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Augmented expression of Polo-like kinase 1 is a strong predictor of shorter cancer-specific overall survival in early stage breast cancer at 15-year follow-up

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Abstract. Polo-like kinase 1 (PLK1) is a serine-threonine kinase that plays a crucial role in the regulation of cell division. In addition, it acts as a modulator of the DNA damage response and as a novel factor in the maintenance of genome stability during DNA replication. The present study aimed to reveal the associations between PLK1 expression and clinicopathological features of patients with breast cancer (BC), particularly patient survival at 5-, 10- and 15-year follow-up. PLK1 expression was evaluated immunohistochemically in routine diagnostic tissue specimens from 83 patients treated radically for stage II BC. Kaplan-Meier analysis revealed a correlation between PLK1 overexpression and long-term survival. High PLK1 immunoreactivity was associated with shorter cancer-specific overall survival (CSOS) and disease-free survival (P=0.00001 and 0.00013, respectively). Multivariate analysis confirmed the negative prognostic significance of PLK1 overexpression for CSOS in all 83 patients (P=0.00030). Furthermore, analogous correlations were observed in both subgroups with and without nodal metastases (P=0.01400 and 0.01200, respectively). The present results indicate that PLK1 expression has a prognostic role in early BC. Immunohistochemical assessment of PLK1 reactivity may potentially become a qualifier for inclusion of PLK1 inhibitor therapy.

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Introduction

Breast cancer (BC) is the second most common malignancy in the world, with estimated 1.67 million newly diagnosed cases and 522,000 associated mortalities worldwide in 2012 (http://globocan.iarc.fr; accessed May 1, 2015). Despite recent efforts to improve the detection rates and treatment of BC, the current situation, as reflected by disease statistics, is not favorable. A better understanding of the tumor's pathobiology will undoubtedly bring novel possibilities for treatment and diagnosis. In an attempt to offer the best medical approach possible for every single patient, modern medicine is evolving towards personalized therapies, which are characterized by a proper balance between the most radical approach possible while avoiding undesired side effects resulting from aggressive treatment. This attitude requires novel ideas regarding drug development and accurate stratification of the patients; therefore, novel prognostic factors are necessary.

Dysregulated cellular division is a key event in cancer initiation and progression. Polo-like kinases (PLKs) are a family of proteins that regulate the cell cycle (1). There are four PLKs with serine-threonine kinase activity in humans (PLK1-PLK4), whereas in PLK5 [which was first described in mice (2)] the kinase domain is truncated and does not possess any catalytic activity (3). However, PLK5 appears to participate in neuronal differentiation and act as a tumor suppressor in brain cancer (3). In addition, PLK1-PLK4, and particularly PLK2, display other roles beyond mitosis regulation (4,5).

PLK1, the best characterized protein of the PLK family, is a serine-threonine kinase that plays a crucial role in the regulation of cell division, centrosome maturation and duplication, assembly of the bipolar spindle, sister chromatid splitting, activation of the anaphase-promoting complex (APC), regulation of mitotic exit and induction of cytokinesis (1,6-14). PLK1 protein comprises two main domains: i) A conserved serine-threonine kinase domain at the N-terminus that is crucial for its kinase activity, and ii) a polo-box domain, a non-catalytic domain that is critical for its spatial distribution in the cells and its molecular interactions with specific substrates (12,13). PLK1

directly phosphorylates cell division cycle 27 protein (a component of the APC) and cyclin B1 (15,16), and together with other important signaling proteins such as p34 kinase, is responsible for mitotic progression (12).

PLK1 acts as a modulator of the DNA damage response and as a novel factor in the maintenance of genome stability during DNA replication (13). In response to DNA damage, the checkpoint kinases ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related protein (ATR) become activated and inhibit entry into mitosis via deactivation of cyclin-dependent kinase 1 (CDK1), which is the crucial kinase that promotes cell division (17). Effective resumption of cell cycle progression (arrested at G2/M) and mitotic entry upon successful repair of DNA damage are based on the activation of PLK1 by Aurora A/Bora-mediated phosphorylation (18,19). Additionally, activated PLK1 is involved in the enzymatic inactivation of WEE1 (a protein kinase that inhibits CDK1) (20) and elimination of claspin, which functions as a key adaptor protein for checkpoint kinase 1 activity (21). Cytophysiological downregulation of WEE1 and claspin by enhanced activity of PLK1 promotes CDK1 activation and leads to mitotic entry (20,21).

High PLK1 expression is observed within intensively proliferating normal tissues such as placenta and colonic epithelium (22), and in various types of cancer, including gastric (23), colorectal (24), hepatocellular (25), prostate (26), breast (27,28), ovarian (29) and non-small cell lung carcinomas (30). Notably, due to its position as a central controller of mitosis, PLK1 has become a potentially valuable target for antiproliferative therapies (31). Emerging experimental results are encouraging, and several anti-PLK1 agents are currently being investigated in clinical trials (32). No predictive factor has been identified thus far that could be used as a reliable qualifier to the potential inclusion of PLK1 inhibitor therapy in BC treatment. Similarly to the case of human epidermal growth factor receptor-2 (HER-2), immunohistochemical analysis of PLK1 expression in BC cells and the subsequent decision of initiating therapy or excluding the patient from therapy may be an alternative. This hypothesis requires verification in extensive, multicentre research studies.

Although great progress has been achieved since the initial characterization of human PLK1 >20 years ago (22,33), there is disagreement among researchers regarding the precise role of this kinase in cancer pathogenesis, and the prognostic significance of its expression in breast tumors has not been clearly established to date.

The present study reports an association between PLK1 expression and patient survival in 5-, 10- and 15-year follow-ups. In addition, an analysis of the correlations between PLK1 expression and other clinicopathological and histopathological features is provided.

Materials and methods

Patients. Tissue samples were acquired from 83 radically treated patients with stage II ductal BC diagnosed between 1993 and 1994 in the Lower Silesian Oncology Centre (Wroclaw, Poland). The patients' mean age was 55.2 years. The study population was selected based on the availability of tissues. All patients underwent surgery (Madden mastectomy) with or without adjuvant treatment [27% of patients were treated by chemotherapy based on the CMF scheme (100 mg/m^2 cyclophosphamide per day, days 1-14; 40 mg/m^2 intravenous methotrexate, days 1 and 8; 500 mg/m^2 intravenous fluorouracil, days 1 and 8; for 6 cyles of 28 days), which is no longer in use]. Following treatment, the patients were under continuous monitoring in the Lower Silesian Oncology Centre. Data regarding relapse and mortality were collected using medical documentation available in the Lower Silesian Oncology Centre. Overall survival (OS), cancer-specific overall survival (CSOS) and disease-free survival (DFS) rates were established for all patients. Table I contains detailed characteristics of the cohort. The present study was approved by the Institutional Review Board of Wroclaw Medical University.

Tumor samples. Tumor specimens were fixed in 10% buffered formalin and embedded in paraffin. All hematoxylin and eosin stained sections were evaluated by two pathologists (P.D. and A.H., Department of Pathomorphology and Oncological Cytology, Wroclaw Medical University, Wroclaw). Tumor stages were assessed according to the tumor-node-metastasis classification system (34). Tumor grades were estimated according to the Scarff-Bloom-Richardson protocol (35), with the Elston-Ellis (36) modification (Table I).

Immunohistochemistry. Immunohistochemical analyses were performed retrospectively on tissue samples collected for routine diagnostic purposes. Formalin-fixed, paraffin-embedded tissue sections were freshly prepared (4 μm thickness; Accu-Cut SRMTM; Sakura, Alphen aan den Rijn, The Netherlands). Immunohistochemistry was performed as previously described (37). For the detection of PLK1, a monoclonal mouse antibody against PLK1 (BD Transduction Laboratories™; BD Biosciences, Franklin Lakes, NJ, USA) was diluted 1:500 in the Antibody Diluent with Background Reducing Components (DakoCytomation; Dako, Glostrup, Denmark). For detection of estrogen receptor (ER), an optimally pre-diluted monoclonal mouse antibody was used (clone 1D5; DakoCytomation; Dako), while for detection of progesterone receptor (PgR), an optimally pre-diluted monoclonal antibody (clone PgR636; DakoCytomation; Dako) was used. For HER-2 detection, a semi-quantitative diagnostic immunohistochemical test was used (HercepTest™ Kit; K5207; DakoCytomation; Dako). Tissue sections were incubated with the above antibodies for 1 h at room temperature. Subsequent incubations involved biotinylated antibodies (15 min, room temperature) and a streptavidin-biotinylated peroxidase complex (15 min, room temperature) (LSAB 2 System-HRP; DakoCytomation; Dako). As a chromogen, 3,3'-diaminobenzidine (DakoCytomation; Dako) was used (10 min, room temperature). All sections were counterstained with Mayer's hematoxylin. In each case, control reactions were included, in which the specific antibody was substituted by a primary mouse antibody (DakoCytomation; Dako), which served as a negative control.

Evaluation of immunohistochemical reaction intensity. The intensity of the immunohistochemical reaction was assessed independently by two pathologists. In doubtful cases, a re-evaluation was performed using a double-headed microscope, (BX45; Olympus, Tokyo, Japan) and staining was discussed until a consensus was achieved.

Table I. Patient and tumor characteristics, and their association with PLK1 immunoreactivity in breast cancer patients.

Patient characteristics	No. (%)	Parameters of PLK1 immunoreactivity			High expression of PLK1 (IRS ≥8)
		%	Intensity	IRS	
All patients	83 (100.0)	0.888	0.204	0.480[b]	0.982[c]
Age (years)[a]					
Mean, 55.2±10.3; median: 55					
Median, 55					
Menopause[c]					
Premenopausal	27 (32.5)	0.316	0.392	0.935	0.296[d]
Postmenopausal	56 (67.5)				
TNM stage according to UICCc		0.244	0.674	0.879	0.570[d]
II A	33 (39.8)				
II B	50 (60.2)				
Tumor size (pT)[a] (mm)		0.540	0.585	0.969	0.280[c]
Mean, 31.0±12.3					
Median, 30					
Nodal metastases (N)[c]		0.232	0.020	0.006	0.037[d]
N^-	47 (56.6)				
N^+	36 (43.4)				
Grading[c]		0.001	0.746	0.057	0.014[d]
G2	59 (71.1)				
G3	24 (28.9)				
ER status[a]		0.182	0.561	0.363	0.464[c]
Negative	22 (26.5)				
Positive	61 (73.5)				
PgR status[a]		0.169	0.374	0.894	0.831[c]
Negative	22 (26.5)				
Positive	61 (73.5)				
HER-2 status[a]		0.735	0.714	0.875	0.646[c]
Negative	64 (77.1)				
Positive	19 (22.9)				
Recurrence[c]		0.394	0.001	<0.001	<0.001[d]
Yes	32 (38.6)				
No	51 (61.4)				

[a]P-value of Kendall's tau rank correlation coefficient. [b]P-value of Pearson's correlation. [c]P-value of Mann-Whitney's U test. [d]P-value of Fisher's test. PLK1, polo-like kinase 1; IRS, immunoreactive score; ER, estrogen receptor; PgR, progesterone receptor; HER-2, human epidermal growth factor receptor-2; TNM, tumor-node-metastasis; UICCc, Union for International Cancer Control classification.

PLK1 expression was evaluated using the semi-quantitative scale of the immunoreactive score (IRS), according to Remmele and Stegner with certain modifications (37,38), which considers the percentage of reactive cells (no staining=0, <25%=1, 25-50%=2, 51-75%=3 and >75%=4) and the intensity of staining (no staining=0, weak=1, intermediate=2 and strong=3), with the final result being the product of both variables. Consequently, nine possible scores (0, 1, 2, 3, 4, 6, 8, 9 and 12) were obtained.

PLK1 expression was only observed in tumor tissues of BC specimens with cytoplasmic localization. Normal breast tissues were characterized by no or weak cytoplasmic PLK1 immunoreactivity.

For subsequent statistical analyses, a two-grade scale system was applied, allocating 0 points for expression of PLK1 <8 (low PLK1 immunoreactivity) and 1 for expression of PLK1 ≥8 (high PLK1 immunoreactivity). Definition of these two groups and determination of the cut-off point is a specific consensus of histopathological observations and statistical analyses.

Evaluation of ER, PgR and HER-2 expression was performed using standard methods described in a previous study (36).

Statistical analysis. Statistical analysis was performed using the Statistica 10.0 software package (StatSoft Inc., Tulsa, OK, USA). OS was defined as the time between primary surgical

treatment and mortality, and it was censored at the last follow-up for those patients who were alive. DFS was defined as the time between primary surgical treatment and date of relapse or mortality, whichever occurred first. DFS was censored at the last follow-up for patients who survived without disease recurrence. CSOS was defined as the time between primary surgical treatment and cancer-associated mortality, and was censored at the last follow-up for surviving patients.

To analyze the associations between PLK1 protein expression and clinicopathological parameters, the Pearson linear correlation coefficient in case of quantitative variables, the Kendall rank correlation in case of ordinal variables, the Pearson χ^2 test of independence in case of categorical variables and the exact Fisher test in case of 2x2 tables, were used. Differences between two groups were tested with the Mann-Whitney U test, while the log-rank test was used for comparison of survival in two groups. The OS rate was estimated by the Kaplan-Meier method, and the influence of explanatory variables on mortality risk was analyzed by Cox proportional hazard regression and logistic regression in case of binary survival. P<0.05 was considered to indicate a statistically significant difference.

Results

PLK1 immunostaining in BC specimens. PLK1 expression defined as IRS >0 was detected in all 83 BC patients. The average IRS was 6.55±3.10, and the median was 6.00. For statistical analysis, augmented immunoreactivity of PLK1 was defined as IRS ≥8 (38 patients, 45.8%), while low immunoreactivity was assigned to IRS=0-6 (45 patients, 54.2%) (Fig. 1).

Association between PLK1 expression and clinicopathological parameters. Overexpression of PLK1 and high intensity of immunohistochemical reaction were significantly correlated with the presence of regional lymph node metastases (P=0.03700 and 0.02000, respectively) (Table I). Disease recurrence was observed more frequently in patients with increased PLK1 expression and with high intensity of PLK1 immunoreactivity (P<0.00100 and P=0.00100, respectively). Paradoxically, increased PLK1 expression and high percentage of $PLK1^+$ cells were associated with lower histological grade (P=0.01400 and 0.00100, respectively). No significant correlations were observed between PLK1 expression and hormone receptor/HER-2 status, primary tumor size, menopausal status or age at the time of diagnosis (Table I).

PLK1 immunoreactivity and patient survival at 5-, 10- and 15-year follow-ups. Univariate logistic regression analysis of PLK1 expression in the context of 5-, 10- and 15-year survival revealed highly negative prognostic significance of PLK1 overexpression in patients with early stage BC in all the follow-up periods analyzed (Table II).

Kaplan-Meier analysis confirmed the correlation of PLK1 overexpression with long-term survival, as high PLK1 immunoreactivity (IRS ≥8) was associated with shorter CSOS and DFS (P=0.00001 and 0.00013, respectively) (Fig. 2A and B). Additionally, high PLK1 immunoreactivity was correlated with shorter CSOS and DFS in patients without local lymph node metastases (P=0.00110 and 0.00900, respectively) (Fig. 2C and D) and in patients with diagnosed nodal metastatic foci (P=0.00900 and 0.03000, respectively) (Fig. 2E and F).

Multivariate Cox regression analysis. In the multivariate Cox regression analysis, two clinicopathological parameters were noticed to have independent prognostic value in patients with early stage BC, namely high expression of PLK1 (P=0.00030) and presence of local lymph node metastases (P=0.00300). Other clinicopathological features had no significance in the multivariate Cox model.

Since lymph node metastases had a significant prognostic impact, multivariate analysis was performed individually in N^- and N^+ patients (Table II). It was demonstrated that, in both lymph node-negative and positive groups, high expression of PLK1 was an independent unfavorable prognostic factor (P=0.01200 and 0.01400, respectively), which confirms the findings of univariate analysis.

Discussion

In the present study, a homogeneous group of patients with stage II invasive ductal BC was investigated with regard to expression levels of PLK1 and patient survival in a 15-year follow-up period. The associations between PLK1 reactivity in BC specimens and the status of HER-2 and steroid receptors were also evaluated.

Overexpression of PLK1 (defined as IRS ≥8) was detected in 45.8% of patients (38 patients), while low immunoreactivity of PLK1 was observed in 54.2% of patients (45 patients). PLK1 expression was only observed in the tumoral compartment of BC specimens, with cytoplasmic localization. With regard to the cytoplasmic expression pattern of PLK1, the present results were similar to those reported by other studies (27,28). By contrast, the findings regarding the cut-off value for high PLK1 immunoreactivity and the incidence of PLK1 overexpression were less concordant (27,28). King *et al* (28) demonstrated PLK1 overexpression in only 11% of analyzed patients, whereas Weichert *et al* (27) reported overexpression in 42.2% cases of BC, a value close to the present observations (45.8%). Likely reasons for these dissimilarities are methodological differences in PLK1 expression assessment between the studies and a highly homogenous study population (stage II, according to the Union for International Cancer Control classification) in the present study (34).

In the current study, overexpression of PLK1 was significantly correlated with the presence of regional lymph node metastases (P=0.03700) and disease recurrence (P<0.00100). Kaplan-Meier analysis confirmed the correlation of PLK1 overexpression with long-term survival, as high PLK1 immunoreactivity was strongly associated with shorter CSOS and DFS (P=0.00001 and 0.00013, respectively). In a multivariate Cox regression analysis, two clinicopathological parameters were observed to have independent prognostic value in patients with early stage BC: High expression of PLK1 (P=0.00030) and presence of regional lymph node metastases (P=0.00300).

Highly negative impact of increased PLK1 expression on patient prognosis was also observed by King *et al* (28), who demonstrated significantly shorter OS of patients with PLK1 overexpression in their analysis of 215 subjects. In addition, a positive correlation between PLK1 expression and the

Table II. Univariate analysis of correlations between immunohistochemical parameters of PLK1 expression and 5-, 10- and 15-year CSOS, and multivariate Cox regression analysis of PLK1 expression and 15-year CSOS in groups with and without lymph node metastases and in the whole cohort of patients.

A, Univariate logistic regression

Parameters of PLK1 expression	5-year survival		10-year survival		15-year survival	
	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)
Positive cells (%)	0.20700	1.85 (0.71-4.87)	0.18800	1.62 (0.79-3.33)	0.11400	1.79 (0.87-3.68)
Intensity	0.01200	3.14 (1.29-7.65)	0.00200	3.33 (1.59-7.01)	0.00040	4.51 (2.01-10.10)
IRS	0.00700	1.35 (1.09-1.67)	0.00080	1.40 (1.16-1.70)	0.00020	1.54 (1.24-1.92)
High expression (IRS ≥8)	0.00500	9.92 (2.01-49.05)	0.00060	7.80 (2.48-24.51)	0.00010	12.18 (3.75-39.62)

B, Multivariate Cox regression analysis of 15-year survival

Clinicopathological parameters	All patients		Without lymph node metastases		With lymph node metastases	
	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)
High expression of PLK1	0.00030	6.13 (2.30-16.33)	0.01200	19.21 (1.91-193.28)	0.01400	4.02 (1.32-12.20)
Tumor size (pT)	0.12100	1.03 (0.99-1.07)	0.05100	1.07 (1.00-1.14)	0.52600	1.01 (0.97-1.06)
Lymph node metastases	0.00300	3.57 (1.55-8.24)	-	-	-	-

CSOS, cancer-specific overall survival; PLK1, polo-like kinase 1; IRS, immunoreactive score; OR, odds ratio; CI, confidence interval; HR, hazard ratio.

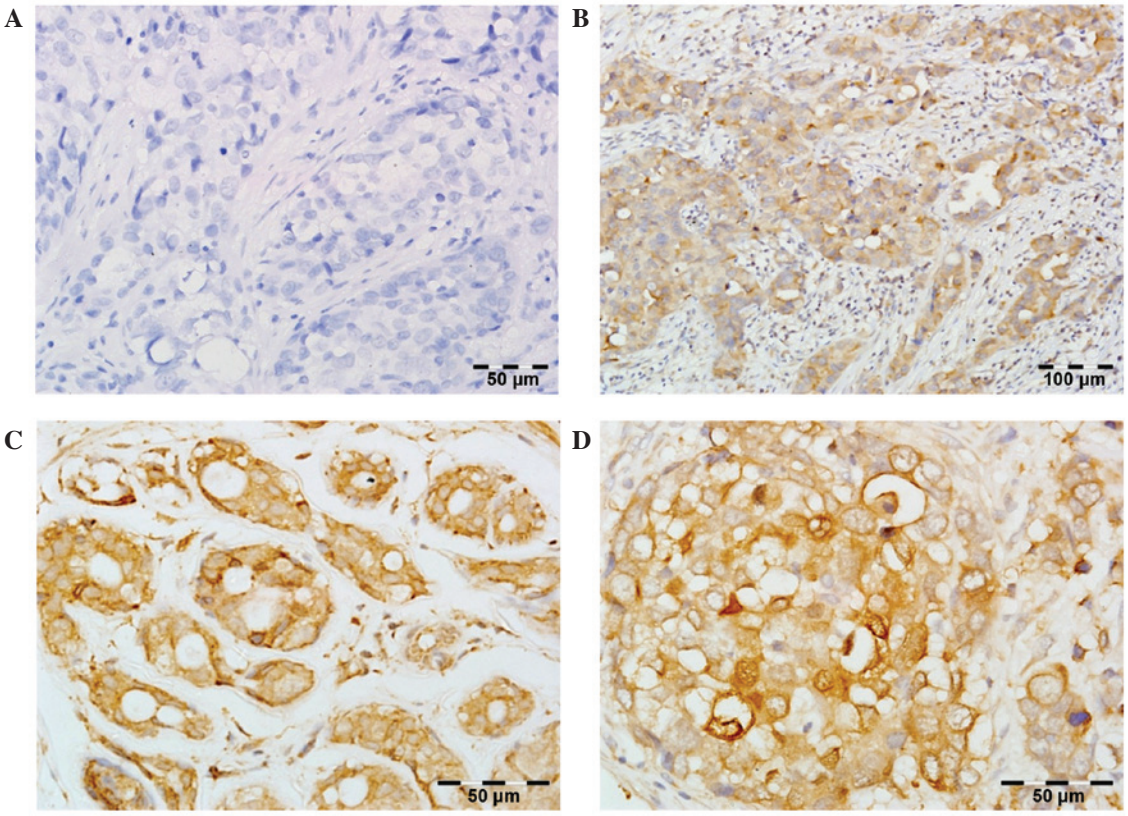

Figure 1. Immunohistochemical analysis of PLK1 expression in BC cells. (A) Lack of PLK-1 expression in BC cells (IRS=0; magnification, x400; hematoxylin staining). (B) Intermediate level of cytoplasmic PLK1 expression in BC cells (IRS=6; magnification, x200; hematoxylin staining). (C and D) High expression of PLK1 in BC cells of two different tumors (IRS=12; magnification, x600; hematoxylin staining). PLK1, polo-like kinase 1; BC, breast cancer; IRS, immunoreactive score.

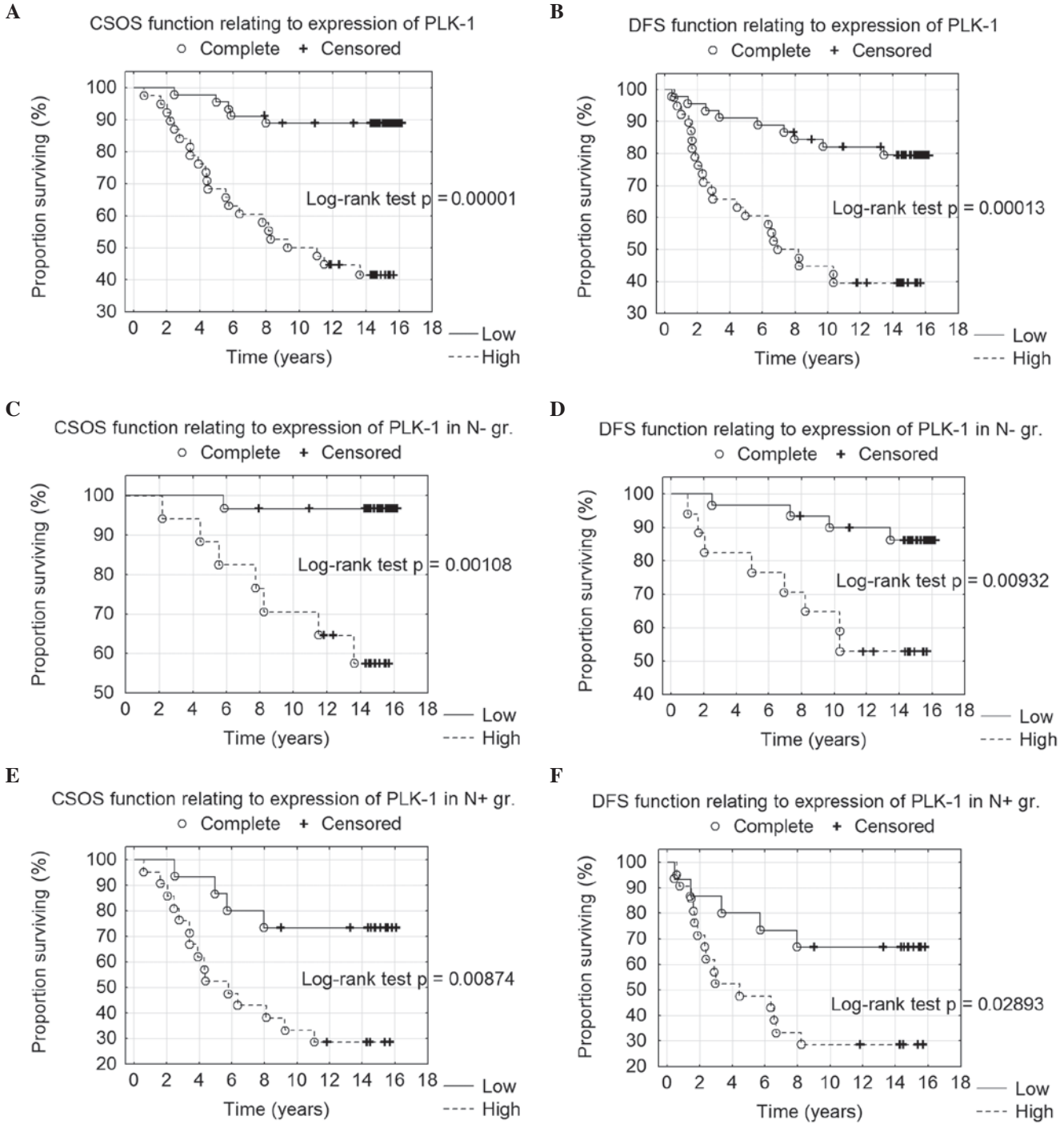

Figure 2. PLK1 immunoreactivity and patient survival. High PLK1 immunoreactivity (IRS ≥8) was associated with shorter (A) CSOS (P=0.00001) and (B) DFS (P=0.00013). High PLK1 immunoreactivity was associated with shorter (C) CSOS (P=0.00110) and (D) DFS (P=0.00900) in patients without regional lymph node metastases. High PLK1 immunoreactivity was associated with shorter (E) CSOS (P=0.00900) and (F) DFS (P=0.03000) in patients with diagnosed nodal metastatic foci. PLK1, polo-like kinase 1; N, lymph nose metastasis; gr., group; CSOS, cancer-specific overall survival; DFS, disease-free survival.

presence of a mutant version of the tumor protein p53 gene was also revealed in that study (28). Weichert *et al* (27) did not confirm the prognostic significance of enhanced PLK1 immunoreactivity in BC cells, and only PLK3 overexpression was observed by the authors to be a negative predictor of OS and recurrence-free survival.

An important point in the interpretation of the present results is the significant correlation between PLK1 overexpression and the presence of regional lymph node metastases, which is commonly accepted as an independent predictor of negative prognosis (38). King *et al* (28) and Weichert *et al* (27) did not observe any significant associations between increased PLK1 immunoreactivity and regional nodal metastases. The absence of associations between PLK1 overexpression and PgR/HER-2 status in the current results are in agreement with those of other authors (27,28). Notably, King *et al* (28) and Weichert *et al* (27) demonstrated that negative ER status and high histological grade correlated with PLK1 overexpression, which was not confirmed in the present study. This is probably due to the highly homogenous population (comprising only early BC patients) in the

current study, whereas the study groups in the aforementioned reports contained patients in all stages of the disease.

Another aspect worth considering is the role of PLK1 expression as a potential marker of cell proliferation, since strong expression of PLK1 (which has been associated with enhanced mitotic activity) is detectable in actively proliferating cells (those in phase G2/M) (39). In the present study and in the study conducted by Weichert *et al* (27), there were cases of BC in which 100% of cells exhibited strong PLK1 immunoreactivity. This observation is difficult to interpret and requires further investigation. PLK1 overexpression is closely associated with the G2/M phase of the cell cycle in *in vitro* models (40). However, such a remarkably high proportion of $PLK1^+$ cells does not necessarily imply that all the positive cells are in the G2/M phase (which is the active phase of proliferation) at the same time. The above observation may indicate a pleiotropic significance of PLK1 in cytophysiology; thus, its expression may not only be a symptom of ongoing cellular divisions, but may also reflect a cellular response to DNA damage in cancer cells and the subsequent attempts to repair it by numerous enzymes, including ATM, ATR and poly(ADP-ribose) polymerase 1 (PARP-1). This postulate is in line with the results of the present study, which identified a positive correlation between enhanced PLK1 and PARP-1 expression in BC cells (data not shown).

Additionally, PLK1 overexpression in cancer cells may result from chromosomal overrepresentation of the *PLK1* gene locus, which leads to increased protein production. This is consistent with the observations of Tirkkonen *et al* (41), who detected chromosomal amplification of the 16p12 region (which contains the *PLK1* locus) in 38% of BC patients, a rate that is close to the 45.8% of tumors overexpressing PLK1 detected in the present study.

In conclusion, there is a significant and independent association between PLK1 overexpression and unfavorable prognosis in the 15-year follow-up of early BC patients. The results of the present study suggest a potential role for PLK1 in the progression of BC. The present findings may aid to generate molecular targeted therapies based on PLK1 inhibitors.

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