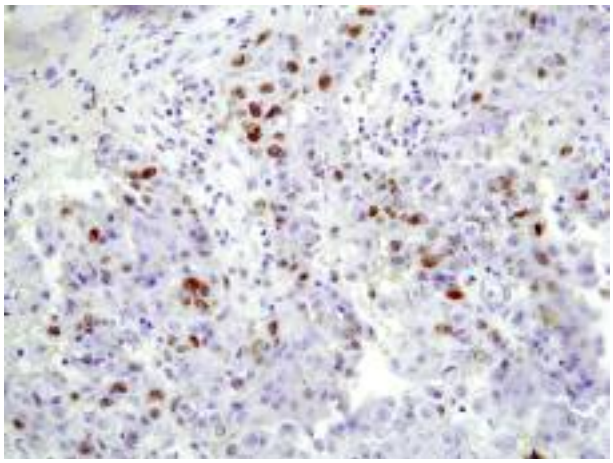


Fig. 4. Positive reaction to Ki-67 (MiB1) antigen in Paget disease cells

Immunoreactivity of steroid receptors, c-erbB2 and P53 protein and Ki-67 proliferative antigen

Positive reaction with monoclonal antibodies against both external (CBE1) and internal (CB11) protein domains was characterised by the presence of linear deposits in the cellular membrane of cancer cells (Fig. 2.). The internal domain expression (reaction with CB11 antibody) was observed in all 69 cases. Additionally, immunopositivity for the external domain (reaction with CBE1 antibody) was revealed in 9 (13%) cases. Immunoreactivity of identical intensity was observed simultaneously in the intraepidermal component of PD and in the breast carcinoma cells. In each case over 80% of cells were stained.

Positive estrogen receptor (ER) staining was observed in 7 (10.1%) PD cases. In each case the reaction was moderate or strong, and at least 10% of cancer cells were stained (grade IV–XII according to Remmele score). Progesterone receptor (PR) immunopositivity was noted only in 2 (2.7%) tumours; the reaction was weak or moderate (grade III or VI according to Remmele score, respectively).

Positive immunohistochemical staining to the protein product of P53 gene, assessed with BP53-12 antibody, was revealed in 43 (62.3%) PD cases. The reaction with PAb1801 antibody was positive in 29 (42.%) cases (Table II). Strong immunoreactivity (grade VI or XII according to Remmele score) with BP53-12 and PAb1801 antibodies was noted in 29 (42%) and in 22 (31.9%) tumours, respectively. The dispersed or granular reaction was limited to the cell nuclei (Fig. 3.).

The rate of cells with positive nuclear staining with monoclonal antibody (MiB1) against the Ki-67 proliferative antigen was assessed independently in the intraepidermal component of PD and in DCIS. Ki-67 index in the epidermal component ranged from 4.0% to 53.3% (mean 19.4%, SD 10.4%; median 19.0%). The mean value of Ki-67 index in DCIS was 14.6% (SD 9.2%, median 13.0%). In the majority of cases (64.1%), proliferative activity of cancer cells, measured as Ki-67 antigen expression in the intraepidermal component, ranged from 10% to 30% (Fig. 4), whereas in DCIS – from 4% to 20% in most cases. The value of Ki-67 index (MiB1) exceeding 25% in the intraepidermal component of PD was associated with worse overall survival as well as 5-year and 10-year survival rate (53.1% and 47.8%, respectively), whereas in the group of PD patients with Ki-67 proliferative index of 25% or less the rates were 73.9% and 71.4%, respectively ($p = 0{,}0637$) (Fig. 5).

Table II. The results of immunohistochemical staining to P53 protein in 69 Paget disease patients

Immunoreactivity (Remmele score)	BP53-12 antibody		PAb1801 antibody	
	Tumours (n)	Tumours (%)	Tumours (n)	Tumours (%)
0	26	37.7	40	58.0
II	2	2.9	2	2.9
III	8	11.6	5	7.2
IV	4	5.8	0	0.0
VI	6	8.7	3	4.3
XII	23	33.3	19	27.6

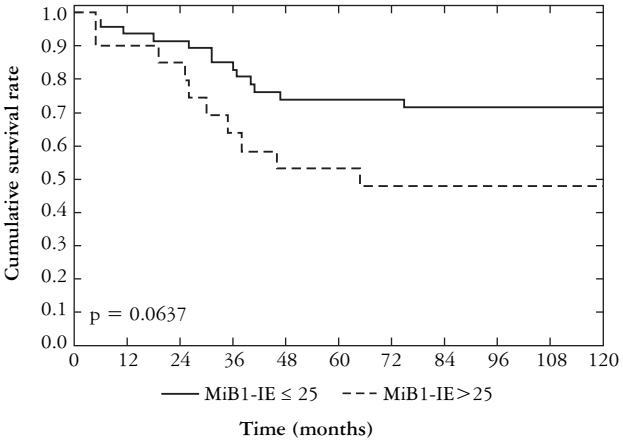
Discussion

In immunohistochemical studies PD cells show typical reaction with antibodies against epithelial membrane antigen (EMA) [17, 18] and cytokeratins, except from cytokeratin CK20 and high molecular weight cytokeratins (34βE12, 34βB4/CK1, 6B10/CK4) [19], and also against CA15-3 and KA-93 antigens [20].

Literature data about the incidence of ER and PR expression in malignant PD cells differ significantly. According to Caliskan *et al*. [7], Dalberg *et al*. [2] and Fu *et al*. [3], ER and PR are expressed in 29–44% and 25–32% of PD cases, respectively. Cohen *et al*. [17] and Liegl *et al*. [14] report that the rate does not exceed 10% of examined tumours (Table III). In the present study, in the group of 69 PD patients, positive nuclear reaction to ER was noted only in 7 (10.1%) tumours. Positive reaction to PR, of week or moderate intensity (grade III or IV according to Remmele score) was observed only in 2 (2.7%) cases. Our results and the results obtained by Cohen *et al*. [17] and Liegl *et al*. [14] support the fact that ER expression in mammary Paget disease cells is exceptionally rare, and even if the reaction is positive, the detected ER is in most cases functionally inactive with no ability to activate PR.

Unlike ER and PR, HER2 (c-erbB2) receptor is overexpressed in the vast majority of PD cases (Table IV). Our results confirm these observations. Positive reaction with monoclonal antibodies against the intracellular domain (CB11) of the protein was noted in all 69 examined cases. In 9 (13%) cases there was additional positive reaction to the extracellular domain of the protein (reaction with CBE1 antibody). HER2 expression of the same intensity was noticed simultaneously in the intraepidermal malignant cells of PD and in breast cancer cells. In each case over 80% of malignant cells were stained.

Our results are consistent with published data and show that mammary Paget disease belongs to breast carcinomas with ER–/PR–/HER2+ phenotype or to breast cancers characterized by coexpression of hormone receptors and HER2 protein (ER+/PR+/–/HER2+ phenotype).



YEARS	1	2	3	4	5	10
MiB1-IE ≤ 25	95.7	91,5	82.8	73.9	73.9	71.4
MiB1-IE > 25	90.0	85.0	63.8	53.1	53.1	47.8

Fig. 5. Overall survival curves in association with Ki-67 (MiB1) index in the intraepidermal component of Paget disease of the nipple

Overexpression of c-erbB2 in PD is the result of *HER2* gene amplification (similarly to ductal carcinoma of the breast) [24-27]. In all 12 cases examined by FISH analysis, Mrhalova and Kodet [25] observed at least 7 copies of the gene occurring in one cancer cell nucleus (precisely 7–26 copies, in the majority of cases >10 copies). In two cases the authors noted polisomy of chromosome 17. Anderson *et al*. obtained similar results, finding strong (+++) immunohistochemical reaction to HER2 in 16 (80%) of 20 examined cases of PD; in all of them the number of gene copies exceeded 5. In four remaining cases neither HER2 expression in IHC method, nor gene amplification in FISH method was found [24]. Tanskanen *et al*. also assumed that the presence of at least 6 gene copies in malignant cells of PD or gene copy clusters in one cell nucleus proves *HER2* gene amplification [26].

According to the recommendations of *College of American Pathologists*, P53 protein belongs to a group of factors which requires intensive laboratory and clinical

Table III. Results of immunohistochemical studies on steroid receptors in Paget disease cells. Number (%) of tumours with positive steroid receptor staining

NO.	NO. OF EXAMINED TUMOURS/TOTAL NUMBER OF PATIENTS	ESTROGEN RECEPTOR	PROGESTERONE RECEPTOR	AUTHOR, YEAR [CITATION NO.]
1	104/114	43 (41%)	33 (32%)	Caliskan, 2008 [7]
2	57-70/223	31/70 (44%)	14/57 (25%)	Dalberg, 2008 [2]
3	58/58	6 (10%)	0	Liegl, 2005 [14]
4	14/14	4 (29%)	4 (29%)	Fu, 2001 [3]
5	19/20	1 (5%)	1 (5%)	Cohen, 1993 [17]
6	69/69	7 (10%)	2 (3%)	Marczyk, 2011

Table IV. HER2 protein overexpression in Paget disease

No. of cases studied	No. of cases with HER2 overexpression	No. (%) of tumours with strong (+++) positive reaction			Author year [citation No.]
		intraepidermal component	intraductal component	invasive component	
12		12/12 (100%)	9/9 (100%)	2/2 (100%)	Keatings, 1990 [21]
10	10 (100%)				Tanskanen, 2003 [26]
19	15 (79%)		12/14(86%)	6/7(86%)	Wolber, 1991 [27]
25	25 (100%)	24/25 (96%)	14/14 (100%)	2/2 (100%)	De Potter, 1994 [22]
14	13 (93%)	13/14* (93%)	13/13 (100%)	13/13 (100%)	Fu, 2001 [3]
23	23 (100%)	23/23 (100%)	18/18 (100%)	18/18 (100%)	Meissner, 1990 [23]
69	69 (100%)	69/69 (100%)	69/69 (100%)	31/31 (100%)	Marczyk, 2011

**In 1 patient there was only Paget disease (intraepidermal component) without DCIS or invasive component.*

studies because of its potential prognostic and predictive value in breast cancer patients. Meanwhile, with respect to PD, there is only one published study, dedicated to P53 protein expression in cancer cells. In the study of Fu *et al*. [3] including 14 patients with PD, positive reaction to this antigen was found in 6 tumours, in the intraepidermal component (6/14, 42.9% of examined tumours), as well as in the intraductal and invasive component of breast carcinoma (6/13, 46.2% of examined tumours). These results are consistent with our observations. Positive nuclear reaction to P53 protein with PAb1801 antibody was observed in 29 (42.0%) tumours, whereas positive reaction to BP53-12 antigen was found in 43 (62.3%) tumours. In 29 (42%) tumours the reaction with antibody against BP53-12 presented strong intensity (grade VI or XII aciordng to Remmele score).

Statistical analysis of survival of 69 PD patients treated in Centre of Oncology in Cracow revealed potential prognostic significance of the evaluation of Ki-67 (MiB1 index). It was shown that patients with MiB1 index, counted in PD cancer cells, exceeding 25% are characterised by lower overall survival rates (p value at the verge of statistical significance). There is only one publication available, by Caliskan *et al*. from the European Institute of Oncology in Milan, dedicated to the analysis of Ki-67 antigen expression in PD cancer cells [7]. The evaluation of survival rates conducted by the authors, in 114 surgically treated patients suffering from mammary Paget disease, did not show correlation between MiB1 index and disease-free and overall 5-year survival rates. It is not possible to make precise comparison between Caliskan *et al*. results and the results derived from our study, because the Italian authors did not disclose precise data on MiB1 index measurement method. The only information given is a threshold of 20% of stained nuclei in malignant cells accepted as positive reaction with MiB1 antibody.

Conclusions

1. Paget disease of the nipple is characterized by overexpression of c-erbB2 (HER2) receptor, whereas steroid (estrogen and progesterone) receptors expression is observed only in 10% of cases.
2. Proliferative activity of cancer cells, expressed as MiB1 index, may be a potential prognostic factor in Paget disease of the nipple.

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