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LOH at 1p31 (ARHI) and proliferation in lymph node-negative breast cancer

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Abstract. *Background*: The mitotic activity index (MAI) is a strong prognosticator in node-negative invasive breast cancer patients. Recently, a correlation between the MAI and specific chromosomal aberrations at chromosome 1p was described.

Methods: Analysis of MAI, immunohistochemical staining patterns for proliferation-associated phosphohistone H3 (PPH3), phosphorylated ERK1/2, p21, cyclin E, Ki67 and cyclin D1 proteins; and prognosis in 158 adjuvant chemotherapy-treated T1-2N0M0 invasive breast cancer patients, analysis of LOH at 1p31 (including ARHI) using the dinucleotide repeats D1S207, D1S430 and D1S464 in 76 patients. Single and multivariate survival analysis was used to evaluate the importance of the various markers tested.

Results: LOH at 1p31 did not correlate with MAI nor provide prognostic information. Phosphohistone H3 was the best prognosticator for patients in all age groups with 20 year distant metastasis free survival of distant metastases 93% vs. 72% respectively ($p = 0.004$, HR = 4.5). In multivariate analysis, phosphohistone H3 < 13 vs. $\geqslant 13$ exceeded the prognostic value of the mitotic activity index.

Conclusions: LOH at 1p31 is common in breast cancer, and correlates with loss of proliferation-associated proteins, but not with MAI, PPH3 or prognosis. PPH3 is the best prognosticator in this study group of adjuvant chemotherapy-treated lymph node-negative breast cancer patients.

Keywords: Breast cancer, proliferation, PPH3, loss of heterozygosity, ARHI

1. Introduction

Breast cancer is the most frequent cancer among women in industrialized countries, and the death rate from breast cancer is persistently high. Traditionally, classification and prognostication are based on lymph node status, which is the strongest prognosticator. However, 20–30% of lymph node-negative patients still die of the disease, necessitating additional prognosticators to select high-risk patients. Proliferative activity is one of the most thoroughly investigated biologic processes for breast cancer prognosis [3,5,6,20,24], and it is an important therapeutic target. Moreover, there is evidence of a relationship between proliferation and response to systemic treatments [8,15]. One strong, yet simple and reproducible, proliferation-associated prognostic factor is the mitotic activity index (MAI) [4,23]. The prognostic value of MAI has been demonstrated in many retrospective and prospective multicenter studies using a fixed threshold (MAI < 10 favorable; MAI $\geqslant 10$ unfavorable) (for a review, see [10]). These studies showed that MAI is reproducible, stable and robust, despite variations in tissue processing, and is a strong prognosticator in node-negative invasive breast cancer 3 in women under 70 years of age, but not in women over 70 [2]. Recently a tight correlation between phosphorylation of histone H3 (=PPH3) and mitotic chromatin condensation has been shown in studies using an antibody selective for the Ser-10 phosphorylated histone H3 (=PPH3).

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PPH3 expression has been characterized in human endometrium, colorectal cancers, ovarian serous adenocarcinomas and breast cancer [7,12,16,17]. In lymph node-negative breast cancer patients under the age of 55 years, PPH3 proved to be a better prognosticator than MAI [21].

Genetic analysis offers opportunities to understand phenotype changes and explore new, perhaps stronger, prognosticators in the clinically important subgroup of relatively young breast cancer patients. Recently, a correlation between the MAI and specific chromosomal aberrations at chromosome 1p were described [14]. Loss of 1p occurred in 43% of breast carcinomas with an MAI $\geqslant 10$ and in only 17% of cancers with an MAI < 10. Deletion of a tumor suppressor gene on chromosome 1p might cause unregulated proliferation of tumor cells and result in a high MAI. A candidate tumor suppressor gene is ARHI (=DIRAS3). Located on 1p31 and described in 1999 as a member of the Ras superfamily of small G proteins, ARHI is frequently inactivated in ovarian and breast tumors [27]. An important characteristic of ARHI is that it is a maternally imprinted gene, so loss of heterozygosity (LOH) of the paternal allele gives rise to total inactivation of the gene. LOH analysis at chromosome 1p31 has shown that ARHI is the center of allelic deletion in some of the breast cancers and cell lines tested [13,18]. Moreover, a recent study showed that, upon cell cycle arrest, both p21 and ARHI increased; while other studies showed that re-expression of ARHI in cancer cells inhibits signaling through Ras/Map and PI3 kinase, upregulates P21 (WAF1/CIP1), downregulates cyclin D1, induces JNK, and inhibits signaling through Stat3 [26]. These data are compatible with the hypothesis that loss of ARHI leads to an increase in cycling cells, perhaps ultimately increasing the final stage of cycling cells (mitosis), and hence, the MAI. To test this hypothesis, we analyzed correlations between immunohistochemical staining patterns of different cell cycle markers, LOH at 1p31, MAI, PPH3 and prognosis in lymph node-negative breast cancer patients.

2. Materials and methods

The study was approved by the Regional Ethics Committee, the Norwegian Social Science Data Service and the Norwegian Data Inspectorate.

2.1. Patients

The archive (from 1984–1989) of the Department of Pathology at the Stavanger University Hospital provided information on a total of 158 operable lymph node-negative T1-2N0M0 invasive breast cancer patients of all ages (median 58, range 22–80 years) with long follow-up of median 176 (range 13–309) months. All patients had been treated with modified radical mastectomy or breast conserving therapy. Postoperative radiation was employed in all patients that underwent breast conserving therapy. Additional adjuvant treatment, including perioperative chemotherapy, postoperative radiation also in selected patients treated with modified radical mastectomy, and endocrine adjuvant treatment (i.e. Tamoxifen) was offered in agreement with national guidelines of the Norwegian Breast Cancer Group at that time.

2.2. Tumor specimen

The post-surgical size of the tumor (pT) was measured in fresh specimens; the tumors were sliced (0.5 cm), fixed in buffered 4% formaldehyde, and embedded in paraffin. Paraffin sections (4 μm) were stained with haematoxylin and eosin (H&E). Histologic type was assessed according to World Health Organization criteria [22]. Grade was assessed according to the Nottingham modification [9,10] based on careful examination by two pathologists with considerable experience in breast pathology, using the criteria MAI 0–5 = 1, MAI 6–10 = 2 and MAI $>$ 10 = 3; nuclear atypia mild = 1, moderate = 2 and marked = 3; and tubular formation majority (i.e., $>$75%) = 1, moderate (10–75%) = 2 and little or none ($<$10%) = 3. Grade was the sum of MAI, nuclear atypia, and tubular formation values. Thus, a sum of 3–5 was Grade I, 6–7 was Grade II, and 8–9 was Grade III. MAI was assessed as previously described [9]. Briefly, all unambiguous mitoses were counted with a conventional transmission light microscope with a 40× objective (450 μm at specimen level) in 10 consecutive neighboring fields of vision in the invasive, most cell-dense area in the periphery of the tumor (representing a total area of 1.59 mm^2 at the specimen level).

2.3. Immunohistochemistry

Tissue Micro-Arrays (TMA) with 2 mm cylinders of 158 breast cancers were used for immunohistochemical analysis. Due to mechanical problems cutting the TMA blocks, some of the cylinders were lost in the different stains. Antigen retrieval methods and antibody dilutions were optimized before beginning the study. To guarantee uniform process-

ing of the cases, all sections were made and immunostained at the same time. Paraffin sections were mounted on silanized slides (S3002, DAKO, Glostrup, Denmark) and dried overnight at 37°C followed by 1 hour at 60°C. 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 3 min at full pressure and cooled for 15 min at 18°C. Immunostaining was performed using an autostainer (DAKO). TBS (with 0.05% Tween 20 (pH 7.6)) was used as rinse buffer. Endogenous peroxidase activity was blocked by peroxidase blocking reagent S2001 (DAKO) for 10 min. Sections were incubated with the following antibodies, dilutions, and time intervals: Ki-67, 1/100, 30 min (MIB-1, DAKO); phosphorylated ERK1/2, 1/600, overnight (4376, Cell Signaling, Danvers, MA, USA); p21, 1/25, overnight (4D10, Novocastra, New Castle, UK); Cyclin D1, 1/100, overnight (SP4, Neomarkers, Fremont, CA, USA), Cyclin E, 1/40, 30 min (13A3, Novocastra), ER, 1/60, 30 min (6F11, Novocastra); PR, 1/150, 30 min (PGR636, NCL); PPH3, 1/1500 (06-570, Upstate, Lake Placid, NY, USA). DAKO antibody diluent (S0809, DAKO) was used, and the antigen-antibody complex was visualized with Peroxidase/DAB (ChemMate Envision Kit K5007, DAKO) after incubation with Envision/HRP rabbit anti-mouse for 30 min and DAB-chromogen for 10 min. Sections were counterstained with H&E, dehydrated, and mounted. Sections of normal breast tissue were included on each processed slide as immunostain controls.

2.3.1. Immunohistochemistry scoring

Antibody staining was evaluated through subjective scoring of positive staining by two independent observers. In cases of disagreement, a consensus score was agreed upon after viewing tissue with a multi-head microscope. For all immunostainings, the number of positive tumor nuclei in the TMA cylinder was estimated, and tumors with $<10\%$ positivity were regarded as negative, while those with $\geqslant 10\%$ positivity were regarded as positive. The PPH3 index was assessed using the same counting protocol as for the MAI, two independent pathologists counted the number of PPH3-positive objects (nuclei and mitoses) in 10 adjacent FOV ($=1.59$ mm^2, with an $\times 40$ objective). Nuclei with fine granular PPH3 staining were not counted, as these cells are not in the G2 phase [21]. If the counts of two observers differed by more than three figures, the count was repeated with a multi-head microscope and a consensus score was obtained.

2.3.2. Loss of heterozygosity

LOH analysis was performed on tissue from 124/158 lymph-node-negative invasive breast cancers. In 34 cases, normal DNA was not obtained. DNA from normal tissue was acquired from previous samples from the same patients or from normal tissue surrounding the tumor. Tumor cells were collected with an automated laser microdissection system (Leica DM LMD, Leica Microsystems GmbH, Wetzlar, Germany). At least 1000 tumor cells were collected from each specimen. DNA from microdissected cells was isolated using the Qiamp DNA Microkit (Qiagen, Fremont, CA, USA) according to the manufacturer's protocol, with an additional incubation of 15 min at 98°C before addition of proteinase K. The quantity/quality of DNA was adequate for amplification in 76 (61%) of the original 124 lymph-node-negative cases. Microsatellite analyses were done using the following dinucleotide repeats: D1S207 Forward: CACTTCTC CTTGAATCGCTT, Reverse: GCAAGTCCTGTTCC AAGTCT; D1S430 Forward: TCCAGATTTAGTGT CATTTCCC, Reverse: CACTTACAGTAACAAGCC CCAG; D1S464 Forward: GCCTAAATTTCTTACA CATCCTAAC, Reverse: TGTTTTAAACACCACAA ATAAATGT.

Each of the dinucleotide repeats produce a polymerase chain reaction (PCR) product with a length <200 bp, that is closely situated to ARHI, and are 70–85% heterozygous. All forward primers were labeled with fluorescein isothiocyanate for detection. PCR was performed on 20 μl reactions containing 5 μl DNA, 2 μl (10× Hot Master Gold Buffer, Eppendorf, Hamburg, Germany) 0.5 μl (10 mM) dNTP, 1 μl (2 μM) forward primer, 1 μl (2 μM) reverse primer, and 0.25 μl (5 U/μl) HotMaster™ Taq DNA Polymerase (Eppendorf). The following program was used for all three PCR reactions: initial denaturation at 94°C for 2 min followed by 35 1-min cycles at 94°C, 2 min at 55°C, 2 min at 65°C, and a final elongation period of 10 min at 70°C. LOH analysis was performed with 2.0 μl PCR product diluted in 23 μl fresh formamid and 0.5 μl size standard (GS500 Liz, Applied Biosystems, Foster City, CA). The Genetic Analyzer 3130xl (Applied Biosystems) with POP7 polymer was used to separate the samples into 36 cm capillaries. GeneMapper Software, version 3.7 (Applied Biosystems), was used for LOH analysis. Allelic imbalance between normal and tumor samples was calculated for each patient. Allelic ratios <0.5 or >1.5 were considered positive for LOH.

2.4. Statistical analyses

Statistical analyses were performed with SPSS version 15.0. Correlations were calculated with chi-square tests. Two-sided Fisher's exact tests were used for two-by-two tables. The main endpoints were distant recurrence and survival relative to breast cancer. For analyzing the probability that patients would remain free of distant metastases, we defined recurrence as any first recurrence at distant sites. All other patients were censored on the date of the last follow-up visit and included deaths from causes other than breast cancer, local or regional recurrences or the development of a secondary primary cancer (including contra-lateral breast cancer). Breast cancer specific survival was defined as any death due to distant metastases (as evident from clinical, radiologic, histological or autopsy data). If the cause of death was unknown, but a metastasis was previously detected, death was considered breast-cancer related unless explicitly stated otherwise. If the status during follow-up indicated a confirmed metastasis without a recurrence date, the date of the follow-up visit was used. Age, time to first distant recurrence, and survival time were calculated relative to the date of primary diagnosis. Survival curves were made using the Kaplan–Meier method. Group differences were evaluated with the log-rank test. The independent importance of potential prognostic variables was evaluated using Cox proportional hazard analysis and expressed as a hazards ratio (HR) with 95% confidence intervals (CI). The prognostic value of proliferation is age dependent [2]. We therefore analyzed the influence of specific age groups (<55, 55–70, >70) on the correlations with ARHI expression.

3. Results

The median follow-up was 176 months (range: 13–309) during which thirty one (20%) developed distant metastases (21 dead of breast cancer related death). The tumor characteristics of the 158 breast cancer patients are representative of node-negative invasive breast cancer in general [2].

3.1. LOH at 1p31

LOH was apparent in 60 (79%) of the cases (Fig. 1). The highest percentage of LOH ($n = 39$, 51%) was

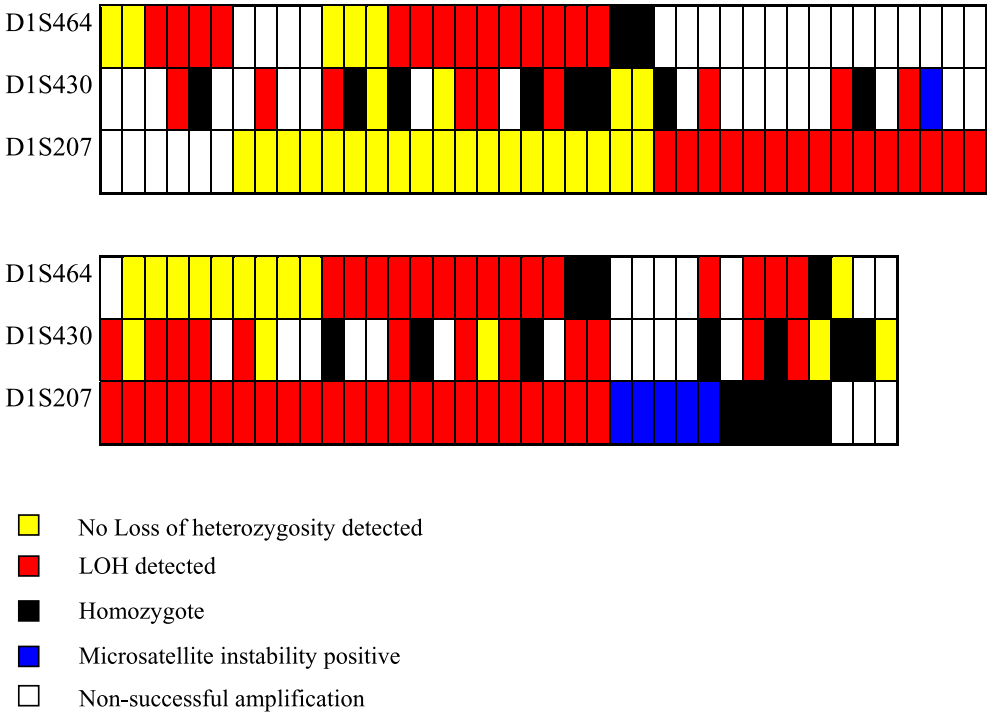

Fig. 1. Overview of the 76 LOH results per sample for each of the three dinucleotide repeats. Samples with microsatellite instability and homozygous samples are not scored as events.

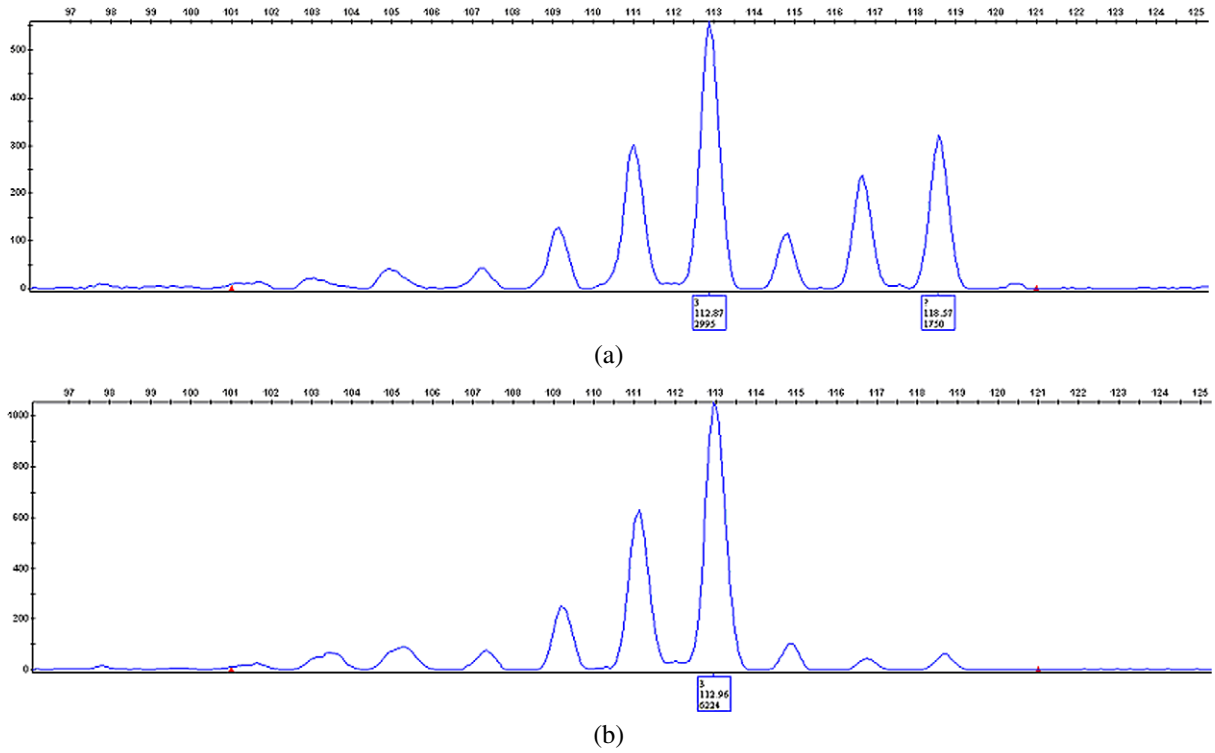

Fig. 2. Example of LOH with D1S207. The upper image demonstrates the pattern found in normal DNA. The lower panel demonstrates the pattern found in tumor DNA of the same patient.

demonstrated at D1S207 (Fig. 2). LOH did not correlate with any of the immunohistochemical staining patterns, MAI2, MAI10, PPH3, or other pathological tumor characteristics. Seventeen (22%) of the 76 cases showed LOH at both the telomeric and the centromeric side of ARHI. In these cases, the ARHI gene was probably inactivated. Consistent with this assumption, 15 (88%) of these cases had a MAI > 2 (see Table 1). The genetic loss of ARHI correlated with only with age, older patients with relatively higher percentage of ARHI loss. Furthermore, 13 of the 17 tumors with ARHI loss were negative for p21. In agreement with this, 11 of 17 tumors were positive for cyclin D1, and 13 of 17 tumors were positive for ERK1/2, but these differences were not statistically significant.

3.2. Survival analysis

In the group of lymph-node-negative patients under 55 years of age, grade, estrogen receptor, MAI10 and PPH3 were the prognostically most important factors (Table 2). Considering all patients (i.e. all age groups) in the multivariate analysis ($n = 99$ with data on tumor diameter, MAI10, PPH3_13, Estrogen receptor, Progesteron receptor and Nottingham grade), PPH3 (Fig. 3) was the only remaining prognostic factor ($p = 0.016$, HR $= 3.7$).

4. Discussion

Many retrospective and prospective studies [3,5,6, 20,24] have shown that the MAI is the strongest prognostic factor in lymph-node-negative invasive breast cancer. Therefore, a biological explanation of the MAI is important, since it could guide us toward a therapeutic intervention that might influence or hinder tumor proliferation. We previously reported that loss of 1p31 correlates with a high MAI, and we hypothesized that this might be due to loss of the ARHI suppressor gene [14]. Although the markers we used are in close proximity to the ARHI gene, loss of ARHI cannot automatically be assumed in cases with LOH at only the telomeric or centromeric side of the gene. Only 17 (22%) cases showed LOH at both the telomeric and the centromeric sides of ARHI, suggesting a loss of ARHI. Cases in which the ARHI gene was lost (defined as

Table 1
Characteristics of patients <56 years of age and tumors in relation to distant metastases free survival

Characteristics		Events/number at risk (%)	Log-rank p-value[1]	HR[2] (95% CI)[3]
Tumor diameter	<2 cm	4/28 (14)	0.11	2.5 (0.8–8.3)
	⩾2 cm	9/32 (28)		
Estrogen receptor	<10%	10/27 (37)	0.039	0.4 (0.1–099)
	⩾10%	6/37 (16)		
Progesteron receptor	<10%	8/28 (29)	0.19	0.5 (0.1–1.5)
	⩾10%	4/26 (15)		
Grade[4]	1	0/13 (0)	0.005	
	2	3/22 (14)		28.7 (0.15–5468.2)
	3	13/36 (36)		
Nuclear atypia[4]	1 (mild)	0/3 (0)	0.042	
	2 (moderate)	2/25 (8)		5.4 (1.2–23.8)
	3 (strong)	14/43 (33)		
Tubular formation[4]	1 (>75%)	0/3 (0)	0.22	
	2 (10–75%)	1/12 (8)		5.0 (0.7–37.9)
	3 < 10%	15/56 (27)		
Mitotic impression	1 (0–5)	3/32 (9)	0.007	
	2 (6–10)	2/10 (20)		2.3 (0.4–14.0)
	3 > 10	11/29 (38)		6.0 (1.7–21.7)
MAI	<10	6/42 (14)	0.016	3.2 (1.2–9.0)
	⩾10	10/29 (34)		
PPH3	<13	0/26 (0)	<0.0001	63.8 (0.7–5601.5)
	⩾13	13/39 (33)		
Ki67*	Negative	3/27 (11)	0.090	3.1 (0.8–12.6)
	Positive	6/19 (32)		
Cyclin D1*	Negative	4/18 (22)	0.72	0.8 (0.3–2.6)
	Positive	11/52 (21)		
Cyclin E*	Negative	5/34 (15)	0.16	2.2 (0.7–6.8)
	Positive	8/32 (25)		
P21*	Negative	13/52 (25)	0.90	0.88 (0.1–6.8)
	Positive	1/4 (25)		
ERK1/2*	Negative	3/11 (27)	0.95	1.0 (0.3–3.7)
	Positive	11/45 (24)		
LOH at 1p31	Absent	1/6 (17)	0.97	0.96 (0.1–8.6)
	Present	4/20 (20)		

[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]for Cox-regression group 1 and 2 were taken together; *for all the immunohistochemical stainings 10% was used as the threshold; <10% negative, ⩾10% positive.

LOH of markers at both ends) showed a higher percentage of MAI > 2, more p21 negativity, and higher positivity for cyclin D1 and p42/44, supporting current ideas about the function of ARHI [26]. Although we observed LOH in only 21% of the cases, in ovarian cancer 40% LOH at 1p31 was demonstrated, and in 10–15% of cases, hypermethylation of the non-imprinted paternal allele was reported as the reason for non-expression of ARHI [25]. Downregulation of ARHI by histone deacetylases in complexes with E2F1 and E2F4 [11] and a lack of ARHI expression in the presence of ARHI mRNA [19] have also been described. LOH at 1p31 and proliferation were not correlated as measured by MAI, cyclin E, Ki67 or PPH3; and although we previously reported a correlation between loss of 1p31 and MAI10, we did not confirm this in the current study. This may reflect the low resolution of Chromosome Genome Hybridization (up to 10 Mb) used in our previous study, which means that we may have searched in the wrong area. Alternative suppres-

Table 2
Overview of the correlations between LOH at 1p31, at one of the three markers, LOH of a telomeric and a centromeric marker around ARHI, with immunohistochemical staining, MAI and other tumor characteristics

Characteristics		LOH at 1p31 at any of the markers absent $n = 16$	LOH at 1p31 at any of the markers present $n = 60$	p-value[1]	LOH at 1p31 including ARHI absent $n = 59$	LOH at 1p31 including ARHI present $n = 17$	p-value[1]
Distant	Absent	13	52	0.4	50	15	1.0
metastases	Present	4	8		10	2	
Age	<56	8	29	1.0	33	4	**0.02**
	56–70	6	22		21	7	
	>70	3	9		6	6	
Tumor diameter	<2 cm	8	28	1.0	31	5	0.1
	⩾2 cm	8	32		28	12	
Estrogen	Positive	11	35	1.0	37	9	0.5
Receptor	Negative	4	14		13	5	
Progesterone	Positive	9	28	0.8	32	5	0.07
Receptor	Negative	6	23		19	10	
Nottingham	1	0	10	0.1	9	1	0.6
grade	2	8	19		21	6	
	3	8	31		29	10	
MAI	⩽2	3	14	1.0	15	2	0.4
	3–9	5	18		17	6	
	⩾10	8	28		27	9	
PPH3	<13	1	14	0.2	10	5	0.7
	⩾13	8	27		26	9	
KI67*	Negative	5	26	0.5	24	7	1.0
	Positive	6	18		19	5	
Cyclin D1*	Negative	7	19	0.6	20	6	1.0
	Positive	9	41		39	11	
Cyclin E*	Negative	5	38	**0.03**	33	10	1.0
	Positive	11	22		26	7	
P21*	Negative	11	44	0.4	42	13	0.7
	Positive	3	5		7	1	
ERK1/2*	Negative	2	10	1.0	8	4	0.5
	Positive	10	45		42	13	

*For all the immunohistochemical stainings 10% was used as the threshold; <10% negative, ⩾10% positive; [1]p-value calculated by two-sided Fisher's exact test.

sor genes at 1p31 are GADD45A (a DNA damage inducible gene), and p18 (a cyclin-dependent kinase inhibitor). Loss of ARHI had no prognostic value, even in subgroup analyses according to age. PPH3 was not only the strongest prognosticator in the subgroup of lymph-node-negative women under 55 years of age, but also when considering all age groups.

In conclusion, LOH at 1p31, is a common event in breast cancer, it is correlated with loss of cell-cycle-associated proteins, but not with MAI, PPH3 or prognosis. Phosphohistone H3 is the best prognosticator in this study group of adjuvant chemotherapy-treated lymph-node-negative breast cancer patients.

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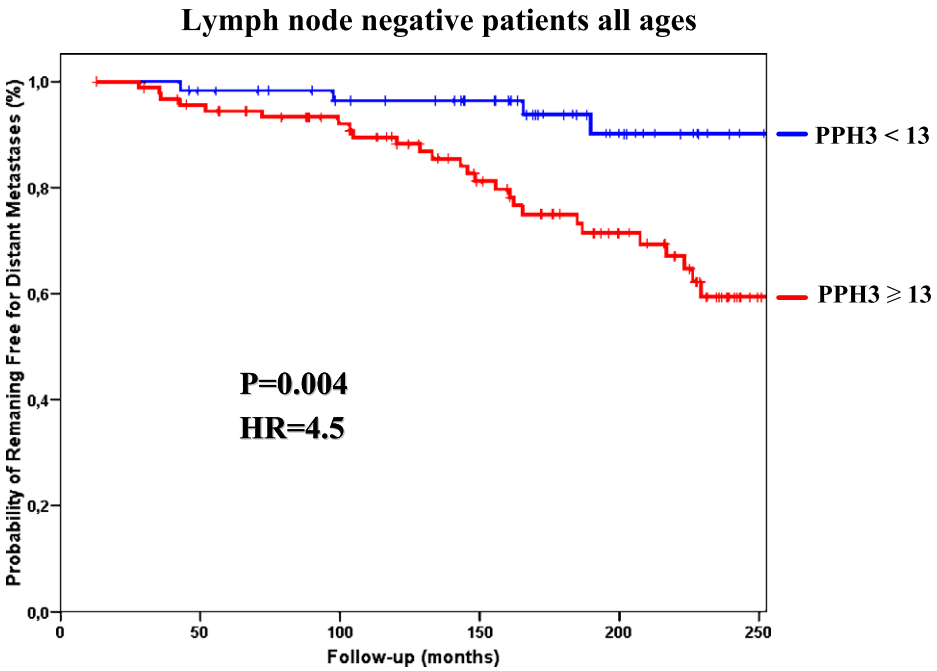

Fig. 3. Long-term recurrence-free survival curves stratified for the PPH3 index (<13 blue line versus ⩾13, red line). *p*, probability of no difference.

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