PML protein expression in hereditary and sporadic breast cancer

P. PLEVOVÁ[1,2]*, J. BOUCHAL[1], M. FIURÁŠKOVÁ[1], L. FORETOVÁ[3], M. NAVRÁTILOVÁ[3], J. ZAPLETALOVÁ[4], R. ČUŘÍK[5], O. KUBALA[6], J. PROKOP[6], Z. KOLÁŘ[1]

[1]Institute of Pathology & Laboratory of Molecular Pathology, Medical Faculty, Palacký University, Olomouc, Czech Republic, e-mail: pavlina.plevova@volny.cz; [2]Department of Medical Genetics, University Hospital, Ostrava, Czech Republic; [3]Department of Cancer Genetics, Masaryk Memorial Cancer Institute, Brno, Czech Republic; [4]Institute of Biophysics, Medical Faculty, Palacký University, Olomouc, Czech Republic; [5]Department of Pathology, University Hospital, Ostrava, Czech Republic; [6]Department of Surgery, University Hospital, Ostrava, Czech Republic

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The PML protein is concentrated in the PML nuclear bodies. Downregulation of the PML protein has been described in various types of cancer and is in accordance with the fact that dysqualification of tumor suppressive functions of the PML protein might promote cancer development. Various differences have been described between sporadic breast cancer and that associated with *BRCA1* and *BRCA2* gene mutations. Expression of the PML protein has not been studied yet. The aim of this study was to determine if there is any difference in PML protein expression in breast cancer of *BRCA1* and *BRCA2* gene mutation carriers compared to sporadic breast cancer and if the PML protein can be used as a prognostic marker. There were 47 breast cancer samples included, 14 and 10 from *BRCA1* and *BRCA2* germline mutation carriers, respectively, and 23 from patients without a *BRCA1/BRCA2* germline mutation. Immunofluorescence staining was used. Downregulation of PML protein expression was found in 2 of 14 (14%), 3 of 10 (30%) and 15 of 47 (31%) cases of breast cancer samples from *BRCA1*, *BRCA2* and no *BRCA1/BRCA2* mutation carriers, respectively ($p_{BRCA1} = 0.019$; $p_{BRCA2} = 0.111$). There was no correlation between PML protein expression and age, histological types, estrogen and progesteron receptor, c-erbB-2 and PCNA expression, TNM classification, disease-free and overall survival. In conclusion, the PML protein is downregulated in approximately 30% of breast cancers cases. Downregulation of PML protein expression was significantly less frequent in *BRCA1* mutation carriers compared to sporadic cases. No correlation was found between PML protein expression and any of the other clinical and laboratory characteristics.

Key words: PML protein; *BRCA1* gene; *BRCA2* gene; breast cancer.

The *PML (*promyelocytic leukemia) protein has a role in the formation and stability of the PML bodies and controls tumor suppressive funtions such as induction of apoptosis, growth arrest and cellular senescence [1, 2]. The PML bodies represent nuclear deposits of various proteins and more than 50 proteins are considered to be bound there either transiently or constitutively [3, 4, 5].

About 5% of breast cancer cases are associated with the hereditary breast and ovary cancer syndrome, the great majority of them occurring in *BRCA1* and *BRCA2* germline mutations carriers [6]. Various differences have been described between breast cancer associated with *BRCA1* and *BRCA2* gene mutations and sporadic breast cancer [7, 8]. Expression of the PML protein was not studied yet. The aim of this study was to determine if there is any difference in PML protein expression in breast cancer from *BRCA1* and *BRCA2* gene mutation carriers compared to sporadic breast cancer and if PML protein expression has any prognostic value.

Materials and methods

Female breast cancer patients were included into the study. Formalin fixed paraffin-embedded tumor samples were studied. There were 47 breast cancer samples included, 14 from *BRCA1* germline mutation carriers, 10 from *BRCA2* germline mutation carriers and 23 from patients without a *BRCA1/*

*Corresponding author

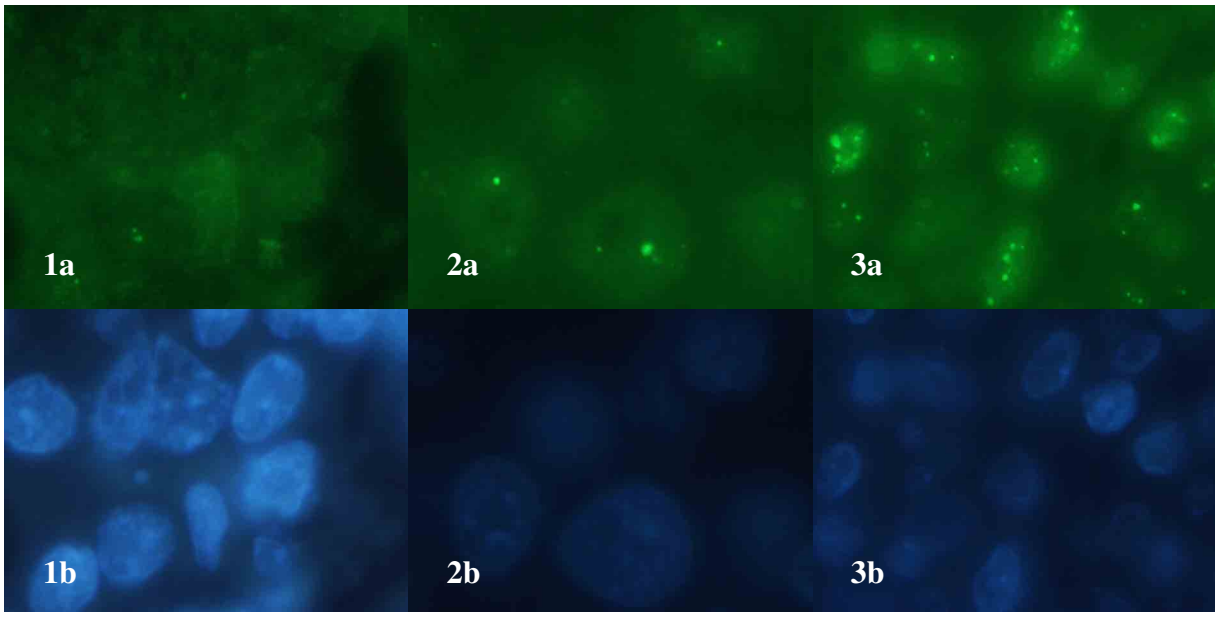

Figure No. 1. PML protein expression in breast cancer samples (1-3a, PML protein expression: 1a, level "1" positivity; 2a, level "2" positivity; 3a, level "3" positivity; 1-3b, dapi; 1000x magnification).

BRCA2 germline mutation. Informed consent was obtained from the patients.

Immunofluorescence staining with anti-PML antibodies was performed. Formalin-fixed paraffin-embedded 4-µm thick tumor tissue sections were deparaffinized and heat-induced antigen retrieval was performed before staining by treatment in a microwave oven (30 minutes at 700 W, in 10 mM citrate buffer, pH 6,0). Unspecific binding sites were blocked by a solution of fat-free dried milk (2,5 mg of dried milk/50 ml PBS). Samples were incubated with rabbit polyclonal antibody against PML (clone H-238, Santa Cruz Biotechnology, USA, dilution 1:200) for 2 hours at room temperature. The samples were washed 3 times in PBS and secondary fluorescent anti-rabbit antibody Alexa-Fluor®488 (Molecular Probes, Oregon, USA) was applied for 1 hour at room temperature. The samples were washed in PBS again and DAPI (Sigma, St. Louis, USA) was applied for 10 minutes. After 2 washes in PBS and one in deionized water the samples were mounted in water medium. The slides were visualized using an Olympus U-RFL-T fluorescent lamp and an Olympus BX50 microscope at 1000x magnification. Images were captured with a Viewfinder imaging system. Captured images were processed using the Adobe Photoshop program.

As regards the anti-PML antibody, we first compared the sensitivity of the H-238 antibody with the well characterized PG-M3 antibody (Santa Cruz Biotechnology, USA). We obtained similar results.

The number and size of the PML bodies was assessed in the nuclei of the cells. Nuclear staining was graded semiquantitatively. Complete absence of the PML bodies was scored "0"; the presence of minute bodies in less than 50% of cells "1"; the presence of the PML bodies of any size in 50-90% of cells or 1-2 minute bodies in 100% of cells "2"; 1-2 large bodies or 3-10 or more minute ones in 100% of cells "3" (Figure 1). The levels "0" and "1" were considered negative, the levels "2" and "3" positive.

Expression of the PML protein was correlated with *BRCA1* or *BRCA2* gene mutation or no *BRCA1/BRCA2* mutation status, with the age of the patients, histology, estrogen and progesteron receptor, c-erbB-2 and PCNA (proliferating cell nuclear antigen) expression, TNM (Tumor, Nodi, Metastasis) classification and disease-free and overall survival.

Estrogen and progesteron receptor, c-erbB-2, and PCNA expression were detected by indirect immunohistochemistry. Before immunostaining, heat-induced antigen retrieval was performed by treatment in a microwave oven. Mouse monoclonal antibodies against estrogen receptor α (clone 1D5, DakoCytomation, Denmark), progesteron receptor (DakoCytomation, Denmark), and PCNA (clone PC10, DakoCytomation, Denmark) and amplification system EnVision™ (DakoCytomation, Denmark) were used. The activity of peroxidase was visualised by DAB. The Herceptest kit (DakoCytomation, Denmark) was used to detect c-erbB-2 expression.

Nuclear expression of estrogen and progesteron receptors and PCNA was assessed semiquantitatively. Samples with less than 10% of positive cells were considered negative (0), those with 10-25% of positive cells – weakly positive (1), those with 25-75% of positive cells – moderately posi-

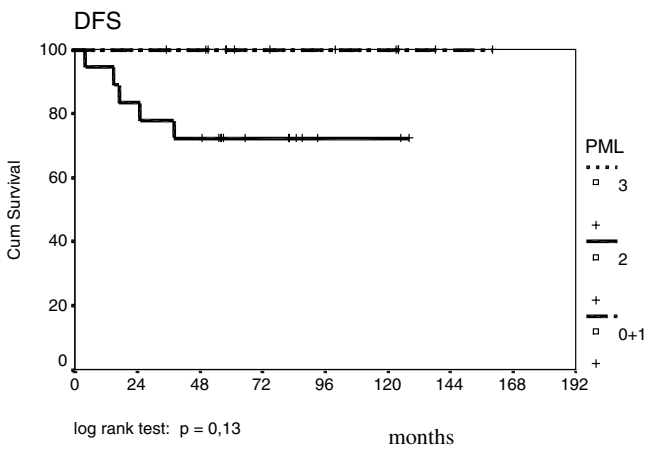

Figure No 2. Disease-free survival of the patients with respect to PML protein expression; Kaplan-Meier analysis (log rank test: p=0.13).

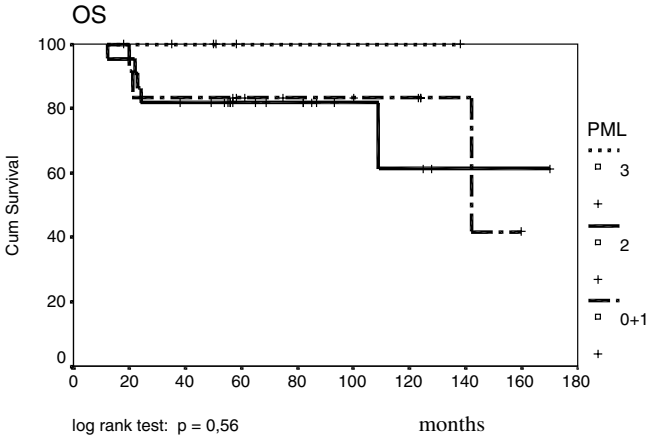

Figure No 3. Overal survival of the patients with respect to PML protein expression; Kaplan-Meier analysis (log rank test: p=0.56).

tive (2) and samples with 75-100% of positive cells – strongly positive (3).

Expression of c-erbB-2 protein was assessed in accordance with the manufacturer's recommendations. Score "0" were cases without membrane positivity or with positivity in less than 10% of cancer cells, score "1+", weak to strong incomplete membrane positivity in more than 10% of cancer cells, score "2+", weak or moderate complete membrane positivity in more than 10% of cancer cells, score "3+", strong complete membrane positivity in more than 10% of cancer cells. The scores "0" and "1+" were considered negative, the scores "2+" and "3+" positive.

Histological typing: Following five categories were classified: ductal carcinoma grade 1 (low grade), 2 (medium grade) and 3 (high grade), lobular carcinoma and mucinous carcinoma.

The *TNM* stage was classified according to the 6th edition of "TNM classification of malignant tumours" [9].

Statistical analysis: Correlation of PML expression with age of the patients was tested using the ANOVA test, with disease-free and overall survival using Kaplan-Meyer analysis and log rank test and with all the other characteristics using the chi-square test.

Results

Expression of the PML protein in breast cancer samples with respect to the *BRCA1/BRCA2* gene mutation status is summarized in Table 1. Tumors from *BRCA1* and *BRCA2* germline mutation carriers expressed the PML protein more often at level "2" compared to tumors from patients without a germline *BRCA1/BRCA2* mutation. The difference reached statistical significance only in the group of tumors from *BRCA1* germline mutation carriers compared to tumors from patients without *BRCA* mutation (p_{BRCA1} = 0.019; p_{BRCA2} = 0.111). There was no statistically significant correlation between the level of PML expression and age of the patients (ANOVA, p=0.59), histological typing (chi-square test,

Table 1. PML protein expression in breast cancer with respect to *BRCA1* and *BRCA2* germline mutation carriership (chi-square test: "*BRCA1* mutation" versus "no mutation", p=0,019; "*BRCA2* mutation" versus "no mutation", p=0,111; "*BRCA1* mutation" versus "*BRCA2* mutation", p=0,346).

Group		PML expression				
		0	1	2	3	Total
BRCA1 mutation	No	1	1	11	1	14
	%	7.10%	7.10%	78.60%	7.10%	100%
BRCA2 mutation	No		3	7		10
	%		30.00%	70.00%		100%
No mutation	No		10	8	5	23
	%		43.50%	34.80%	21.70%	100%
Total	No	1	14	26	6	47
	%	2.10%	29.80%	55.30%	12.80%	100%

Table 2. Correlation of PML protein expression with age of the patients (For 2 patients, the data were not available; ANOVA, p=0.59).

PML expression	No	Age				
		Minimum	Maximum	Median	Mean	Standard deviation
0+1	14	31	56	47	44.6	8.53
2	25	22	74	45	46.5	11.61
3	6	28	59	42	41.7	11.94
Total	45	22	74	45	45.3	10.68

Table 3. Correlation of PML protein expression with histological findings (chi-square test, p=0.73).

PML expression		Histology – carcinoma					
		ductal grade 1	ductal grade 2	ductal grade 3	lobular	mucinous	Total
0+1	No		7	6	2		15
	%		46.7%	40.0%	13.3%		100%
2	No	1	8	15	1	1	26
	%	3.8%	30.8%	57.7%	3.8%	3.8%	100%
3	No		1	4	1		6
	%		16.7%	66.7%	16.7%		100%
Total	No	1	16	25	4	1	47
	%	2.1%	34.0%	53.2%	8.5%	2.1%	100%

Table No 4. Correlation of PML protein expression with estrogen receptor expression (2 samples could not be scored; chi-square test, p=0.19).

PML expression		Estrogen receptor				
		0	1	2	3	Total
0+1	No	5	2	4	4	15
	%	33.3%	13.3%	26.7%	26.7%	100%
2	No	12	5	4	4	25
	%	48.0%	20.0%	16.0%	16.0%	100%
3	No	4		1		5
	%	80.0%		20.0%		100%
Total	No	21	7	9	8	45
	%	46.7%	15.6%	20.0%	17.8%	100%

Table No 5. Correlation of PML protein expression with progesteron receptor expression (4 samples could not be scored; chi-square test, p=0.09).

PML expression		Progesteron receptor				
		0	1	2	3	Total
0+1	No	2	2	4	5	13
	%	15.4%	15.4%	30.8%	38.5%	100%
2	No	12	2	7	4	25
	%	48.0%	8.0%	28.0%	16.0%	100%
3	No	3	2			5
	%	60.0%	40.0%			100%
Total	No	17	6	11	9	43
	%	39.5%	14.0%	25.6%	20.9%	100%

p=0.73), TNM classification (chi-square test, p=0.76, 0.52 and 0.71, respectively), PCNA expression (chi-square test, p=0.63), disease-free survival (log rank test, p=0.13) and overall survival (log rank test, p=0.56). Cases with negative PML expression (i.e. expression at the "0" or "1" levels) expressed more often estrogen and/or progesteron receptor and c-erbB-2 than PML positive ("2" and "3" level) cases. However, the difference did not reach statistical significance (chi-square test; estrogen receptor, p=0.19; progesteron receptor, p=0.09; c-erbB-2, p=0.076).

Discussion

Severe downregulation of PML expression was detected in 31% of breast cancer samples in our study. PML downregulation might represent a selective advantage for tumor cells. It is in accordance with the needs of tumor cells to escape physiological processess of apoptosis, growth arrest and cellular senescence.

Gurrieri and co-workers found partial or complete loss of PML protein expression in 8 (21%) and 12 (31%) of 38 breast carcinomas, respectively [10]. They used immunohistochemistry to detect the protein and complete loss was defined as undetectable levels of PML and partial loss being defined by two or fewer PML nuclear bodies per cell. The percentage of tumors with complete loss was relatively high in this study compared to our results. We have detected complete loss of PML expression in only 1 tumor from a patient with a germline *BRCA1* gene mutation. The tumors classed as level "1" expression were characterized by one or two weak signals in less than 50% of the cells in our study. The likely explanation for this discrepancy might be the used method with respect to the higher sensitivity of immunofluorescent staining used in our study. The other explanation might be false positivity caused by unspecific binding of the antibody. In any case, the level "1" expression was characterized as severe downregulation of PML expression in our study and was considered negative.

Tumors from patients with germline *BRCA1* and *BRCA2* mutations expressed the PML protein most often at the "2" level. This association was statistically significant in *BRCA1* mutation carriers. This fact suggests different mechanisms of tumor development in *BRCA* germline mutation carriers. This finding can be added to the list of known differences between *BRCA1*-associated tumors compared to sporadic ones, including a high proportion of medulary and ductal

Table No 6. Correlation of PML protein expression with c-erbB-2 expression (chi-square test: p=0.331; correlation of PML with c-erB-2 level 0+1 (c-erbB-2 negative) versus 2+3 (c-erbB-2 positive), p=0.076).

PML expression		c-erbB-2				
		0	1	2	3	Total
0+1	No	4	3	7	1	15
	%	26.7%	20.0%	46.7%	6.7%	100%
2	No	9	7	8	2	26
	%	34.6%	26.9%	30.8%	7.7%	100%
3	No	2	4			6
	%	33.3%	66.7%			100%
Total	No	15	14	15	3	47
	%	31.9%	29.8%	31.9%	6.4%	100%

Table No 7. Correlation of PML protein expression with PCNA expression (4 samples could not be scored; chi-square test, p=0.63).

PML expression		PCNA			
		1	2	3	Total
0+1	No	1	6	5	12
	%	8.3%	50.0%	41.7%	100%
2	No	4	11	9	24
	%	16.7%	45.8%	37.5%	100%
3	No		4	1	5
	%		80.0%	20.0	100%
Total	No	5	21	15	41
	%	12.2%	51.2%	36.6%	100%

Table No 8 a-c. Correlation of PML protein expression with TNM classification (the data were not available for all the patients; chi-square test, p=0.76, 0.52 and 0.71 for T, N, M, respectively).

a)

PML expression		T (Tumor)				
		1	2	3	4	Total
0+1	No	3	7	1	1	12
	%	25.0%	58.3%	8.3%	8.3%	100%
2	No	5	8	4	3	20
	%	25.0%	40.0%	20.0%	15.0%	100%
3	No	2	2	2		6
	%	33.3%	33.3%	33.3%		100%
Total	No	10	17	7	4	38
	%	26.3%	44.7%	18.4%	10.5%	100%

b)

PML expression		N (Nodi)			
		0	1	2	Total
0+1	No	8	3	1	12
	%	66.7%	25.0%	8.3%	100%
2	No	12	7	1	20
	%	60.0%	35.0%	5.0%	100%
3	No	2	4		6
	%	33.3%	66.7%		100%
Total	No	22	14	2	38
	%	57.9%	36.8%	5.3%	100%

c)

PML expression		M (Metastasis)		
		0	1	Total
0+1	No	9	1	10
	%	90.0%	10.0%	100%
2	No	17	2	19
	%	89.5%	10.5%	100%
3	No	6		6
	%	100%		100%
Total	No	32	3	35
	%	91,4%	8,6%	100%

grade 3 carcinomas, frequent loss of estrogen and progesteron receptor and c-erbB-2 expression, a high frequency of p53 somatic mutations, DNA non-diploidy with a high S-phase fraction, increase in MIB-1 staining grades and a lower frequency of cyclin D1 overexpression [7, 8, 11–13].

A statistically significant association between complete loss of PML expression and progression to lymph nodes was found by Gurrieri et al. [10]. In our study, no correlation between PML expression and any of the other studied clinical parameters was found.

Loss of the PML protein is a frequent event in human cancers of various histologic origins. Besides breast cancer, PML protein expression was found to be lost in certain tumor types such as lung, prostate, CNS, germ cell and thyroid tumors, non-Hodgkin's lymphomas and nasopharyngeal carcinoma [10, 14–19].

The PML protein is expressed in normal tissues, with the highest levels of the protein being found in postmitotic, differentiated cell types, such as endothelial cells, epithelia, and tissue macrophages, especially activated ones [20].

In conclusion, we found downregulation of PML protein expression in approximately 30% of breast cancer samples. Tumors from germline *BRCA1* mutation carriers expressed the PML protein statistically significantly more often at the moderate level compared to tumors from patients without germline *BRCA1/BRCA2* mutations where downregulation of PML expression was seen more often. There was no other

correlation between PML expression and any of the laboratory and clinical characteristics.

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