Fine needle aspiration cytology of benign breast disease. Markers of apoptosis and proliferation*

J. ŚWIĄTECKA[1], P. LAUDAŃSKI[2], J. DZIĘCIOŁ[3]

[1]Department of Gynecology, e-mail: jswiatecka@wp.pl, [2]Department of Gynecological Endocrinology, and [3]Department of Anatomy, Medical University of Bialystok, 15-276 Bialystok, Poland

Received May 14, 2003

Aim of the study was to compare the fine needle aspiration cytology findings of benign breast lesions with incidence of proliferation markers and apoptosis.

This study included 37 patients with palpable breast lumps, referred for USG guided FNA. FNAC were prospectively classified as C2-benign, C4-suspicious of malignancy, and C5-malignant. The specimens were simultaneously stained for Ki-67, MPM2, Bcl2 and P53. The diagnoses in group-C2 were following: simple cyst, multiple cysts, simple cyst with apocrine metaplasia, inflammatory cyst, benign dysplasia (BD) and benign solid tumors. The final diagnoses, after histopathological verification, in cases of primary classification as C4 and C5 were as follow: proliferative fibroadenoma (FAp) and breas cancer, respectively. Great majority of C2/BD aspirates were negative for proliferative antigens Ki-67 and PCNA. These antigens were detected in part of benign solid tumors, as anticipated in suspicious solid tumor, and in all of cancer aspirates. Bcl-2 immunopositive cells were detected approximately in one quarter of C2/BD, nearly in half of C2 solid tumors and in one C4/FAp. Most of diagnosed specimens were P53-negative.

Immunocytodetection of Ki67, MPM2, Bcl2, P53 might be promising, supportive method in the classification of benign breast lesions. FNAC increases the reliability of diagnosis when complemented by immunocytochemical staining. It could be helpful procedure of establishing more accurately the biology of these lesions and possibly serve as an essential factor in clinical follow-up. Nevertheless, further study on larger group of patients comparing cytological and histopathological diagnosis is required to estimate reliability of its predictive value.

Key words: benign breast disease, fine-needle aspiration, Ki-67, Bcl-2, p53, PCNA

Increased emphasis has been placed on breast cancer screening over the last several years. Although mammography (MRTg) is an excellent tool for the detection of breast cancer, not all cancers can be distinctly imaged mammographically. In young patients with dense breast the false-negative rate of MTRg may approach 25%. In older women with increase amount of fatty tissue within the breasts, the false negative rate is much lower, but still remains at the level of about 5% to 6%. Because MRTg is imperfect and is not routinely performed in all women, manual breast examination by physicans, patient's self-examination as well as mammasonography (MUSG) must all also remain an integral part of breast examination and screening. On the other hand, although there is an increasing incidence of breast cancer, most breast lumps are benign. Over 75% of patients attending specialized breast disease centers do so for nonmalignant breast lesions [4, 16].

Fine needle aspiration cytology (FNAC) has been shown to be of value in the pre-operative assessment of breast disease. FNAC has been used in palpable and non-palpable lesions detected by clinical examination, by MRTg screening and MUSG. In most cases of primary malignant epithelial neoplasms and benign breast lesions, fine-needle aspiration provides reliable diagnosis due to their characteristic cytologic features. Because inadequate sampling may result in a false-negative cytology diagnosis, the time-

*The research was supported by the Grant No. 4-29 756 from the Medical University of Bialystok.

tested rule is that a negative cytologic diagnosis is not definitive unless it is fully supported by negative clinical and mammographic findings [4].

The use of FNA has been shown to reduce the number of benign surgical biopsies [15]. Free-hand FNA and USG guided FNA, especially used as a part of triple test, has been proven to be accurate for the detection of breast carcinomas. It is a rapid and reliable means of breast cancer diagnosis. It usually considers sufficient grounds on which to proceed directly to appropriate treatment. However, its utility in the classification of benign breast lesions is insufficient [18].

The balance between programmed cell death i.e. apoptosis, proliferation and redifferentiation, is of important significance in the pathogenesis and progression of breast cancer. Apoptosis is considered to play a critical role in tumorigenesis. It has been shown that apoptosis is controlled by both pro-oncogenes such as bcl-2, c-myc and tumor-suppressor genes as p53 [24–26]. When normal mammalian cells are subjected to stress signals (e.g. hypoxia, radiation, DNA damage or chemotherapeutic drugs) p53 is activated, additionally to its activation, degradation of the p53 protein is blocked. One of the first cell death-regulating genes to be identified was bcl-2, an anti-apoptotic gene that appears to block a distal step in an evolutionarily conserved pathway crucial to apoptotic cell death [10]. The principal intracellular effectors of apoptosis are a family of cysteine proteases with homology to interleukin-1b converting enzyme and the nematode cell death protease CED-3. High level of the Bcl-2 protein protect cells from early death by apoptosis, through preventing the activation of caspases that carry out the process. Surprisingly in the study of KRAJEWSKI et al [10], caspase-3 immunointensity was higher in 86% of breast cancers, both in "in situ" as well as in infiltrating adenocarcinomas, when compared with the adjacent normal mammary epithelium of the same specimens. Regardless of the deregulation in the apoptotic process it seems that high proliferation rate of cancer cells is another hallmark of cancer. Proliferation markers such as: PCNA, MP-mitotic protein, protein MPM2 or nuclear antigens MIB-1 and Ki67 are the most often studied [9]. Proliferating Cell Nuclear Antigen (PCNA) a marker determining growth fraction is a 36 kD nuclear protein which is directly involved in DNA synthesis. The distribution of PCNA in cell cycle increases through G1, peaks at the G1/S-phase, decreases through G2 and reaches low immunohistochemically undetectable levels in M-phase and quiescent cells (G0) [21]. Ki-67 is the most widely used marker to assess proliferative fractions, and significant direct correlation between Ki-67 and PCNA in breast cancer was reported [21]. The measurement of biological markers in breast cancer as prognostic indicators has been utilized in practice for many years. With the widespread production of polyclonal and monoclonal antibodies, it is now possible to evaluate many of markers in tissue sections and cytological aspirates. The immunocytochemical detection of the proliferative antigens as well as p53 and bcl-2 genes products is often applied for the monitoring of therapeutic process of breast cancer disease [11, 12, 21, 25].

The aim of our study was to indicate the presence of immunopositive cells in FNA aspirates from benign breast lesions for the following antigens: Ki-67, PCNA, Bcl2, P53.

Material and methods

Patients. The study group consisted of 37 female patients in the age between 19 and 63 years with a mean age of 41 years, all with palpable breast lump. In two cases only USG guided FNA was done due to age less than 20 years. The triple test had been done in remaining 35 patients.

Immunocytochemical identification of PCNA, Ki-67, p-53 gene product, bcl-2 gene product. Immunocytodetection of p-53 and bcl-2 gene products as well as PCNA and Ki-67 antigen were performed after cytofixation of aspirate with the Cytofix (Merck). The antibody binding was detected by the biotin-streptavidin-peroxidase method using Universal LSAB+kit HRP with DAB-3,4-diaminodenzidine as a chromogen [23]. Data were expressed as a percentage <10%, <10% and <50%, <50% of immunopositive cells and intensity of reaction was expressed using scale: + low, ++ middle, +++ high. The following antibodies were used: PCNA monoclonal mouse antibody, clone PC10 Isotype IgG2a DAKO; Ki-67 monoclonal mouse antibody, clone Ki-S5 Isotype IgG1 DAKO; Bcl-2 oncoprotein: monoclonal mouse antibody, clone 124, chromosomal translocation t(14, 18) Isotype IgG1 kappa DAKO; p53 Protein monoclonal mouse antibody, clone DO-7 Isotype IgG2b kappa DAKO. Proliferative index >30% was evaluated as high.

All patients provided written informed consent for participation in the study, the study protocol was approved by the local Ethical Committee.

Results

Forty six fine needle aspirates from 37 female patients with palpable breast lump were evaluated. Five out of 37 patients had multiple FNA-s (1 patient with multiple cysts 2+3, 1 patient with 2 cysts, 1 patient with 2 solid tumors primary classified as C2 and C4, 1 patient with cancer cells + simple cyst, 1 patient with benign dysplasia C2 +2 solid tumors classified as C2). Remaining 32 patients had single FNA.

The patients age ranged between 19 and 63 years. In benign condition the mean age was 39.9 years while in malignant cases it was 53.3 years.

The cytological diagnosis, after H+E staining, using Cy-

Table 1. Immunocytodetection of Ki-67 and PCNA antigens, bcl-2 and p53 proteins in FNA cytology slidesfrom palpable breast masses with cytological diagnosis as follow: C2 BD – benign dysplasia, C2 in solid tumors, C4 in solid tumor, C5

FNAC diagnosis	Ki-67 no. of cases	% of posit. cells	PCNA no. of cases	% of posit. cells	Bcl-2 no. of cases	% of posit. cells	p53 no. of cases	% of posit. cells
C2 BD	1/22	<10%	3/22	<10%	4/22 1/22	<10% 10%< <50%	1/22 1/22	>50% <10%
C2 solid tumors*	2/5	<10%	2/5	<10%	2/5	<10%	0/5	negative
C4 solid tumor**	1/1	10%< <50%	1/1	10%< <50%	1/1	>50%	0/1	negative
C5***	3/3	>50%	1/3 2/3	ca.100% >50%	1/3 2/3	ca.100% negat.	1/3	10%< <50%

After histopathological verification: *1 patient – fibroadenoma, **1 patient – proliferative fibroadenoma, ***3 patients – cancer.

tological Classification of the National Health Service Breast Screening Program NHSBSP (C1 – inadequate, scanty or acellular specimen or poor preparation, C2 – benign, C3 – atypia probably benign, C4 – suspicious of malignancy, C5 – malignant [15]) were as follow:

C1 – 2 cases, qualified after second FNA as C2.

C2 – simple cyst, multiple cysts, simple cyst with apocrine metaplasia, inflammatory cyst (*inflammatio chronica partim purulenta*) n=10 (number of patient) a=15 (number of aspirates), benign dysplasia n=22 a=22 respectively, benign solid tumor n=4 a=5 respectively.

C4 – suspect (solid tumor) n=1 a=1 respectively.

C5 – cancer cells n=3 a=3, respectively (Tab. 1, Fig. 1, 2, 3 and 4).

The cysts aspirates after centrifugation were stained with H+E and all of them contained: epithelial cells, macrophages and sparse lymphocytes. There were no or occasional epithelial glandular cells in aspirates. On account of that, the simple cysts preparations were not immunocytochemically tested. Histopathological verification was performed in all patients with primary diagnosis described as C5 and in patient with solid tumors classified as C2 and C4 with high proliferative index. Final diagnosis was as follow: breast cancer in 3 patients with C5 cytology and fibroadenoma and proliferative fibroadenoma in patient primary classified as C2 and C4, respectively.

The immunodetection of proliferative antigens was observed in all of cancers aspirates (Fig. 1B and 2B). Number of Ki-67 and PCNA immunopositive neoplastic cells was more than 50% in both cases. Most of benign aspirates were negative for both studied proliferative antigens (Fig. 1A and 2A). Ki-67 and PCNA antigens were detected in proliferative fibroadenoma (percentage of immunopositive cells ranged between 10% and 50%) and in 2 out of 5 benign solid tumors (<10% for both studied antigens). Bcl-2 oncoprotein was detected in 7 out of 27 benign preparations, classified as C2, in proliferative fibroadenoma/C4 and in 1 case of carcinoma (Fig. 4A, B). In these cancer cells immunostaining was very strong and observed at near 100% of cells. Finally most of benign aspirates (excluded 2 out of 22 (Fig. 3A)), all of solid tumors (diagnosed as C2 and C4) were negative for p53 immunoreaction (Fig. 3B). In only 1 out of 3 cancer slides p53 immunoreaction was observed (range of positive cells between 10% and 50%.) (Tab. 1).

Discussion

The impact of the rising incidence of breast disease has led to the demand for reliable, cost effective and quick diagnostic tests. For this reason the last decade has shown a large increase in the usefullness of cytological techniques in the diagnosis of breast lesions [3].

The most important for cytopathologist in breast disease are accurate and reliable experiences of FNAs. For such reports to be clinically useful, and for the triple approach to diagnosis of breast lesions to be effective, clinicians need to be able to assimilate the likely outcome of specific cytological reporting categories with clinical and radiological features [18]. Fine needle aspiration cytology (FNAC) of the breast is a rapid and reliable means of diagnosis of carcinoma but the classification of benign lesions in FNAC is less precise. In some cases, cytological diagnosis may be difficult because of the presence of certain cytological characteristics, which suggest a proliferative/indeterminante epithelial lesion, i.e. a cytological "gray zone" [1, 14, 17].

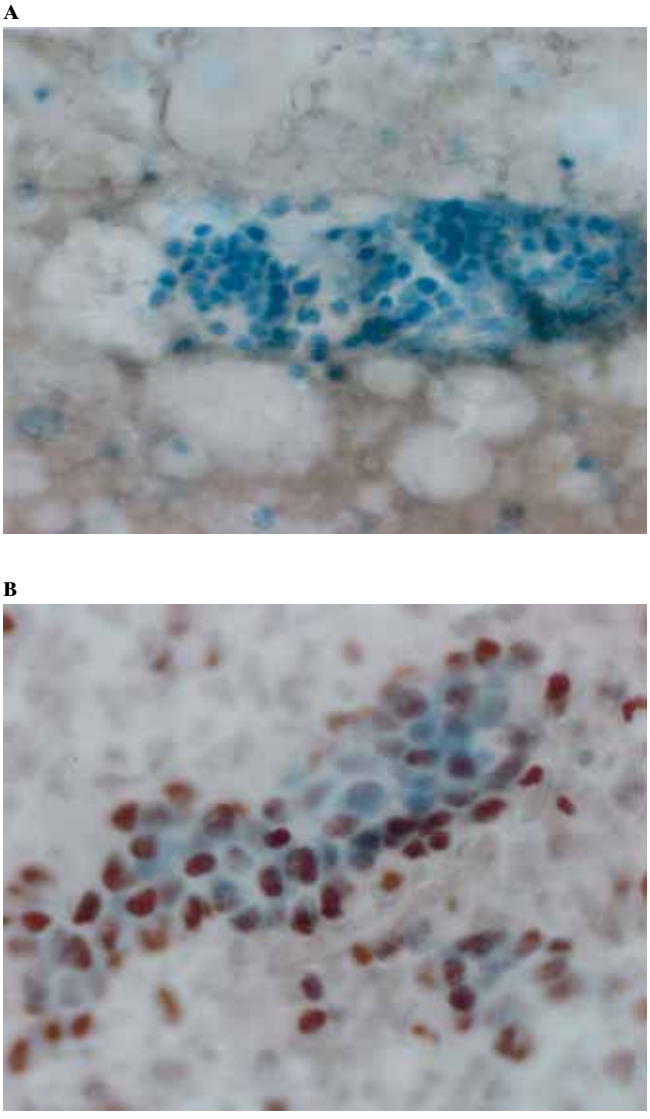

Figure 1. Immunocytochemically detection of Ki-67 antigen (Ki-67 monoclonal mouse antibody, clone Ki-S5 Isotype IgG1 DAKO in breast FNA preparations from: A. benign disease – negative staining, B. cancer cells – positive staining.

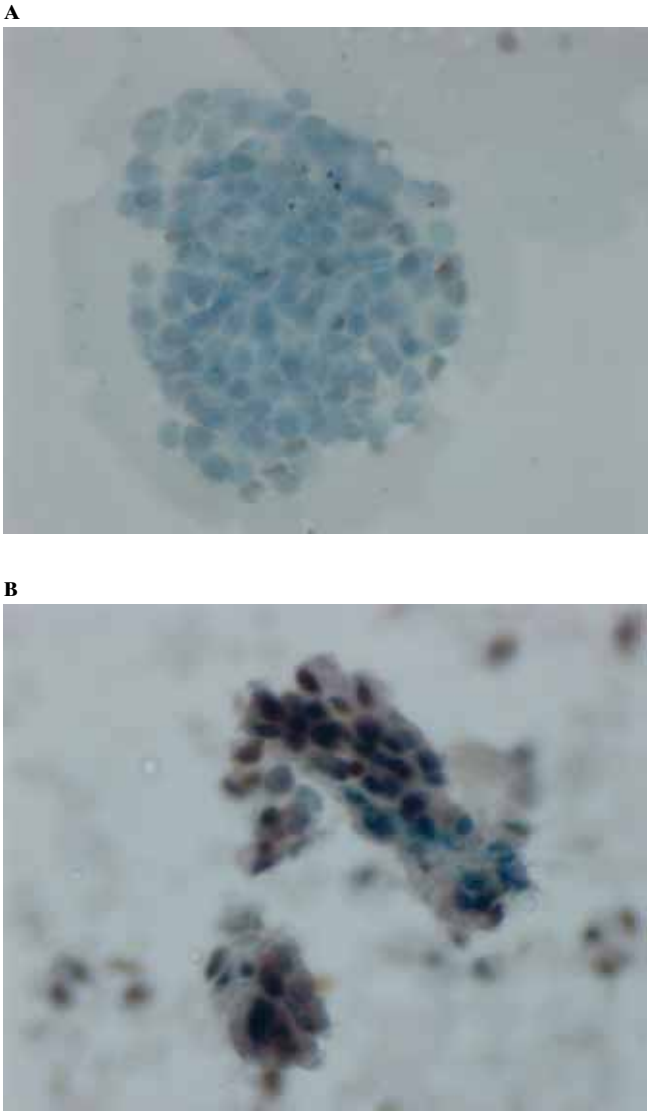

Figure 2. Immunocytochemically detection of PCNA antigen (PCNA monoclonal mouse antibody, clone PC10 Isotype IgG2a DAKO) in breast FNA preparations from: A. benign disease – negative staining, B. cancer cells – very strong positive staining.

The gray zone in breast FNAC is a broad spectrum that include proliferative fibrocystic disease, sclerosing adenosis and even malignancy. Diagnosing gray zone pathology as atypical in FNAC causes no delay in treatment as excisional biopsy is recommended for all unequivocal cases [17]. For this reason other adjunct methods would be helpful to establish both: equivocal cytological diagnosis and clinical algorithms to deal with patients with Benign Breast Disease (BBD).

The growth rate of neoplasm is determined by the differences between apoptosis, proliferation rate and necrosis of cancer cells. Apoptosis is regulated in a complex manner, with several proteins, among them some promoting apoptosis (p53 protein, Bcl-X_s, Bax), whereas others are antiapoptotic (Bcl-2, Bcl-X_1) [7, 8]. Dysregulation of normal programmed cell death mechanisms plays an important role in the pathogenesis and progression of breast cancer, as well as in responses of tumors to therapeutic intervention [10, 24]. Apoptosis-related genes (bcl-2, p53) are also involved in the progression of breast cancer [24–26]. Both may have certain predictive values, but the clinical data are still not resolved [6, 24]. Bcl-2 can prevent apoptosis induction in cancer cells by a wide variety of stimuli, including chemotherapeutic drugs and gamma irradiation; neurotrophic factor withdrawal from neurons; cytotoxic cytokines such as tumor necrosis factor-alpha; fas-ligand and transforming

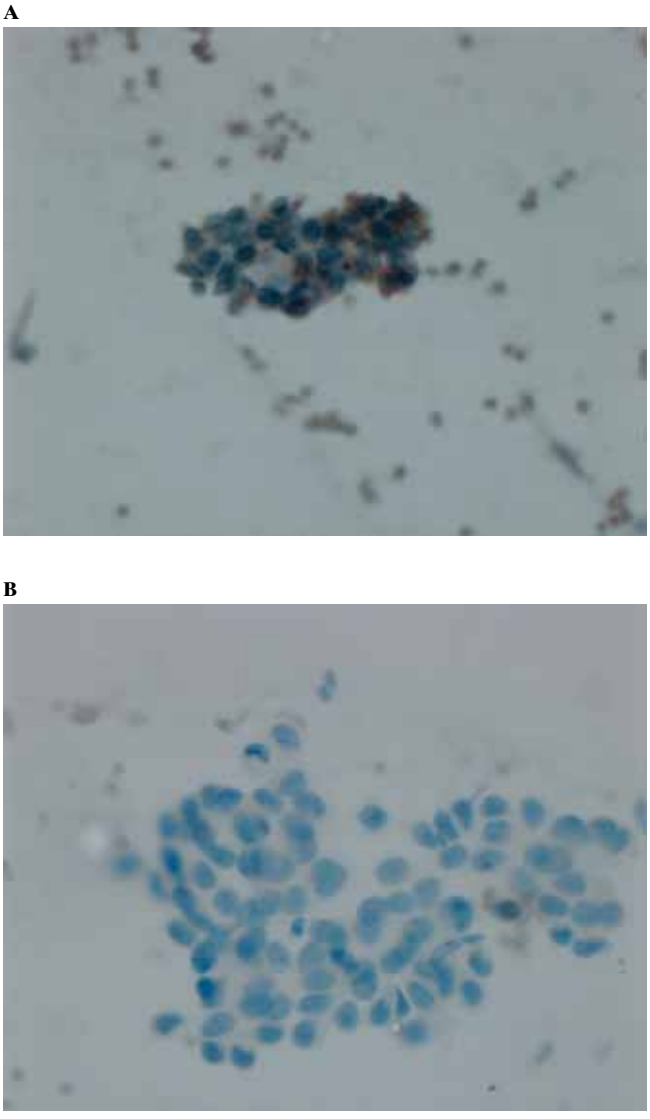

Figure 3. Immunocytochemically detection of p53 protein (p53 Protein monoclonal mouse antibody, clone DO-7 Isotype IgG2b kappa DAKO) in breast FNA preparations from: A. benign disease – strong positive staining, B. cancer cells – negative staining.

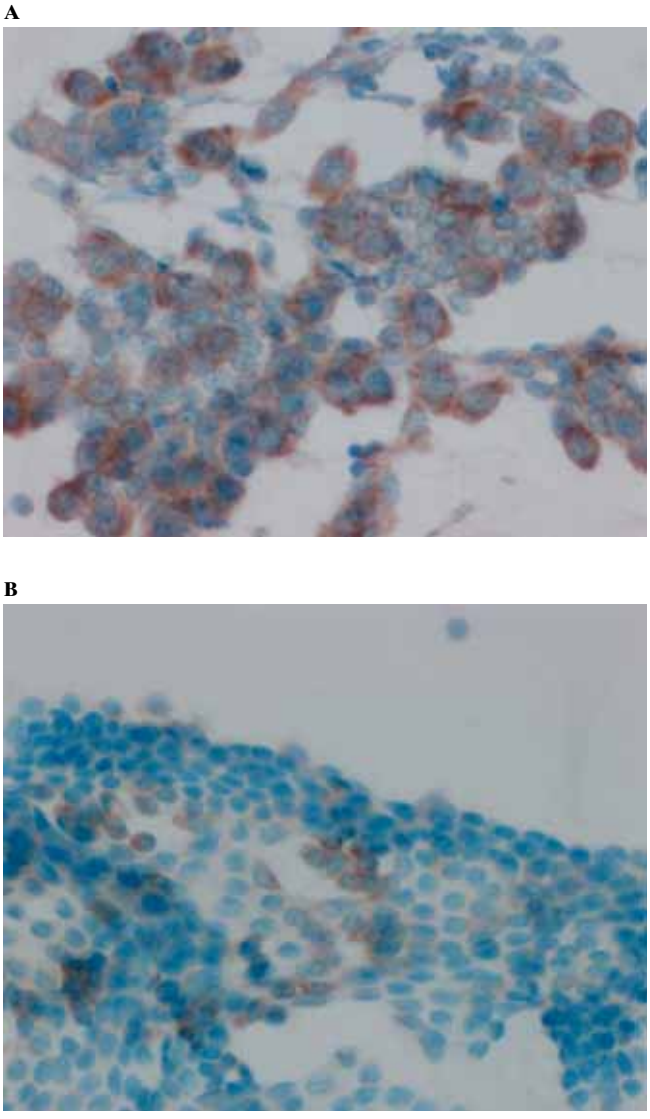

Figure 4. Immunocytochemically detection of bcl-2 oncoprotein (bcl-2 oncoprotein: monoclonal mouse antibody, clone 124, chromosomal translocation t(14, 18) Isotype IgG1 kappa DAKO) in breast FNA preparations from: A. benign disease – strong positive staining, B. benign disease – positive staining.

growth factor-beta; heat shock; calcium ionophores and chemicals that induce oxidative injury. The ability to prevent or delay cell death triggered by multiple mechanisms has suggested that the Bcl-2 protein controls a late event in final common pathway involved in programmed and apoptotic cell death [10]. Expression of bcl-2, not well expressed in preinvasive breast lesions, was found in most of normal ductal epithelial cells, ductal hyperplasia, atypical ductal hyperplasia (ADH), and atypical lobular hyperplasia/lobular carcinoma *in situ* (LCIS). Bcl-2 immunopositive cells were also detected in more than 70% of intraductal carcinomas *in situ* (DCIS), and was found to be expressed in 45% of invasive carcinomas. Higher bcl-2 expression was observed even in normal epithelial cells when compared to intraductal and invasive cancerous cells. Furthermore, bcl-2 positivity in intraductal lesions was significantly higher than in invasive cancerous lesions [22, 24]. In our work bcl-2 immunopositive cells were detected in 5 out of 22 (22.7%) benign lesions, 2 of 5 (40%) benign C2 evaluated solid tumors and in proliferative fibroadenoma aspirates. Percentage of immunopositive cells in benign aspirates, when excluding proliferative fibroadenoma, were not higher than 50%. Surprisingly, in 1 out of 3 studied cancer cases nearly 100% of cells were stained for bcl-2. ZHANG and SIZIOPIKOU with coworkers have not observed p53 nuclear accumulation in normal breast epithelial cells, ductal hyper-

plasia, ADH, or LCIS lesions. More than 20% of intraductal cancerous lesions and 30% of invasive cancerous lesions were positive for p53 expression. An inverse relationship was shown between bcl-2 and p53 expression in invasive carcinomas [22, 24]. In agreement with these studies we did not observe p53 positive cells in 20 of 22 benign lesions preparations (90.9%) and in all of fibroadenomas. In 1 out of 3 cancer cases there were relatively high (10%> >50%) level of p53 immunopositive cells. Study of ROHAN and coworkers revealed an association of p53 accumulation with increased risk of progression of benign breast disease to breast cancer (adjusted odds ratio [OR]=2.55; 95% confidence interval [CI]=1.01–6.40) and hence a close follow up and earlier intervention may be recommended in women with benign breast disease and p53 accumulation [19].

In normal human breast the highest percentage of Ki-67 positive cells was found in mammary lobules type-1 (Lob1.), with the progressive reduction in the more differentiated Lob2. and Lob3. Breast ER-alpha (estrogen receptor) and PgR (progesterone receptor) positive epithelial cells were negative for Ki-67, while cells positive for Ki-67 did not express steroid receptors [20]. MIDULLA and coworkers, in retrospective studies concerning immunocytochemical expression of cellular markers such as Ki-67 and Bcl-2 in FNAC from breast proliferative lesions, have postulated a correlation between Ki-67 expression and malignancy. Additionally, according to these authors, immunocytochemical Ki-67 expression may be helpful in the differentiation of cytologically suspicious/indeterminate breast lumps [13, 14]. In our study, the majority of benign FNAC preparations were PCNA and Ki-67 negative, 86% and 95%, respectively. Only in proliferative fibroadenoma (after histological verification) percentage of immunopositive cells for both proliferative antigens ranged between 10% and 50%. As expected, in all carcinoma FNAC preparations high level of both PCNA and Ki-67 positive cells were observed.

Immunocytochemical detection of Ki67, MPM2, Bcl-2, P53 might be a promising diagnostic method in the classification of benign breast lesions. FNAC complemented by using immunocytochemical staining increases the reliability of diagnosis. It could be a helpful procedure of establishing more exactly the biology of these lesions and essential factor in clinical follow-up. Nevertheless, further study, on the larger group of patients, comparing immunocytological parameters of breast FNAs with postoperative immunohistological verification is required to estimate its predictive value.

References

[1] AL-KAISI N. The spectrum of "gray zone" in breast cytology. A review of 186 cases of atypical and suspicious cytology. Acta Cytol 1994; 38: 898–908.

[2] EISSA S, LABIB R, KHALIFA A, SWELAM N, KHALIL F et al. Regulators of apoptosis in human breast cancer. Clin Biochem 1999; 32(5): 321–326.

[3] GROVES AM, CARR M, WADHERA V, LENNARD TWJ. An aduit of cytology in the evaluation of nipple discharge. A retrospective study of 10 years' experience. The Breast 1996: 5: 96–99.

[4] HAJDU SI, GASTON JP. Aspiration cytology of breast. Clin Lab Med 1991; 11(2): 357–368.

[5] IOACHIM EE, MALAMOU-MITSI V, KAMINA SA, GOUSSIA AC, AGNANTIS NJ. Immunohistochemical expression of Bcl-2 protein in breast lesions: correlation with Bax, p53, Rb, C-erbB-2, EGFR and proliferation indices. Anticancer Res 2000; 20(6B): 4221–4225.

[6] JANSEN RL, JOOSTEN-ACHJANIE SR, VOLOVICS A, ARENDS JW, HUPPERETS PS et al. Relevance of the expression of bcl-2 in combination with p53 as a prognostic factor in breast cancer. Anticancer Res 1998; 18(6A): 4455–4462.

[7] KANDOUZ M, SIROMACHKOVA M, JACOB D, CHRETIEN MARQUET B, THERWATH A et al. Antagonism between estradiol and progestin on Bcl-2 expression in breast-cancer cells. Int J Cancer 1996; 68: 120–125.

[8] KASTAN MB, ONYEKWERE O, SIDRANSKY D, VOGELSTEIN B, CRAIG RW. Participation of p53 protein in the cellular response to DNA damage. Cancer Res 1991; 51: 6304–6311.

[9] KĘDZIA W, GOŹDZICKA-JÓZEFIAK A, KĘDZIA H, SPACZYŃSKI M. Immunohistochemical staining of proliferative activity of c-erb-B2, EGF-R as well as p53 in vaginal praecancer and cancer cells from HPV-16 positive and negative patients. Gin Pol 2000; 71: 63–69 (In Polish).

[10] KRAJEWSKI S, KRAJEWSKA M, TURNER BC, PRATT C, HOWARD B et al. Prognostic significance of apoptosis regulators in breast cancer. Endocr Relat Cancer 1999; 6(1): 29–40.

[11] MAKRIS A, POWLES TJ, ALLRED DC, TROTT PA, ASHLEY SE et al. Quantitative changes in cytological molecular markers during primary medical treatment of breast cancer: a pilot study. Breast Cancer Res Treat 1999; 53: 51–59.

[12] MAKRIS A, POWLES TJ, ALLRED DC, TROTT PA, ASHLEY SE et al. Changes I hormone receptors and proliferation markers in tamoxifen treated breast cancer patients and relationship with response. Breast Cancer Res Treat 1998; 48: 11–20.

[13] MIDULLA C, DE IORIO P, NAGAR C, PISANI T, CENCI M et al. Immunohistochemical expression of p53, nm23-HI, Ki67 and DNA ploidy: correlation with lymph node status and other clinical pathologic parameters in breast cancer. Anticancer Res 1999; 19(5B): 4033–4037.

[14] MIDULLA C, PISANI T, DE IORIO P, CENCI M, DIVIZIA E et al. Cytological analysis and immunocytochemical expression of Ki67 and Bcl-2 in breast proliferative lesions. Anticancer Res 2002; 22(2B): 1341–1345.

[15] MUCCI B, PALMER M, ATHEY G, SCARISBRICK G, SMITH D et al. Finee-nedle cytology of breast specimens: a training aid. The Breast 1996; 5: 251–253.

[16] O'GRADY LF. Nonmalignant breast disease. In: O'Grady LF, Lindfors KK, Howell LP, Rippon MB, editors. A practical Approach to breast disease. Little, Brown and Company, Boston New York Toronto London, 1995: 9–22.

[17] OZKARA SK, USTUN MO, PAKSOY N. The gray zone in breast fine needle aspiration cytology. How to report on I? Acta

Cytol 2002; 46: 513–518.

[18] ROBINSON IA, MCKEE G. Cytological performance in palpable versus stereotactically sampled breast lesions. The Breast 1996; 5: 415–417.

[19] ROHAN TE, HARTWICK W, MILLER AB. Immunohistochemical detection of c-erB-2 and p53 in benign breast disease and breast cancer risk. J Natl Cancer Inst 1998; 90: 1262–1269.

[20] RUSSO J, AO X, GRILL C, RUSSO IH. Pattern of distribution of cells positive for estrogen receptor alpha and progesterone receptor in relation to proliferating cells in the mammary gland. Breast Cancer Res Treat 1999; 53(3): 217–227.

[21] SCHONBORN I, MINGUILLON C, MOHNER M, EBELING K. PCNA as a potential prognostic marker in breast cancer. The Breast 1994; 3: 97–102.

[22] SIZIOPIKOU KP, PRIOLEAU JE, HARRIS JR, SCHNITT SJ. bcl-2 expression in the spectrum of preinvasive breast lesions. Cancer 1996; 77(3): 499–506.

[23] ŚWIĄTECKA J, DZIĘCIOŁ J, ANCHIM T, DĄBROWSKA M, PIETRUCZUK M et al. Influence of estrogen, antiestrogen and UV-light on the balance between proliferation and apoptosis in MCF7 breast adenocarcinoma cells. Neoplasma 2000; 47: 15–24.

[24] ZHANG GJ, KIMIJIMA I, ABE R, KANNO M, KATAGATA N et al. Correlation between the expression of apoptosis-related bcl-2 and p53 oncoproteins and the carcinogenesis and progression of breast carcinomas. Clin Cancer Res 1997; 3: 2329–2335.

[25] ZHANG GJ, KIMIJIMA I, TSUCHIYA A, ABE R. The role of bcl-2 expression in breast carcinomas (Review). Oncol Rep 1998; 5(5): 1211–1216.

[26] ZHANG GJ, KIMIJIMA I, WATANABE T, KANNO M, SAGARA H et al. Correlation between apoptotic index, bcl-2 protein expression and progression and prognosis in breast carcinoma. Gan To Kagaku Ryoho 1998; 25 Suppl 3: 415–421.