

Comparing the prognostic value of PTEN and Akt expression with the Mitotic Activity Index in adjuvant chemotherapy-treated node-negative breast cancer patients aged <55 years¹

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Abstract. *Background:* The prognostic value of the PI3K/Akt/mTOR pathway and PTEN in invasive breast cancer (IBC) is controversial. Cell proliferation, especially the Mitotic Activity Index (MAI), is strongly prognostic in lymph node-negative (LNneg) invasive breast cancer. However, its prognostic value has not been compared with the value of Akt and PTEN expression. *Material and methods:* Prognostic comparison of Her2Neu, p110alpha (*PIK3CA*), Akt, mTOR, PTEN, MAI and cell-cycle regulators in 125 LNneg patients aged <55 years with cyclophosphamide, methotrexate, and 5-fluorouracil (CMF)-based adjuvant systemic chemotherapy. *Results:* Twenty-one (17%) patients developed distant metastases = DMs (median follow-up: 134 months). p110alpha correlated ($p = 0.01$) with pAkt but only in PTEN-negatives; pAkt correlated ($p = 0.02$) with mTOR. PTEN-negativity correlated with high MAI, high grade and ER-negativity ($p = 0.009$). The MAI was the strongest prognosticator (Hazard Ratio = HR = 2.9, $p = 0.01$). Her2Neu/p110 α /Akt/mTOR features have no additional prognostic value to the MAI. PTEN had additional value but only in MAI < 3 (39/125 = 31%; 8% DMs). 19/39 = 49% of the MAI < 3 patients have combined MAI < 3 / PTEN+ with 0% DMs, contrasting 15% DMs in MAI < 3 / PTEN- ($p = 0.03$). *Conclusions:* In T₁₋₃N₀M₀ adjuvant CMF-treated breast cancer patients aged <55 years, MAI was the strongest survival predictor. The PI3K/Akt/mTOR pathway and cell-cycle regulator characteristics had no additional prognostic value, but PTEN has. Patients with combined MAI < 3 & PTEN-positivity had 100% survival. The small subgroup of MAI < 3 patients that died were PTEN-negative.

Keywords: Breast cancer, cell proliferation, Mitotic Activity Index, Akt pathway, PTEN

1. Introduction

Breast cancer is one of the most common cancers and a leading cause of cancer-related mortality in

women in the Western world. About 60% of affected women are lymph node-negative, and many receive adjuvant systemic treatment (AST). Treatment guidelines usually recommend no AST in “low risk” patients, often determined by tumor grade and oestrogen receptor status [15]. This means that the majority of young lymph node-negative patients are treated with AST, although only 15–25% develops distant metastases without AST. Moreover, the reproducibility of grading tumors is far from perfect, even among experts [11]. Use

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of accurate and reproducible treatment selection criteria is critical for reducing over-treatment, while not promoting under-treatment.

Proliferative activity represents one of the biologic processes most thoroughly investigated for its prognostic value in breast cancer [3–5,21,26,27]; it is certainly an important therapeutic target. Moreover, some analyses have shown a relationship between proliferation and response to systemic treatments [7,18]. One of the strongest, simplest and most reproducible proliferation-associated prognostic factors is the Mitotic Activity Index (MAI = number of mitoses per 1.59 mm²) [1,9,21]. Its prognostic value has been shown in many retrospective and prospective studies using a fixed threshold (MAI < 10 favorable outcome; MAI ≥ 10 unfavorable outcome) [3]. A recent prospective, multicentre, long-term follow-up study showed that the MAI is the strongest prognostic factor for node-negative invasive breast cancer [3].

Using Comparative Genomic Hybridization, we previously found that amplification of chromosome 3q26 was considerably stronger than the MAI in prognosticating distant metastases [17]. This led us to hypothesize that over-expression of the protein coded by the gene *PIK3CA* (the p110alpha catalytic subunit of a PI3-kinase class 1a), which is located on 3q26, could be the underlying cause of the poor prognosis associated with this amplification [17]. P110alpha is an important component of phosphoinositide 3-kinase (PI3-kinase), a growth factor-activated transforming lipid and protein kinase. PI3-kinases of the 1a class have multiple effectors and are involved in cell motility, invasion and apoptosis inhibition [25]. In breast cancer, p110alpha plays an important role in transmitting growth factor signals into the cell via the Akt pathway.

The PI3K/Akt/mTOR regulatory pathway of protein translation involved in the regulation of cell proliferation (through downregulation of p27 and thereby up-regulation of cyclinE), growth, differentiation, migration, and survival has been investigated by others, but with conflicting results. One study reported that low PTEN expression sensitises tumor cells to inhibitors of the PI3kinase/Akt pathway [16]. Another study found no correlation with overall survival [24]. A third study concluded that Akt/mTOR would be a promising therapeutic target [22]. It has also been suggested that Akt immunoprofiling could predict patients' response to endocrine therapies, e.g. Tamoxifen [20]. Lately, other functions for nuclear expression of both PTEN and Akt have also been described [6,30].

One criticism that can be raised against these Her2neu-PI3K-PTEN-Akt-mTOR pathway studies is

that they have included a mix of cancers with a variety of biological characteristics: small and large tumors, node-negative and -positive patients, and patients of all ages. Considerable hormonal changes take place with age, and these changes influence proliferation and other relevant factors. The analysis of such biologically variable groups is scientifically less desirable and may explain the differences in the prognostic values and correlations obtained for the Akt and PTEN pathways components. Moreover, a comparison of the value of Akt and PTEN expression with the MAI, one of the strongest prognosticators and predictors in node-negative breast cancer under 55 years, and other cell-cycle regulators [3], has as yet not been reported.

We have therefore tested the hypothesis, that expression of Her2neu-p110alpha-PTEN-Akt-mTOR, cell-cycle regulators (p27, cyclinE) and MAI were correlated and that certain combined expression patterns were strongly prognostic. To this end, the expression pattern of the Her2neu-PI3K-PTEN-Akt-mTOR pathway and several cell-cycle regulators, as well as classical prognostic factors like the oestrogen receptor (OR), progesterone receptor (PR) and the MAI were analyzed, in a homogeneous group of lymph node-negative invasive breast cancer patients under 55 years of age, all treated with adjuvant systemic chemotherapy.

2. Materials and methods

2.1. Patients

All aspects of this study were approved by the Regional Ethics Committee, the Norwegian Social Science Data Service and the Norwegian Data Inspectorate.

The archive of the Department of Pathology at the Stavanger University Hospital provided paraffin embedded material from a total of 1169 breast tumor patients (treated between 1978 and 1994). The following patients were excluded from further study: 81 patients with carcinoma *in situ* and 88 with extensive carcinoma *in situ* and a microinvasive component less than 1 mm, which is ineligible for MAI evaluation. Another 11 had a previous history of breast cancer, 19 had a recurrence within 6 months of follow-up and probably had unnoticed metastatic disease at the time of diagnosis, and 41 had a follow-up of less than 6 months because patients moved or died of other diseases. Patients with Paget's disease, $n = 18$, were also excluded, as

were 23 patients with bilateral breast cancer, 2 males, and 11 other rare non-cancerous breast malignancies. From 14 patients, no material was available, and 29 patients were lost to follow-up. This left a total of 832 patients, of which 442 were lymph node-negative and 186 were aged <55 years. Following the protocol of the Norwegian Breast Cancer Group, only 125 of these 186 lymph node-negative patients received systemic adjuvant chemotherapy (cyclophosphamide, methotrexate, and 5-fluorouracil (CMF)). All patients were treated with modified radical mastectomy or breast-conserving therapy (always with adequate axillary lymph node dissection with at least 10 nodes examined). Locoregional radiotherapy was given to patients that underwent breast-conserving therapy or had medially localized tumors.

The post-surgical size of the tumor was measured in fresh specimens; the tumors were cut in slices of 0.5 centimeter thick, fixed in buffered 4% formaldehyde and embedded in paraffin. Four micrometer thick paraffin sections were cut and stained with haematoxylin and eosin (H&E). Histological type and grade were assessed by three pathologists (JB, EG, KK) with considerable experience in breast pathology, according to the World Health Organization criteria [29]. Grade was carefully assessed according to the Nottingham modification [12,13], using MAI 0–5 = 1, 6–10 = 2 and >10 as 3; nuclear atypia as mild = 1, moderate = 2 and marked = 3; and tubular formation as majority (>75% = 1), moderate (10–75% = 2) and little or none (<10% = 3). The grade is the sum of tubular formation + nuclear atypia + MAI class, where Grade 1: (sum = 3, 4 or 5), Grade 2 (sum = 6 or 7) and Grade 3 (sum = 8 or 9).

The MAI was assessed as described elsewhere [2]. In short, all unambiguous mitoses were counted in ten consecutive neighboring fields of vision in the most cellular area (representing a total area of 1.59 mm² at the specimen level).

2.2. Immunohistochemistry

Antigen retrieval and antibody dilution were optimized before the study started. To ensure uniform handling of the samples, all sections were processed at the same time. Paraffin sections of 4 micrometer thickness, adjacent to the H&E sections used for the MAI and histological assessment, were mounted onto silanized slides (#S3002, DAKO, Glostrup, Denmark) and dried overnight at 37°C followed by 1 hour at 60°C. The sections were deparaffinized in xylene and rehydrated in

a graded series of alcohol solutions. Antigen retrieval was performed by pressure cooking in 10 mM TRIS / 1 mM EDTA (pH 9.0) for three minutes at full pressure and cooled for 15 minutes at 18°C.

Immunostaining was performed using an autostainer (DAKO). TBS (#S1968, DAKO) with 0.05% Tween 20 (pH 7.6) was used as the rinse buffer. Endogenous peroxidase activity was blocked by peroxidase blocking reagent (#S2001, DAKO) for 10 minutes and the sections were incubated with the antibodies at the following dilutions and time intervals: oestrogen receptor = OR 1 : 400, 30 minutes (#RM-9102-S, Neomarkers, LabVision, Fremont, CA, USA); progesterone receptor = PR 1 : 400, 30 minutes (#RM-9001-S, Neomarkers); Her2neu, Herceptest (DAKO); p110alpha 1 : 200, overnight (#4254, Cell Signaling, Danvers, MA, USA); PTEN 1 : 300, 30 minutes (clone 6h2.1, Cascade Biosciences, Winchester, MA, USA); phospho-Akt 1 : 300, overnight (Ser473) (587F11, Cell Signaling); phospho-mTOR 1 : 800, overnight (Ser2488) (49F9, Cell Signaling); p27 1 : 100, 30 minutes (SX53GB, DAKO); cyclin-E 1 : 40, 30 minutes (13A3) (Novocastra, NewCastle, United Kingdom). DAKO antibody diluent #S0809 was used, and the immune complex was visualized by Peroxidase/DAB (#K5007, ChemMate Envision Kit, DAKO) with incubation of Envision/HRP, Rabbit anti-mouse (ENV) for 30 minutes and DAB + chromogen for 10 minutes. The sections were counterstained with Haematoxylin, dehydrated and mounted. Controls for the immunostaining were stained normal breast tissue control sections and positive control sections on each slide, next to positive normal cell compartments (if available) within the test sections.

2.3. Scoring of immunohistochemistry results

Three independent observers (EJ, HS and IS) scored the staining intensity in the invasive epithelial cancer cells, if scores differed between the two observers a consensus score was obtained under a session with a multihead microscope. Her2neu scoring was performed according to the protocol provided with the FDA approved Herceptest (DAKO). For pAkt and PTEN, cytoplasmic staining and nuclear staining were evaluated separately, while for p110alpha, and phospho-mTOR only cytoplasmic staining was assessed, as follows: 0 = no staining or weak staining in less than 10% of tumor cells, 1 = weakly positive in more than 10% of the tumor cells, 2 = moderately positive and 3 = strongly positive. Nuclear staining

Table 1
 Characteristics of patients and tumors in relation to recurrence-free survival

Characteristic		Recurrence-free survival		
		Events/ at risk (%)	Log-rank ¹ <i>P</i> -value	HR ² (95% CI ³)
Patient age in years	25–45	14/63 (22)	0.05	0.4 (0.16–1.0)
	45–56	7/62 (11)		
Tumor diameter	<2 cm	7/63 (11)	0.07	2.3 (0.9–5.6)
	≥2 cm	14/62 (23)		
Oestrogen receptor	Pos	10/66 (15)	0.36	0.7 (0.3–1.6)
	Neg	11/59 (19)		
Progesterone receptor	Pos	12/78 (15)	0.32	0.6 (0.3–1.5)
	Neg	9/47 (19)		
Grade	1	1/20 (5)	0.02	2.4 (0.28–20.8)
	2	5/45 (11)		
	3	15/60 (25)		
Nuclear atypia	1 (Mild)	1/5 (20)	0.33	0.7 (0.09–6.1)
	2 (Moderate)	6/52 (11)		
	3 (Strong)	14/68 (21)		
Tubular formation	1 (>75%)	0/8 (0)	0.11	6.4 (0.9–47.9)
	2 (10–75%)	1/16 (6)		
	3 (<10%)	20/101 (20)		
Mitotic impression	1 (0–5)	5/58 (9)	0.01	2.2 (0.52–9.1)
	2 (6–10)	3/19 (16)		
	3 (>10)	13/48 (27)		
MAI	<10	9/78 (11)	0.01	2.9 (1.22–6.9)
	≥10	12/47 (25)		
MAI	<3	3/39 (8)	0.02	2.6 (0.65–10.6)
	3–9	6/39 (15)		
	≥10	12/47 (25)		
MAI	<3	3/39 (8)	0.02	3.9 (1.13–13.2)
	≥3	18/86 (21)		
Her2neu	0	12/60 (48)	0.58	0.8 (0.3–1.9)
	1+	8/50 (40)		
	2+	1/9 (7)		
	3+	0/6 (5)		
p110alpha	Negative	0/2 (0)	0.24	2.0 (0.8–5.3)
	Weak	6/55 (11)		
	Moderate	13/57 (23)		
	Strong	2/9 (22)		
PTEN-negativity	Absent	5/56 (9)	0.06	2.5 (0.9–6.9)
	Present	16/69 (23)		
PTEN in cytoplasm	Absent	9/44 (20)	0.4	0.7 (0.3–1.6)
	Present	12/81 (15)		
PTEN in nucleus	Absent	12/54 (22)	0.13	0.5 (0.2–1.2)
	Present	9/71 (13)		
phospho-AKT in cytoplasm	Negative	2/9 (22)	0.49	1.4 (0.3–6.3)
	Weak	13/60 (22)		
	Moderate	6/46 (15)		
	Strong	0/8 (0)		

Table 1
(Continued)

Characteristic		Recurrence-free survival		
		Events/ at risk (%)	Log-rank ¹ <i>P</i> -value	HR ² (95% CI ³)
phospho-AKT in nucleus	Absent	11/50 (22)	0.22	0.6 (0.2–1.4)
	Present	10/73 (14)		
phospho-mTOR	Weak	16/81 (20)	0.28	0.6 (0.2–1.6)
	Moderate	5/42 (12)		
	Strong	0/2 (0)		
Cyclin-E	≤10%	12/82 (15)	0.03	2.6 (1.09–6.2)
	>10%	9/32 (28)		
P27 in cytoplasm ⁴	≤50%	17/77 (22)	0.02	0.2 (0.1–0.9)
	>50%	2/38 (5)		
P27 in nucleus ⁴	≤50%	10/44 (23)	0.2	0.6 (0.2–1.4)
	>50%	9/71 (13)		

¹ KM: Kaplan–Meier survival estimates.

² HR: Hazard ratios: values greater than one indicate an increased risk for the second (or third) category compared to the first category.

³ CI: Confidence Interval.

⁴ 115 cases had adequate sections that could be evaluated, with 19 distant recurrences.

intensity for PTEN and pAkt was scored as absent or present (weak or strong staining). PTEN staining was also scored for the number of negative tumor cells, if any. “PTEN-negativity” in Table 1 is defined by a combined nuclear and cytoplasmic PTEN negativity in the tumor cells.

For cyclin-E, oestrogen receptor and progesterone receptor staining, the percent of positive tumor nuclei was estimated, and all tumors with more than 10% positive tumor cells were considered positive. For p27, the threshold for positivity in both cytoplasm and nucleus was set at 50%.

2.4. Statistical analyses

SPSS (Statistical Package for the Social Sciences) for Windows version 14.0 was used. Correlations between variables were calculated using the Chi-square test. For survival analysis, the main endpoint was distant metastasis-free survival. To analyze the probability that patients would remain free of distant metastases, we defined recurrence as any first recurrence at a distant site. All other patients were censored on the date of the last follow-up visit or death from causes other than breast cancer, local or regional recurrences or the development of any secondary primary cancer (including contra-lateral breast cancer). If the status during follow-up indicated a confirmed metastasis without a date of recurrence, the date of that follow-up visit was

used. Age, time to first recurrence and survival time were calculated relative to the date of primary diagnosis. For the MAI, previously established prognostic thresholds (<6, 6–10, 11 and higher [12], <10 versus ≥10, and MAI < 3, 3–9 and ≥10 [3]) were studied. Kaplan–Meier survival curves were constructed and differences between groups were tested by the log-rank test. The relative importance of potential prognostic variables was tested using Cox-proportional hazard analysis (forward, Wald) and expressed as a Hazard Ratio (HR) with 95% confidence intervals. The log rank and the Hazard Ratio between Grade 1 and the other grade subcategories could not be calculated as there were no events in the Grade 1 category, therefore Grade 1 and Grade 2 were grouped and tested against Grade 3; likewise, for nuclear atypia mild and moderate cases were taken together and tested against marked atypia. For Tubular Formation, <10% versus >10% were grouped, and for p110alpha negatives and weakly positives were grouped and analyzed against moderately positives and strongly positives. For the same reason phosphorylated-Akt, and Her2neu, the moderately positives and the strongly positives were grouped.

3. Results

The median follow-up time was 134 months (range: 10–311). 21 (17%) patients developed distant metas-

Table 2

Overview of correlations between the histopathological, immunohistochemical features and proliferation. Red: negative correlation; green: positive correlation; grey: no correlation (with $P = 0.05$ as threshold for significance)

	T-size	OR	PR	Grade	MAI10	PTEN	Her2neu	p110alpha	Akt	mTOR	p27	CyclinE
T-size	X											
OR	-	X										
PR	-	+	X									
Grade	+	-	-	X								
MAI10	+	-	-	+	X	-						
PTEN	0	+	+	-	-	X						
Her2neu	0	0	0	0	0	0	X					
p110alpha	0	0	0	0	0	0	0	X				
Akt	0	0	0	+	+	+	0	+	X			
mTOR	0	0	0	0	0	0	0	+	+	X		
p27	0	+	0	-	-	+	0	0	0	0	X	
CyclinE	+	-	-	+	+	-	0	+	0	0	-	X

tases. Table 1 shows the distant metastases recurrence-free survival results of the patients stratified on the basis of morphological and immunohistochemical characteristics. Grade, MAI, cyclinE and p27 were significant prognosticators ($p < 0.05$). Table 2 summarizes the correlations between all the features analyzed.

PTEN-positive staining was either cytoplasmic, nuclear or combined nuclear-cytoplasmic. Figure 1A shows a typical PTEN-negative tumor and a positive tumor with cytoplasmic and nuclear PTEN staining. PTEN cytoplasmic and/or nuclear staining are strongly correlated, and PTEN expressions are correlated with MAI < 10 , low grade, OR positivity, PR positivity, high p27 expression (both cytoplasmic and nuclear) and low cyclin-E expression. PTEN negativity (defined as no cytoplasmic or nuclear staining) was strongly correlated with MAI ≥ 10 , high grade, ER negativity, PR negativity, low p27 expression (both cytoplasmic and nuclear) and higher cyclin-E expression. Although all these factors are weak predictors of poor outcome, the presence of PTEN-negative tumor cells itself is prognostically just not significant ($p = 0.06$). Although nuclear and cytoplasmic PTEN expression correlated, there were more cytoplasmic than nuclear PTEN-positive cancers: 13/54 nuclear PTEN-negative cancers were cytoplasmic PTEN-positive, contrasting with only 3/44 cytoplasmic negatives which had nuclear PTEN expression. Most tumors with nuclear PTEN staining also show nuclear Akt staining ($p = 0.003$). Tumors with nuclear PTEN expression had a lower MAI than PTEN nuclear-negative tumors (MAI = 8 vs. 16; $p = 0.003$). PTEN expression in the nucleus alone was not prognostic.

Twelve percent of the cancers showed moderate (2+) or strong (3+) Her2neu staining (Fig. 1B), but the great majority was negative (0) or only weakly positive (1+). Her2neu positivity (= 2+ and 3+ together) did not correlate with expression of any of the Akt-pathway members, MAI, or other histopathological features.

A positive example of p110alpha staining is shown in Fig. 1C. Expression of p110alpha correlated with cytoplasmic pAkt expression ($p = 0.01$) and with mTOR expression ($p = 0.007$), but only in those tumors with PTEN-negative tumor cells. p110alpha expression had no prognostic value.

Phospho-Akt (ser473) was expressed simultaneously in the cytoplasm and nucleus (Fig. 1D). Cytoplasmic pAkt over-expression correlated with p110alpha expression, high grade and high MAI, but not with survival. Strong cytoplasmic pAkt expression correlated with strong cytoplasmic mTOR expression.

Phospho-mTOR (ser2488) expression (Fig. 1E) correlated with p110 α and pAkt expression, as mentioned above, but not with MAI, expression of any of the cell-cycle regulators or histopathological features. Additionally, mTOR expression had no prognostic value.

Low p27 expression in both the nucleus and the cytoplasm (Fig. 1F) correlated with high grade, ER negativity, high cyclin-E expression and high MAI. Tumors with cytoplasmic p27 expression $< 50\%$ have a worse prognosis ($p = 0.02$).

Strong cyclin-E expression (Fig. 1G) correlated with large tumors, high grade, ER and PR negativity, low p27 expression (both cytoplasmic and nuclear) and high MAI. Cyclin-E expression $> 10\%$ was associated with a worse prognosis ($p = 0.03$).

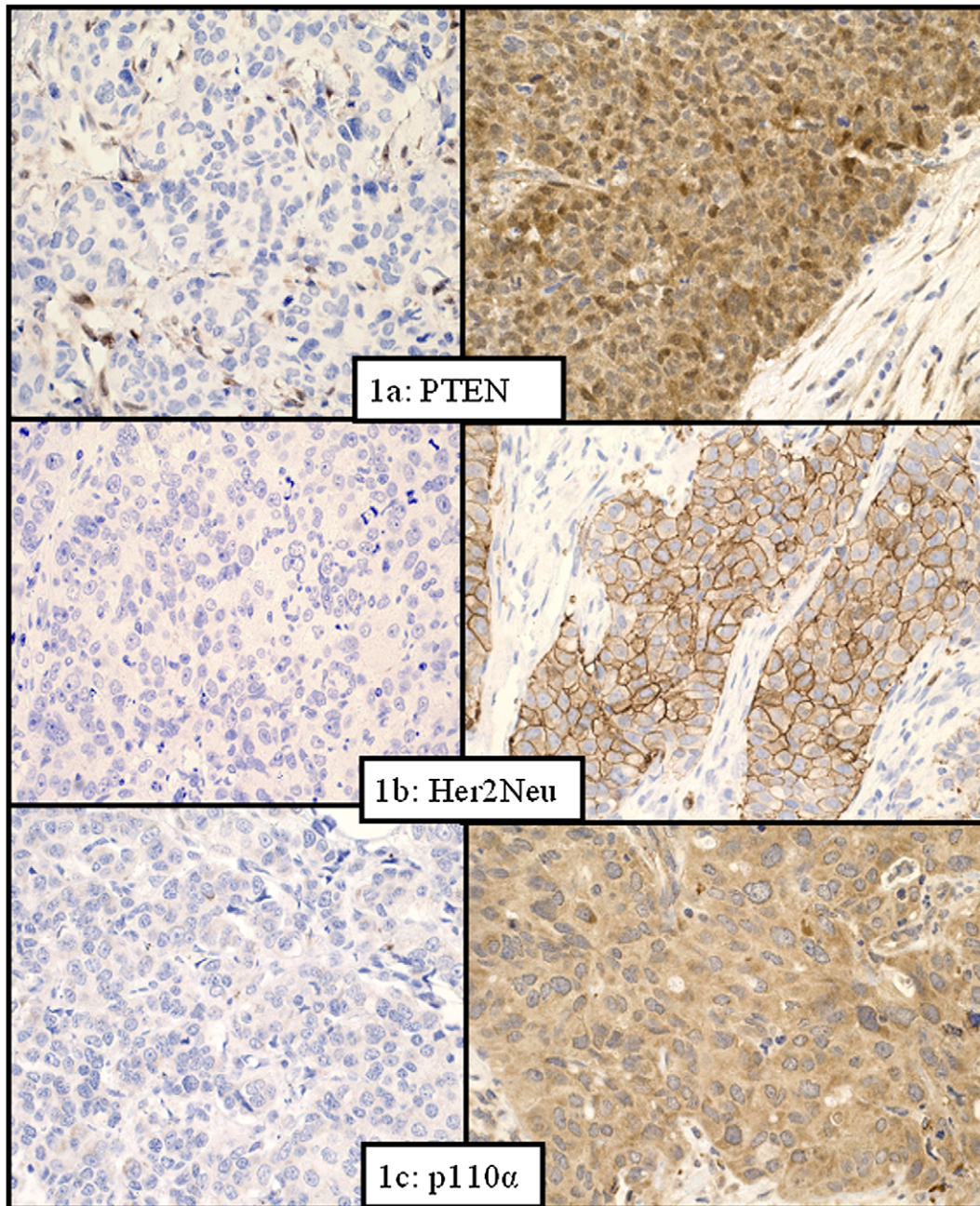


Fig. 1. Representative immunohistochemical samples. At the left, a negative or weakly positive sample is shown. (A) A typical PTEN-positive tumor with both cytoplasmic and nuclear staining. (B) A strongly positive (3+) Her2neu stained-tumor. (C) A tumor showing strong positive staining for p110alpha. (D) Simultaneous cytoplasmic and nuclear staining for pAkt. (E) Strong staining for mTor. (F) A tumor with a high percentage of p27 staining in both the cytoplasm and the nucleus. (G) Cyclin-E nuclear staining in more than 10% of tumor cells.

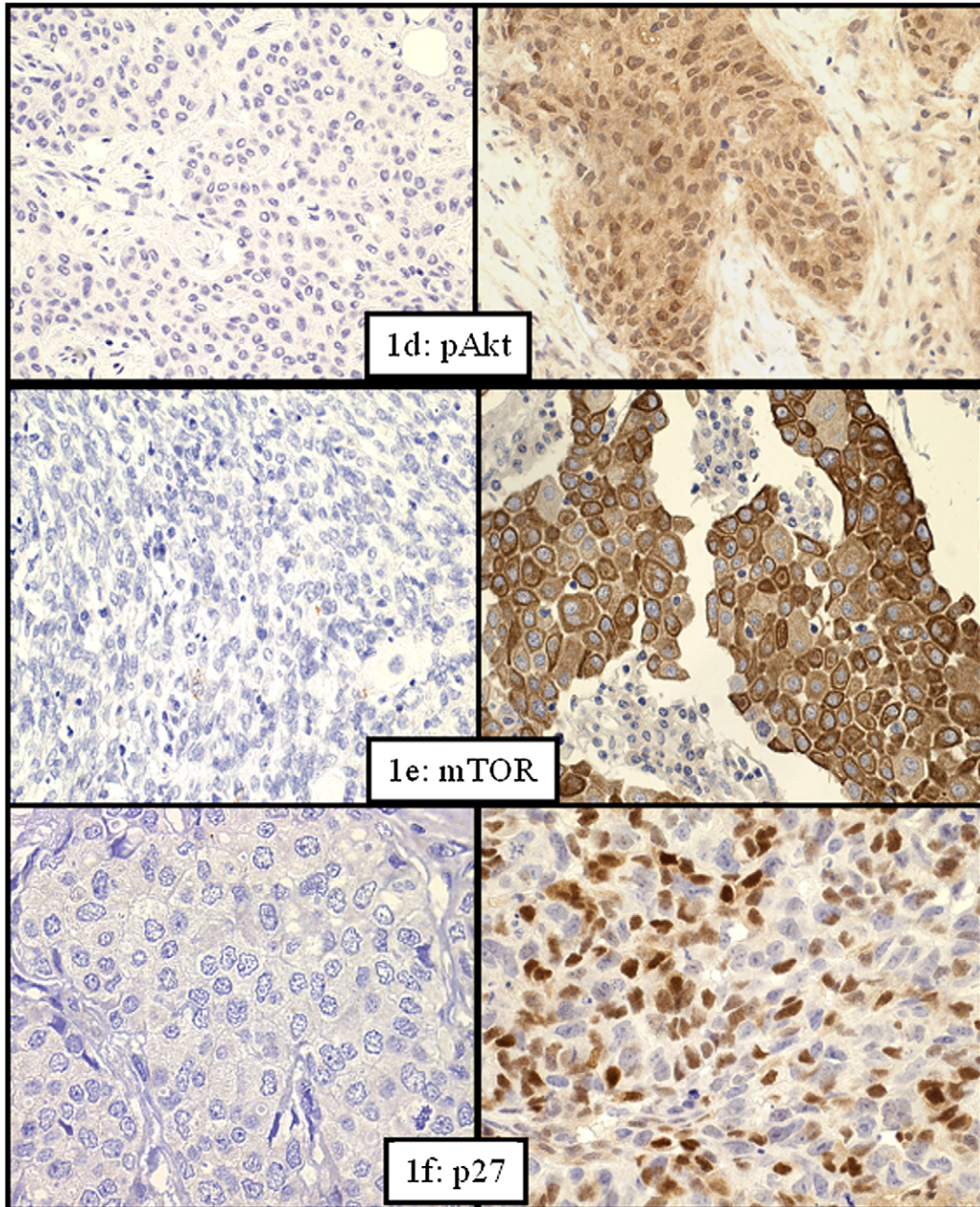


Fig. 1. (Continued).

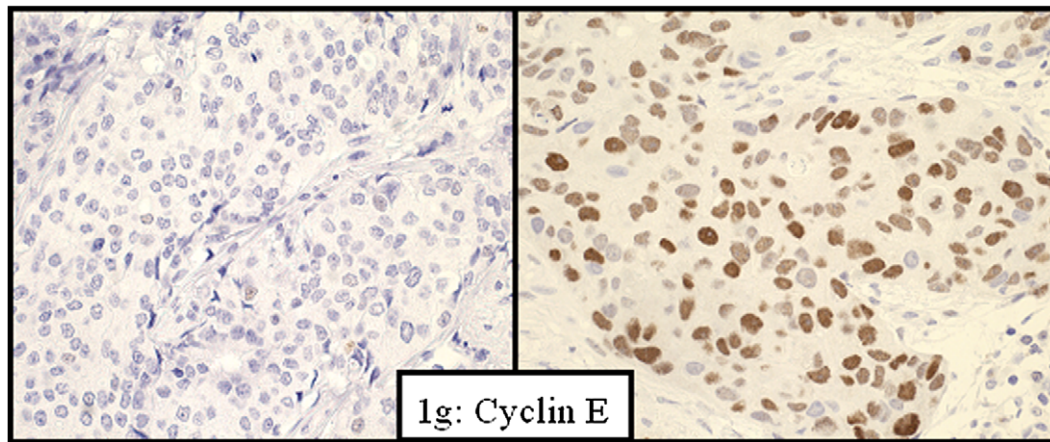


Fig. 1. (Continued).

3.1. Prognostic value of combined Akt-PTEN positivity

Seventy-six percent of the 29 patients with both nuclear PTEN-negativity and pAkt-negativity remained free of distant recurrence, contrasting with 90% of the tumors with both nuclear PTEN and Akt expression ($p = 0.08$).

With multivariate analysis, a MAI with a threshold of <10 versus ≥ 10 (HR = 2.9, $p = 0.01$) was the strongest prognosticator. Of all other features analyzed, only PTEN expression had prognostic value in addition to the MAI, but only in patients with a MAI < 3 (PTEN negativity defined as the absence of both cytoplasmic and nuclear expression). Of the 125 patients in the study, 39 (31%) had a MAI < 3 , with only 3/39 (8%) having distant metastases. 19/39 (49%) of the MAI < 3 patients had combined MAI < 3 / PTEN positivity with 0% distant metastases, contrasting with 15% distant metastases for MAI < 3 / PTEN negativity ($p = 0.045$). The predicted favourable outcome for cancers with combined MAI < 3 / PTEN positivity is also clear from the fact that only 2/39 patients had a loco-regional recurrence (which were cured after resection). Interestingly this group of patients does not consist only of grade 1 patients but also grade 2, both OR positive and negative and tumors smaller and bigger than 2 cm.

The prognostic value of the MAI also overshadowed the prognostic value of any of the cell-cycle regulators studied.

4. Discussion

In this study of systematically adjuvant chemotherapy-treated patients, the MAI with the classical threshold of 10 was the strongest prognosticator for distant metastasis-free survival. This is in agreement with many earlier retrospective [10,23,28], and a recent large prospective multicenter long-term follow-up study of 516 lymph node-negative breast cancer patients under 55 years, not treated with any form of adjuvant systematic treatment [3].

The Akt pathway has become an important clinical target due to the development of kinase inhibitors that reduce tumor growth. The pathway includes Her2neu, p110alpha, PTEN, pAkt and mTOR (amongst others) and is often depicted as a linear model with only PTEN as an outside inhibitor of the pathway. The immunohistochemistry results presented here are in agreement with the linear model of the Akt pathway, as p110alpha expression correlates with cytoplasmic pAkt in the absence of PTEN expression, and cytoplasmic pAkt correlates with cytoplasmic mTOR. However, none of the Akt pathway members had prognostic value. PTEN was prognostic, but only in cancers with low proliferation (MAI < 3). In this group of patients, PTEN may therefore be a more suitable therapeutic target than the many regulators and effectors in the Akt pathway. Lack of PTEN expression is reported to occur in 1–48% of patients [8,14,24] (we found 33% in this study), which means that there are differences in either patient selection or methodologies used. This requires further investigation.

The biological role of both pAkt and PTEN in the nucleus is still unclear, but very few cancers were exclusively positive for expression of these proteins in

the nucleus and negative for expression in the cytoplasm. The expression of the Akt and PTEN proteins was strongly correlated. Cytoplasmic PTEN is required for apoptosis through its phosphorylation of pAkt and upregulation of p27, whereas nuclear expression of PTEN [6] is linked to regulation of growth suppression through cell cycle arrest, by down-regulation of cyclin D1 and prevention of the phosphorylation of MAPK. Indeed, a significant difference in the mean number of mitoses was found between those with and those without nuclear PTEN staining (8 vs. 16, $p < 0.001$). These results suggest that PTEN expression is directly related to mitoses through downregulation of p27 and upregulation of cyclin-E.

As for the role of nuclear pAkt, an inhibitory effect on tumor invasiveness was recently reported as pAkt promotes ubiquitination and degradation of the nuclear factor of activated T cells family (NFATs). NFATs are targets of alpha(6)beta(4) integrin signalling and are involved in promoting the invasive potential of cancer cells [19].

We conclude that the MAI is the strongest univariate and multivariate predictor of outcome in the T₁₋₃N₀M₀ CMF-treated breast cancer patients <55 years. Of all the Akt pathway factors studied, only PTEN has prognostic value to add to the MAI: combined MAI ≤ 3 and PTEN positivity identifies a group of 15% of all patients with an excellent prognosis when treated with adjuvant chemotherapy. Other Akt pathway parameters have no prognostic value. This suggests that the biological role of both Akt and PTEN is more complex than often is depicted in the linear pathway model making successful targeted therapies development far more difficult. It is important to use biologically homogeneous groups of breast cancers in prognostic Akt/PTEN studies.

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