Prognostic comparison of the proliferation markers (mitotic activity index, phosphohistone H3, Ki67), steroid receptors, HER2, high molecular weight cytokeratins and classical prognostic factors in $T_{1-2}N_0M_0$ breast cancer

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The proliferation factors: mitotic activity index (MAI), phosphohistone H3 (PPH3) and Ki67 have strong prognostic value in early breast cancer but their independent value to each other and other prognostic factors has not been evaluated.
In 237 $T_{1-2}N_0M_0$ breast cancers without systemic adjuvant treatment, formalized MAI assessment and strictly standardized, fully automated quantitative immunohistochemistry (IHC) for Ki67, PPH3, estrogen (ER) and progesterone receptor (PR), HER2, cytokeratins-5/6 and -14, and automated digital image analysis (DIA) for measuring PPH3 and Ki67 were performed. Section thickness was measured to further control IHC measurements. All features were measured in the periphery of tumors. The different proliferation assessments and other well-established clinicopathological and biomarker prognostic factors were compared.
DIA-Ki67 added prognostically to PPH3. None of the other biomarkers or clinicopathological variables added prognostically to this PPH3/Ki67 combination. However, when PPH3 is replaced by MAI the prognostic value is nearly the same.
In early operable node negative breast cancer without adjuvant systemic treatment, Ki67 with a threshold of 6.5% assessed by digital image analysis in the periphery of the tumor is prognostically strong. The combination of either PPH3/Ki67 or MAI/Ki67 overshadowed the prognostic value of all other features including Ki67 alone.

Key words: breast cancer, proliferation, automation, Ki67, phosphohistone H3, mitosis.

Introduction

Breast cancer is the most frequent female malignancy in the western world [1]. Treatment has developed considerably over the past decades. Several prognostic and predictive factors have been introduced to improve therapeutic decision-making [2, 3]. Guidelines from Adjuvant!Online (AO), Sankt Gallen (SG) or the Norwegian Breast Cancer Group (NBCG) often combine conventional predictors to estimate relapse and mortality risk and classify the patients into low, intermediate, or high-risk groups [2]. Such factors identify 80% or more of all lymph node negative patients (LN-neg) as high risk, while only 15-20% of the patients die from metastatic disease when left untreated [4]. The use of these guidelines therefore means serious overtreatment.

Proliferation assessed by either mitotic activity index (MAI), phosphohistone H3 (PPH3) or Ki67 has a strong prognostic value [5-7]. Lymph node negative breast cancer patients with a high proliferation index in general have a 3-6 times higher risk of dying from distant metastases than those with low proliferation [5]. Mitotic activity index has an accuracy in LN-neg considerably exceeding that of Adjuvant!Online and the Norwegian national breast cancer treatment guidelines and identifies patients who would have benefitted from adjuvant systemic treatment (AST), but were regarded as low-risk groups by Adjuvant! or the NBCG guidelines, and vice versa [8].

We have recently tested the reproducibility and prognostic value of different Ki67 measurement techniques which are widely used. The measurement techniques varied from interactive counts to fully automated image analysis. The results showed that counts of Ki67 positive cells by different pathologists were poorly reproducible. Interactive point-weighted counting of Ki67 by morphometric techniques were much more reproducible, but automated digital image analysis (DIA) was the most reproducible and prognostically strongest [9].

In the present study we compared, in operable node negative breast cancers of women aged less than 71 years without systemic adjuvant treatment, the prognostic value of the MAI, Ki67 and PPH3. For the MAI, formalized and strictly protocolized measurement was performed as described in the national Dutch MMMCP multicentre prospective evaluation. For Ki67 and PPH3, fully automated and standardized tissue processing, antigen retrieval and immuno-histochemical staining were done using strict standard operating procedures, while measurement was done by automated digital image analysis (using the previously established optimal prognostic threshold of 6.5% Ki67 and PPH3 positive cells). The results of the MAI, PPH3 and Ki67 were also compared with other well-established and validated prognosticators (estrogen and progesterone receptors (ER, PR), HER2 (neu) and cytokeratin-5/6).

Material and methods

Patients

The study was approved by the Regional Ethics Committee, the Norwegian Social Science Data Service, and the Norwegian Data Inspection. The results are presented in accordance with the reporting recommendations for tumor marker prognostic studies criteria [10].

Paraffin-embedded material from 384 consecutive invasive node negative breast cancer patients less than 71 years old with operable breast cancer treated between 1990 and 1997 from the Department of Pathology at the Stavanger University Hospital (Stavanger, Norway) was used. The following patients were excluded: patients who received adjuvant treatment (n = 90), those with carcinoma *in situ* only or extensive carcinoma *in situ* with a small micro-invasive component < 0.5 mm that was ineligible for MAI evaluation (n = 18), patients with recurrence within 6 months of follow-up (n = 3), those with < 6-month follow-up (n = 5), and patients with Paget's disease (n = 1), bilateral breast cancer (n = 4), or other previous malignancies (n = 2). Material was technically inadequate for 21 patients, leaving 240 $T_{1-2}N_0M_0$ patients for analysis. There was no significant difference in age or tumor size in the 240 patients when compared to the original 384 patients. All patients were treated with modified radical mastectomy (n = 131) or breast-conserving therapy (n = 109), always with adequate lymph node dissection (at least 10, median 13 nodes). Locoregional radiotherapy was administered to patients who underwent breast-conserving therapy or had medially localized tumors.

Pathology

The post-surgical size of the tumor was measured on the fresh specimens. Tumors were cut into 0.5 cm slices, fixed in 4% buffered formaldehyde, and embedded in paraffin. Paraffin sections were cut into highly standardized 4-μm sections for hematoxylin-eosin (HE). Histological type was assessed according to World Health Organization criteria [11]. Grade (Grade 1 = 3, 4, or 5; Grade 2 = 6 or 7; Grade 3 = 8 or 9) was assessed according to the Nottingham modification [12], calculated as the sum of tubule formation (> 75% = 1, 10-75% = 2, and < 10% = 3), nuclear atypia (mild = 1, moderate = 2, and marked = 3), and MAI class (0-5 = 1, 6-10 = 2, and > 10 = 3).

Mitotic activity index assessment

The MAI was assessed as described in detail elsewhere [5, 8]. Briefly, all unambiguous mitoses were counted in 10 consecutive neighboring fields of vision (FOV) in the most cell-dense area (1.59 mm^2 at specimen level), usually in the periphery of the tumor (the so-called

growing zone). For details of the counting method, see [7]. The MAI has been shown to be reproducible and insensitive to variations in tissue processing [13-15].

Sections for immunohistochemistry

Four-micrometer thick paraffin sections adjacent to the HE sections used for assessment of MAI, histology and immunohistochemistry (IHC) were mounted onto Superfrost Plus slides (Menzel, Braunschweig, Germany) and dried overnight at 37°C followed by 1 h at 60°C. To ensure uniform handling of samples, all sections were made by the same person, on the same microtome with constant room temperature and constant rotation speed of the microtome, and processed simultaneously for IHC. We have shown before that the coefficient of variation of the section thickness is low and not a factor significantly influencing the prognostic value of Ki67 expression [9].

Immunohistochemistry

The immunohistochemical methods used have been carefully tested and compared with other methods to select the most optimal procedures, as described elsewhere [6]. In short, antigen retrieval and antibody dilution were optimized prior to the study onset. Sections were deparaffinized in xylene and rehydrated in decreasing concentrations of alcohol. Antigen was retrieved with a highly stabilized retrieval system (ImmunoPrep, Instrumec, Oslo, Norway) using 10 mM TRIS/1 mM EDTA (pH 9.0) as the retrieval buffer. Sections were heated for 3 min at 110°C followed by 10 min at 95°C and cooled to 20°C.

Rabbit polyclonal anti-phosphohistone H3 (ser 10) (Upstate #06-570; Lake Placid, NY) was used at a dilution of 1 : 1500. Ki67 (clone MIB-1, DAKO, Glostrup, Denmark) was used at dilution 1 : 100. ER (clone SP1, Neomarkers/LabVision, Fremont, CA, USA) was used at a dilution of 1/400. PR (clone SP2, Neomarkers/LabVision) was used at a dilution of 1/1000.

For HER2 assessments, the HercepTest kit (DAKO) was used according to the manufacturer's FDA-approved procedures. HercepTest 2+ and 3+ cases were retested with the PathVysion (Vysis, Downers Grove, IL, USA) assay following the manufacturer's FDA-approved protocols. Only HER2 amplified cases were regarded as positive. Cytokeratin 5/6 (Clone D5/16 B4, Dako, Glostrup, Denmark) at a dilution of 1/100 and cytokeratin 14 (Clone LL002, Novocastra, Wetzlar, Germany) at a dilution of 1/40 were used. For lymph vessel invasion, the same protocol was used as described before [16]. Briefly, the sections were incubated with a primary antibody cocktail of p63 (Dako, Glostrup, Denmark, clone 4A4) and D2-40 (Dako, clone D2-40). The primary antibodies were diluted to a final dilution of 1 : 1200 and 1 : 200 respectively. In all protocols the Dako antibody diluent (S0809) was used.

Anti-phosphohistone H3 was incubated for 60 min at 22°C. All other antibodies were incubated for 30 min at 22°C. The EnVision™ Flex detection system (Dako, K8000) was used for visualization. Sections were incubated for 5 min with peroxidase-blocking reagent (SM801), 20 min with the EnVision™ FLEX/HRP Detection Reagent (SM802), 10 min with EnVision™ FLEX DAB+ Chromogen (DM827)/EnVision™ FLEX Substrate Buffer (SM803) mix and 5 min with EnVision™ FLEX Hematoxylin (K8008). The slides were then dehydrated and mounted. All immunohistochemical stainings were performed using a Dako Autostainer Link 48 instrument and EnVision™ FLEX Wash Buffer (DM831).

Due to the small size of the invasive cancer left after recutting of the paraffin blocks, Ki67 could not be assessed in 3 cases, leaving 237 cases for analysis.

Automated digital image analysis of Ki67 and PPH3

We have described before how subjective counts and computerized interactive morphometry were done, but Ki67 and PPH3 expression assessment by the fully automated VIS digital image analysis (DIA) system (Visiopharm, Hørsholm, Denmark), using similar image processing principles as described before [6], was much more reproducible and also stronger prognostically [9]. Reference is made to that original detailed description and a brief treatise will follow here. Depending on the tumor diameter, two to ten square areas of each 1.59 mm^2 with subjectively the highest Ki67 index were scanned at 20× magnification. A mask of tumor cells was semi-automatically created. Inside this mask blue (negative) and brown Ki67 positive nuclei were segmented using a Bayesian classifier. The Ki67 index was calculated using the areas of classified blue and brown nuclei. The square with the highest Ki67 index was used as the final result. A similar technique was used for PPH3. Not surprisingly, the reproducibility of the DAI-Ki67 and PPH3 counts by the automated digital image analysis on different days by different observers on 10 randomly selected cases was close to perfect ($R^2 = 0.99$).

Data analysis

For survival analysis, the main end points were distant metastases occurrence and overall distant metastases-related survival. To determine the probability that patients would remain free of distant metastases, we defined recurrence as any first recurrence at a distant site. Patients were censored from the date of the last follow-up visit for death from causes other than breast cancer, local or regional recurrences, and the development of a second primary cancer, including contralateral breast cancer. If a patient's status during follow-up indicated a confirmed metastasis without a re-

Table I. Recurrence-free and breast cancer specific survival results of the different features analyzed

Characteristic		Recurrence				Disease-related mortality			
		Events/ at risk	AW* (%)	Log rank P-value	HR (95% CI)*	Events/ at risk	AW* (%)	Log rank P-value	HR (95%CI)*
Age	< 55	20/104	81			15/104	86		
	≥ 55	16/133	88	0.41	0.8 (0.4-1.5)	13/133	90	0.43	0.8 (0.4-1.6)
Tumor	≤ 2 cm	26/203	87			19/203	91		
	> 2 cm	10/34	72	0.02	2.3 (1.1-4.8)	9/34	75	0.01	2.7 (1.2-6.0)
ER*	pos	23/196	88			17/196	91		
	neg	13/41	68	0.001	3.0 (1.5-6.0)	11/41	73	0.001	3.5 (1.6-7.4)
PR*	pos	18/162	89			13/162	92		
	neg	18/75	76	0.008	2.4 (1.2-4.5)	15/75	80	0.006	2.7 (1.3-5.7)
HER2*	neg	27/211	87			22/211	90		
	pos	9/26	65	0.002	3.1 (1.5-6.7)	6/26	77	0.05	2.4 (1.0-5.8)
Grade	1	1/80	99			1/80	99		
	2	17/106	84		11.4 (1.5-86.0)	13/106	88		8.7 (1.1-66.9)
	3	18/51	65	< 0.001	24.7 (3.3-185.3)	14/51	73	< 0.001	20.7 (2.7-157.3)
MAI*	0-5	5/144	97			4/144	97		
	6-10	7/29	76		6.6 (2.1-21.1)	5/29	83		5.9 (1.6-22.0)
	> 10	24/64	63	< 0.001	10.8 (4.1-28.4)	19/64	70	< 0.001	17.2 (4.0-73.7)
Nuclear atypia	mild	1/33	97			1/33	97		
	mod.	13/128	90		2.7 (0.4-21.0)	9/128	93		2.0 (0.3-15.8)
	marked	22/76	71	0.003	7.3 (1.0-54.0)	18/76	76	0.002	6.6 (0.9-49.7)
Tubule formation	> 75%	1/24	96			1/24	96		
	10-75%	3/64	95		0.8 (0.09-8.1)	1/64	98		0.3 (0.02-4.9)
	< 10%	32/149	79	0.006	4.1 (0.6-30.0)	26/149	83	0.004	3.6 (0.5-26.6)
MAI†	0-9	12/173	93			9/173	95		
	≥ 10	24/64	63	< 0.0001	5.9 (3.0-11.9)	19/64	70	< 0.0001	5.9 (2.7-13.0)
LVI*	no	27/193	86			20/193	90		
	yes	9/44	80	0.18	1.7 (0.8-3.6)	8/44	82	0.19	1.8 (0.8-4.2)
TNP*	no	27/209	87			21/209	90		
	yes	9/28	68	0.01	2.5 (1.2-5.3)	7/28	75	0.02	2.6 (1.16.2)

Table I. Cont.

CHARACTERISTIC		RECURRENCE				DISEASE-RELATED MORTALITY			
		EVENTS/ AT RISK	AW* (%)	LOG RANK P-VALUE	HR (95% CI)*	EVENTS/ AT RISK	AW* (%)	LOG RANK P-VALUE	HR (95%CI)*
Basal-CK*	neg	26/210	88			19/210	99		
	pos	10/27	63	< 0.001	4.0 (1.9-8.3)	9/27	67	0.001	4.3 (1.9-10.0)
Ki67-DIA‡	< 6.5%	3/121	98			1/121	99	< 0.0001	27.5
	≥ 6.5%	33/116	72	< 0.0001	12.2 (3.7-39.8)	27/116	77		(3.7-202.7)
Ki67-DIA§	<15%	11/165	93			9/165	95	< 0.0001	4.5 (2.0-10.0)
	≥ 15%	25/72	65	< 0.0001	5.4 (2.7-11.0)	19/72	74		
PPH3†	< 13	6/153	96			5/153	98		30/8464
	≥ 13	30/84	64	< 0.0001	10.6 (4.4-25.6)	23/84	73	< 0.0001	8.9 (3.4-23.5)

** AW – alive and well; HR – hazard ratio; CI – confidence interval; ER – estrogen receptor; PR – progesterone receptor; MAI – mitotic activity index; PPH3 – phosphobistone H3; LVI – lymph vascular invasion; TNP – triple negative phenotype tumor; CK – cytokeratin; BLC – basal-like carcinoma*

† per 10 HPF, 1.59 mm² at specimen level in the periphery of the tumor

‡ 6.5% is the threshold found with receiver operating curve and CART analysis, in the current study of node negative breast cancer patients

§ 15% is the threshold used by the Norwegian Breast Cancer Group for lymph node positive estrogen receptor positive breast cancer

currence date, the follow-up visit date was used. Age, time to first recurrence, and survival time were calculated relative to the primary diagnosis date. For the MAI, three sets of previously established prognos-tic thresholds [12] (< 6, 6-10, ≥ 11, < 10 versus ≥ 10; and < 3, 3-9, and ≥ 10) were examined. The prognostic thresholds were 6.5% for Ki67 and 13 per 1.59 mm² at specimen level for PPH3. Kaplan-Meier survival curves were constructed, and between-group differences were tested using the log-rank test. The relative importance of potential prognostic variables was tested using Cox-proportional hazard analysis and expressed as a hazard ratio (HR) with a 95% confidence interval (CI).

Results

Thirty-six out of the 237 patients included in the study (15%) developed distant metastases and 28 (12%) died. Table I shows the univariate survival results.

With multivariate survival analysis, Ki67 prognostically overshadowed the following variables: age, tumor diameter, grade, ER, PR, HER2, CK5/6, CK14, triple negative phenotype tumor, basal-like cell type, lymph vessel invasion. PPH3 was however prognostically strongest, and DIA-Ki67-6.5% added prognostically to PPH3 (Table II). The PPH3/Ki67 combination therefore overshadowed all other features studied. Women with PPH3 < 13 and DIA-Ki67 < 6.5% have an excellent 10-year survival of 99%, even without adjuvant systemic therapy. If PPH3 is < 13, but DIA-Ki67 ≥ 6.5%, the overall survival still is 90%. When PPH3 ≥ 13 the mortality is high even when Ki67% is low (Table III). Table IV shows the prognostic interaction of MAI < versus ≥ 10, and DIA-Ki67-– 6.5%. In patients with MAI < 10, DIA-Ki67 < 6.5% identifies a group with an excellent prognosis, but patients with Ki67 ≥ 6.5% have a significantly increased risk of dying from distant metastases (p = 0.001, hazard ratio = 14.8). In patients with MAI ≥ 10, low Ki67 hardly occurs and has no additional prognostic value. Ki67 therefore is prognostically useful in patients with low proliferation according to either MAI or PPH3, but not in those with high MAI and PPH3 values.

Discussion

The current study shows that the proliferation features MAI, PPH3 and Ki67 (the latter two assessed by digital image processing) have strong prognostic value. PPH3 and MAI are strongly correlated, which is biologically understandable. DIA-Ki67 with a threshold of 6.5% is the strongest prognosticator of all Ki67 features, added prognostically to PPH3, and this combination overshadowed all other features studied.

As to the question why the proliferation markers MAI and PPH3 are prognostically stronger than Ki67,

Table II. Multivariate comparison of all features shows that Ki67 by digital image analysis with a threshold of 6.5%, in combination with PPH3 (with a threshold of 13) is the strongest prognostic combination explaining all other features

VARIABLES IN THE EQUATION								
		BETA	STANDARD ERROR	WALD	SIGNIFICANCE	HAZARD RATIO	95% CONFIDENCE INTERVAL FOR HAZARD RATIO	
							LOWER	UPPER
Step 1	**PPH3 < 13 vs. ≥ 13**	2.2	0.49	19.6	< 0.0001	8.9	3.4	23.5
Step 2	**PPH3 < 13 vs. ≥ 13**	1.2	0.52	4.9	0.03	3.2	1.1	8.9
	DIA-Ki67 – 6.5%	2.6	1.08	5.6	0.02	12.8	1.5	107.0

VARIABLES NOT IN THE EQUATION IN STEP 2

FEATURE	PROBABILITY OF NO DIFFERENCE
age < 45, 45-55, > 55 years	0.59
Tumor diameter ≤ 2, > 2 cm	0.33
MAI 0-2, > 2	0.26
MAI 0-9, ≥ 10	0.51
MAI 0-5, 6-10, > 10	0.56
grade	0.50
estrogen receptor	0.23
progesterone receptor	0.16
HER2 negative, positive	0.87
basal cell like negative, positive	0.31
triple negative, positive	0.85
cytokeratin 5/6 negative, positive	0.29
cytokeratin 14 negative, positive	0.13
lymph vessel invasion	0.73

it is important to remember that Ki67 stains nuclei of cells in all phases of the cell cycle, i.e. G1-, S-, G2- and M-phase cells. However, many of these cells will go into the G0 phase or end in apoptosis as a result of DNA damage. In contrast, MAI exclusively identifies cells in the M phase and most of these cells will reach cell division. Likewise, PPH3-positive cells also have a much higher likelihood of dividing than Ki67 positive cells, as PPH3 stains only very late G2- and M-phase cells [17, 18].

The clinical use of the additional prognostic value of DIA-Ki67 in patients with PPH3 < 13 may depend on the attitude of the treating medical oncologist and the patient. Medical oncologists in the USA may regard a 10% risk of dying from metastatic disease too high to NOT give adjuvant systemic treatment. In north-west Europe, this risk is at the border of what often is regarded as just acceptable, as systemic chemotherapy in women < 55 years old can have serious side effects.

Unfortunately, digital image analysis equipment is not yet widely available in pathology laboratories. This will most likely change in the years to come, with the advent of digital pathology. Until this has become a reality, pathologists could send their Ki67 stained sections to specialized laboratories which have the necessary computerized equipment. Alternatively, interactive morphometry assessment of Ki67 might be an inexpensive alternative [9]. Subjective counts not supported by point-weighted sampling, in our view, are not a defendable option as the determinations between pathologists vary too much.

In conclusion, in node negative breast cancer patients not undergoing adjuvant systemic treatment, PPH3 or MAI combined with Ki67 assessed by digital image analysis is prognostically strong, and therefore of potentially high clinical relevance.

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Table III. The additional prognostic value of DIA-Ki67-6.5% is in the patients with a low PPH3 (< 13), not in the patients with PPH3 ≥ 13

CASE PROCESSING SUMMARY					
PPH3	**DIA-KI67**	**TOTAL N**	**N OF EVENTS**	**CENSORED**	
				N	%
PPH3 < 13	< 6.5%	114	1	113	99.1
	≥ 6.5%	40	4	36	90.0
	overall	154	5	149	96.8
PPH3 ≥ 13	< 6.5%	8	1	7	87.5
	≥ 6.5%	75	22	53	70.7
	overall	83	23	60	72.3
overall	overall	237	28	209	88.2

Overall comparisons

PPH3 < 13 vs. ≥ 13		χ^2	DF	SIG.
PPH3 < 13	Log Rank (Mantel-Cox)	5.901	1	0.015
PPH3 ≥ 13	Log Rank (Mantel-Cox)	0.707	1	0.400

Test of equality of survival distributions for the different levels of DIA-Ki67 – 6.5%.

Table IV. The additional prognostic value of DIA-Ki67-6.5% is in patients with MAI < 10

CASE PROCESSING SUMMARY							
MAI < 10 VS. ≥ 10		**KI67 BY DIA 6.45%**		**TOTAL N**	**N OF EVENTS**	**CENSORED**	
						N	%
dimension 0	MAI < 10	dimension 1	Ki67 ≤ 6.45	119	1	118	99.2
			Ki67 > 6.45	55	8	47	85.5
			overall	174	9	165	94.8
	MAI ≥ 10	dimension 1	Ki67 ≤ 6.45	2	0	2	100.0
			Ki67 > 6.45	61	19	42	68.9
			overall	63	19	44	69.8
	overall	dimension 1	overall	237	28	209	88.2

Overall comparisons

MAI <10 vs. ≥ 10	χ^2	DF	SIG.
MAI < 10 Log Rank (Mantel-Cox)	10.9	1	0.001
MAI ≥ 10 Log Rank (Mantel-Cox)	0.60	1	0.44

Test of equality of survival distributions for the different levels of Ki67 by DIA 6.45%.

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